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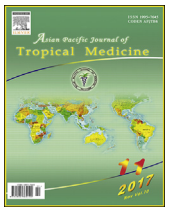

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Efficient hepatoprotective activity of cranberry extract against CCl_4-induced hepatotoxicity in Wistar albino rat model: Down-regulation of liver enzymes and strong antioxidant activity

Fahad Hussain[1], Arif Malik[1✉], Ujala Ayyaz[1], Hassan Shafique[1], Zohaib Rana[2], Zahid Hussain[3✉✉]

[1]*Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Raiwind Road, 55150, Lahore, Pakistan*

[2]*Department of Biochemistry, University of Agriculture, Faisalabad, 38040, Pakistan*

[3]*Department of Pharmaceutics, Faculty of Pharmacy, Universiti Teknologi MARA, Puncak Alam Campus, Bandar Puncak Alam, 42300, Selangor, Malaysia*

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ABSTRACT

Objective: To investigate the hepatoprotective efficacy of cranberry extract (CBE) against carbon tetrachloride (CCl_4)-induced hepatic injury using *in-vivo* animal model.
Methods: The hepatoprotective efficacy of CBE (200 and 400 mg/kg) was investigated against CCl_4 (4 mL/kg)-induced hepatotoxicity, elevated liver enzymes [ALT (alanine aminotransferase), AST (aspartate aminotransferase), and alkaline phosphatase (ALP)], and total protein (TP) contents in the serum. Moreover, CBE-aided antioxidant defense against hepatotoxic insult of CCl_4 was measured by evaluating a number of anti-oxidative biomarkers including reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) in the serum by using spectrophotometric analyses.
Results: Results showed that the exposure of experimental animals to CCl_4 did induce significant hepatotoxicity compared to the non-induced (untreated) group. The oral administration of CBE demonstrated a significant dose-dependent alleviation in the liver enzymes (AST, ALT, and ALP), increased antioxidant defense (GSH, SOD, and CAT), and reduced MDA levels in the serum of treated animals compared to the animals without treatment. The resulting data showed that the administration of CBE decreased the serum levels of ALT, AST, and ALP compared to the CCl_4-induced group.
Conclusions: The resulting data evidenced that CBE exhibits promising hepatoprotective potential against the chemical induced hepatotoxicity, maintains homeostasis in liver enzymes, and can provide significant antioxidant defense against free radicals-induced oxidative stress.

1. Introduction

Liver cancer is the most typical fifth type of cancer in men and seventh most prevalent type in women that is caused by viral and some exogenous environmental toxins [1]. Excessive exposure of environmental toxins, alcohol and overdoses of drugs may lead to various liver diseases such as cirrhosis, hepatitis, fibrosis, etc. Liver is the major organ of our body that is involved in detoxification and metabolism to maintain homeostasis in body against external noxious challenges. This detoxification process is very important because otherwise the exogenous chemicals might cause over production of free radicals that are harmful to liver normal functions [2,3]. The ingestion of CCl_4 in rat modal causes necrosis that further lead to steatosis, fibrosis, and cirrhosis and finally transformed into the hepatocellular carcinoma [2].

According to the World Health Organization, 85% countries including Africa and Asia are using plant-derived natural medicines due to the presence of many bioactive compounds that act as antioxidant in biological system. The synthetic drugs might have various side effects due to the fact that the focus of scientists is getting shifted to the plants-derived herbal medicines. CAT and GPx are involved in the conversion of O^{-2} to hydrogen

First author: Fahad Hussain, Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Raiwind Road 55150, Lahore, Pakistan.
E-mail: fahad.hussain5712@gmail.com
✉Corresponding author. Professor Arif Malik, Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Raiwind Road, 55150, Lahore, Pakistan.
E-mail: drarifmalik.uol@gmail.com
✉✉Corresponding author. Dr. Zahid Hussain, Department of Pharmaceutics, Faculty of Pharmacy, Universiti Teknologi MARA, Puncak Alam Campus, Bandar Puncak Alam, 42300, Selangor, Malaysia.
Tel: +60 102483089
E-mail: zahidh85@yahoo.com
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peroxide and catalyze into water to provide protection against reactive oxygen species (ROS) activity [4]. Different types of bioactive compounds are present in plants such as flavonoids, rutins, glycosides, terpenoids, tannins and alkaloids that act as antioxidant during stress condition and play crucial protective roles such as antitumor, anti-inflammatory, anti-mutagenic and immune-modulating against the CCl_4 liver toxicity [5].

Cranberry consists of several bioactive compounds such as fructose, vitamin C, flavonoids, anthocyanidins, catechins, triterpenoids and phenolic compounds that are numerously used due to their healthcare benefits and against different types of cancer [6]. Flavonoids in cranberry prevent the mitochondrial damage, fragmentation, loss of membrane integrity and scavenge free radicals, superoxide radicals, hydroxyl radicals and lipid peroxidation [7]. According to the results of previous studies, flavonoids of cranberry extract (CBE) exhibit promising potential to reduce the elevated level of ALT (alanine aminotransferase), AST (aspartate aminotransferase) and prevent accumulation of lipid membrane droplets in rats liver models [8]. CBE powder is also used in food to improve antioxidant defense of the body. *Vaccinium macrocarpon* (American cranberry) consists of several polyphenol compounds that act as anti-tumorigenic, anti-inflammatory, anti-mutagenic, and anti-carcinogenic. The concentration of these polyphenols increases in cranberry with the time of ripening and as well as the size of the fruit. Six aglycones are present in the anthocyanins. The second most abundant compound present in CBE is phenolic acid such as hydroxybenzoic acid and hydroxycinnamic acid. But the major quantity of benzoic acid is present. Only three types of hydroxybenzoic acid such as 2,4-dihydroxybenzoic, *p*-hydroxybenzoic and *o*-hydroxybenzoic are present in lower quantity. Similarly, the hydroxycinnamic also contain various sub-contents such as *p*-coumaric, ferulic acid, sinapic and caffeic. The third most abundant bioactive compound are terpenes on which very less work has been done compared to the polyphenolic composition. Flavonols are present in cranberry fruit but more quantity is present in elderberry [4].

Previous researchers suggested that CCl_4 is the most commonly used chemical method to induce hepatic injury [9]. They suggested that the formation of trichloromethyl radical is the most noxious free radicals produced by the CCl_4. CCl_4 is a multifactorial toxic agent that is involved in the production of free radicals, lipid peroxidation, alteration in genes expression, binding to the macromolecules, and cause loss of calcium homeostasis [10]. In the body, CCl_4 is converted into the reactive metabolite in the presence of oxygen and cytochrome P450 CYP (2E1), CYP 2B, and CYP3A. The reactive metabolites of CCl_4 such as CCl_3 and CCl_3OO react with polyunsaturated fatty acid and convert them into the saturated fatty acid which comprises the integrity or permeability of cell membrane and cause lipid peroxidation and initiate leaking process [2]. In this study, we have investigated the hepatoprotective efficacy of CBE against CCl_4-induced hepatic injury using a rat model.

2. Materials and methods

2.1. Experimental animals

Adult Wister male albino rats (160–200 g) were purchased from the National Institute of Health (Islamabad, Pakistan). The animals were kept at 25 °C and fed with regular diet and controlled under 12 h light–dark period and humidity (RH 60%). The animal study and handling protocols were approved by the Ethics Committee for the Scientific Research at the University of Lahore.

2.2. Plant extract

The standardized extract of cranberry was purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). Different doses of CBE (200 and 400 mg/kg) were prepared.

2.3. Induction of hepatic injury

The hepatic injury was induced by using a previous method [11], with minor modifications. Briefly, a dose of 4 mL/kg of CCl_4 was administered orally once a day for a period of 10 d to induce hepatic toxicity in all the experimental animals.

2.4. Experimental design

The experimental animals were randomly divided into the five groups (n = 6). First group was normal animals without inducing hepatic injury and is used to measure the physiologic concentrations of various biomarkers (liver enzymes). The second group was induced with CCl_4 and was undergone no treatment. The third, fourth and fifth groups were induced with CCl_4 and treated with CBE (200 mg/kg), CBE (400 mg/kg), and Clavazine (Commercial drug), respectively.

2.5. Collection of blood and separation of serum specimens

At the end of treatment period, all the experimental animals were sacrificed and their blood samples (5 mL) were collected from the heart. The serum samples were separated from the blood samples by centrifugation at 1 500 r/min for 10 min. The serum samples were then stored at −60 °C until further biochemical analyses.

2.6. Biochemical assays

2.6.1. Determination of total protein (TP) and liver enzymes

The hepatoprotective efficacy of different doses of CBE was evaluated by determining the TP contents and liver enzymes [AST, ALT, and alkaline phosphatase (ALP)] in the serum specimens of all experimental animals compared to the untreated group. The levels of AST, ALT and TP were measured using spectrophotometric analysis [12]. However, the level of ALP was measured using the previously established method [13] using Randox kits (Randox Laboratories Ltd., Crumlin, UK).

2.6.2. Determination of antioxidant defense

The antioxidant activity of CBE (200 and 400 mg/kg) was evaluated by investigating the levels of the reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) in the serum specimens of all experimental groups.

The levels of GSH were measured using the Ellman's method [14]. Ellman's reagent reacts with GSH to yield a chromophore and

oxidize GSH. The CAT levels in the serum samples of all the experimental animals were determined using Aebi's method [15] at 230 nm.

The level of SOD activity was measured in the serum samples of all experimental animals using the Nishikimi method [16] which was later modified by Kakkar [17]. Briefly, 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.052 mol/L), 0.3 mL of nitro blue tetrazolium (300 μmol/L), 0.1 mL of phenazine methosulphate (186 μmol/L) and 0.2 mL of dihydronicotinamide adenine dinucleotide (NADH) (750 μmol/L) were added to 0.1 mL of the serum sample. The reaction was started by adding NADH followed by the incubation at 30 °C for 90 s. After that, 0.1 mL of glacial acetic acid was added to terminate the reaction. Then, 4.0 mL of n-butanol was introduced to the reaction mixture. The reaction mixture was stand for 10 min, followed by the centrifugation at 1 500 r/min to separate the upper butanol layer from the sample reaction mixture. The occurrence of chromogen was measured at 560 nm. The level of lipid peroxidation was determine by the measuring the level of MDA using a previously established method [18].

2.7. Statistical analysis

A CoStat computer package (version 6.4) (Co Hort software, Monterey, CA) was used to statistically analyze the data. The results are reported as mean ± S.D. A P value of ≤ 0.05 was considered as significant.

3. Results

3.1. Effect of CBE on CCl_4-induced hepatotoxicity and liver enzymes

The hepatoprotective efficacy of CBE was determined by evaluating the levels of ALT, AST, ALP, and TP in the serum samples of all experimental animals.

3.1.1. Effect of CBE on the serum levels of ALT and AST

Results indicated that ingestion of CCl_4 in Wistar albino rats caused a significant ($P < 0.05$) increase in the levels of ALT and AST in the serum specimen compared to the untreated group (Table 1). Results also indicated that treatment of CCl_4-intoxicated rats with CBE resulted in a significant ($P < 0.05$) dose-dependent down-regulation in the serum levels of ALT and AST which demonstrates the hepatoprotective potential of CBE. The decrease in the levels of ALT and AST were more pronounced in animals administered with 400 mg/kg. It was also observed that the hepatoprotective activity of CBE particularly at a dose of 400 mg/kg was comparable to Clavazin (a strong hepatoprotective agent).

3.1.2. Effect of CBE on serum levels of ALP and TP

The effect of different doses (200 and 400 mg/kg) of CBE was also determined on the levels of ALP and TP in the serum of all experimental animals and the results are shown in Table 1. Our results indicated that the exposure of animals to CCl_4 cause significant ($P < 0.05$) elevation in the ALP levels and a decrease in TP levels. The administration of CBE caused a significant ($P < 0.05$) decrease in the levels of ALP and increased in the levels of TP at a dose of 400 mg/kg. A significant decrease in the levels of ALP and increase in the levels of TP showed promising hepatoprotective potential of CBE.

3.2. Antioxidant activity of CBE against CCl_4-induced oxidative stress

The hepatoprotective activity of CBE was also evaluated by measuring its antioxidant activity by determining the levels of GSH, SOD, CAT, and MDA in the serum of all experimental animals.

3.2.1. Effect of CBE on GSH level in the serum

The resulting data indicated that level of GSH was significantly ($P < 0.05$) decreased in CCl_4-intoxicated rats compared to the untreated animals (Table 2). The administration of CBE reversed the concentration of GSH to the normal level which indicates its strong antioxidant efficacy. Results showed that the levels of GSH were significantly ($P < 0.05$) increased to (6.32 ± 0.41) mg/dL (in animals treated with 200 mg/kg) and (6.63 ± 0.38) mg/dL (in animals treated with 400 mg/kg) when treated with different concentrations of CBE compared to the CCl_4-intoxicated rats [(2.33 ± 0.35) mg/dL].

3.2.2. Effect of CBE on SOD activity in the serum

In this study, we have also evaluated the antioxidant activity of different doses of CBE (200 and 400 mg/kg) by determining the level of SOD in the serum of all experimental animals (Table 2). Results showed that intoxication of rats with CCl_4 causes significant ($P < 0.05$) down-regulation in the serum levels of SOD [(38.25 ± 2.94) mg/dL] compared to the untreated animals [(80.32 ± 4.51) mg/dL] (Table 2) which indicates substantial hepatotoxicity induced by CCl_4. However, the oral administration of CBE was resulted in increased concentrations of SOD in the CBE-treated animals. We observed a dose-dependent significant ($P < 0.05$) increase in the SOD levels in animals treated with different concentrations of CBE (Table 2). The antioxidant activity of CBE was comparable to the Clavazin.

Table 1
Effect of cranberry extract (CBE) on liver enzymes.

Tested groups	ALT (mU/mL)	AST (mU/mL)	ALP (mU/mL)	TP (mg/dL)
Untreated (normal)	28.25 ± 4.26	26.99 ± 4.66	91.52 ± 4.35	5.88 ± 0.41
CCL_4 + no treatment	125.26 ± 12.25*	101.20 ± 9.66*	125.36 ± 7.25*	3.26 ± 0.32*
CCL_4 + CBE200	39.25 ± 7.26$^\#$	36.25 ± 3.25$^\#$	102.31 ± 4.91$^\#$	5.26 ± 0.34$^\#$
CCL_4 + CBE400	35.25 ± 5.66$^\#$	31.25 ± 4.25$^\#$	91.25 ± 4.02$^\#$	5.59 ± 0.27$^\#$
CCL_4 + Clavazin	30.26 ± 3.26$^\#$	32.26 ± 3.56$^\#$	111.26 ± 5.37$^\#$	5.23 ± 0.26$^\#$

Treatment with CBE resulted in dose-dependent decreases in the serum levels of ALT, AST, and ALP and increase in the serum concentration of TP in Wistar albino rats. Results are reported as mean ± S.D. A $^*P < 0.05$ represents significant difference between untreated and CCl_4-induced group. A $^\#P < 0.05$ represents significant difference between CCl_4-induced group and CBE and Clavazin treated groups.

Table 2
Effect of cranberry extract (CBE) on antioxidant defense.

Tested groups	GSH (mg/dL)	SOD (mg/dL)	CAT (μmol/mol)	MDA (nmol/mL)
Untreated (normal)	8.01 ± 0.57	80.32 ± 4.51	34.26 ± 2.25	4.62 ± 0.51
CCL_4 + no treatment	2.33 ± 0.35*	38.25 ± 2.94*	17.12 ± 0.62*	9.62 ± 0.84*
CCL_4 + CBE200	6.63 ± 0.38#	62.26 ± 3.22#	25.36 ± 1.59#	5.95 ± 0.28#
CCL_4 + CBE400	6.32 ± 0.41#	68.26 ± 2.17#	33.24 ± 1.43#	5.32 ± 0.39#
CCL_4 + Clavazin	6.20 ± 0.32#	72.32 ± 4.81#	36.25 ± 1.31#	4.12 ± 0.18#

Treatment with cranberry extract resulted in dose-dependent increases in the serum levels of GSH, SOD, and CAT and decrease in the serum concentration of MDA in Wistar albino rats. Results are reported as mean ± S.D. A $^{*}P < 0.05$ represents significant difference between untreated and CCl_4 induced group. A $^{\#}P < 0.05$ represents significant difference between CCl_4-induced group and CBE and Clavazin treated groups.

3.2.3. Effect of CBE on CAT levels in the serum

We have also observed that the intoxication of Wistar albino rats with CCl_4 caused substantial ($P < 0.05$) decrease in the serum levels of CAT [(17.12 ± 0.62) μmol/mol] compared to the untreated rats [(34.26 ± 2.25) μmol/mol] (Table 2). However, the administration of CBE resulted in a significant ($P < 0.05$) increase in the serum levels of CAT to (25.36 ± 1.59) and (33.24 ± 1.43) μmol/mol in animals treated with 200 and 400 mg/kg, respectively. These results indicate a strong anti-oxidative potential of CBE.

3.2.4. Effect of CBE on MDA levels in the serum

In this study, we have also evaluated the serum levels of MDA in all experimental animals (Table 2). Our results indicated that the level of MDA were moderately reduced [(5.95 ± 0.28) nmol/mL] in animals treated with 200 mg/kg of CBE; however, the level of MDA was significantly decreased in the animals treated with 400 mg/kg of CBE [(5.32 ± 0.39) nmol/mL] compared to the CCl_4-intoxicated animals [(9.62 ± 0.84) nmol/mL] (Table 2). These results clearly evidenced a dose-dependent strong anti-oxidative and hepatoprotective efficacy of CBE.

4. Discussion

CCl_4 is a hepatotoxic compound causing severe liver injury. It undergoes metabolism by the action of cytochrome p450 that is present in endoplasmic reticulum of liver cells and leads to the production of unstable and complex metabolites of CCl_4 [19] which may cause hepatotoxicity. CCl_4 is activated in the presence of cytochrome p450 (CYP 2E1), and (CYP2B, CYP3A) both are marginally involved in the transformation of CCl_4 to its metabolites such as trichloromethyl ($CCl_3^{\cdot}$) free radicals that can also convert into trichloromethyl peroxy radical (CCl_3OO^{-}) in the presences of oxygen. These metabolites of CCl_4 are very reactive. CCl_3 free radical covalently binds to the biomacromolecules and $CCl_3O_2^{\cdot}$ involves in lipid peroxidation to dissolve the polyunsaturated fatty acid and change into small fragment called MDA or 4-hydroxynonenal. The reactivity of these free radicals alters the integrity or permeability of cell membrane due to oxidation of polyunsaturated fatty acid in cellular membranes. This causes leakage of liver enzymes such as ALT, AST and ALP into the blood circulation [20].

Therefore, in CCl_4-induced hepatotoxicity, the normal liver functions are affected which include substantial increase in the levels of liver enzymes such as ALT, AST, ALP, and TP [21]. The levels of these liver enzymes and TP concentration are the main biochemical markers which indicate the status of liver function. Low levels of these biomarkers are normally present in the blood; however, in case of hepatotoxicity or injury to liver, levels of these biomarkers elevated in the blood [21]. Therefore, the levels of ALT, AST, ALP and TP in the blood are directly related to the extent of the liver tissue damage [21]. However, our results indicated that treatment of CCl_4 intoxicated rats with CBE reversed the situation and resulted in a significant downregulation in the serum levels of these liver enzymes. These results clearly indicate the strong hepatoprotective activity of CBE.

The hepatic tissue destruction caused by free radicals generated by the exposure of chemical toxin (CCl_4) also cause substantial oxidative stress and produce ROS in the cellular systems [22]. The mechanism of liver injury induced by ROS is to change the liver pathology. CCl_4 are metabolized into their metabolite such as $CCl_3^{\cdot}$ and CCl_3OO^{-} free radicals in the presence of cytochrome p450 in liver and kidney [2]. These ROS bind to the macromolecules protein, carbohydrate, lipid, and DNA and cause severe oxidative stress that may lead to cell death or regeneration [23]. Lipid peroxidation is the measure of thiobarbituric acid reactive substance [24] and MDA is a direct biochemical marker of oxidative stress induced by chemicals, drugs, or external noxious injuries [24]. The attack of free radicals on polyunsaturated membrane lipids cause the production of MDA which is measured as the product of free radical injury on membrane lipid [24]. Our results indicated that the treatment of experimental animals with CBE cause significant decrease in the levels of MDA in the serum. These results indicated hepatoprotective efficacy of CBE.

To prevent cellular destruction, body adopts various physiologic anti-oxidative regulatory mechanisms. GSH is capable of preventing damage to important cellular components caused by ROS such as free radicals, peroxides, lipid peroxides, and heavy metals [25]. The intoxication with CCl_4 causes reduction in the synthesis and functioning of GSH; however, our results indicated administration of CBE provides a strong anti-oxidative defense against oxidative stress induced by ROS, even in the continuous exposure to the chemical toxins. Our results are also in line with previous study [26].

Other physiologic regulatory mechanisms to protect against oxidative stresses induced by ROS include SOD and CAT. The SOD is a group of metalloenzymes whose function appears to be protection of cells from the toxic effects of the endogenously generated superoxide radicals [27]. The CAT enzyme catalyzes the decomposition of hydrogen peroxide to water and oxygen and is a very important enzyme in protecting the cell from oxidative damage induced by ROS [28]. They are present in

the serum to protect against cellular injuries caused by oxidative stress. Our results have indicated that the intoxication of Wistar albino rats with CCl_4 causes significant downregulation in the serum levels of these anti-oxidative enzymes. However, interestingly, our results also suggested that treatment of CCl_4 intoxicated rats with CBE is resulted in significant up-regulation in the expression of these antioxidants in the serum of rats. These results clearly revealed the anti-oxidative capacity of the CBE against CCl_4-induced hepatotoxicity in Wistar albino rats.

CCl_4 is one of the most commonly used toxins to induce hepatotoxic injury. Therefore, in this study, we ingested CCl_4 orally to Wistar albino rats for the purpose of inducing hepatotoxicity. The hepatoprotective efficacy of CBE was investigated by evaluating its potential to alleviate the elevation of liver enzymes (ALT, AST, and ALP) and enhance the antioxidant defense against oxidative stress by measuring GSH, SOD, CAT, and MDA levels in the serum. We observed that CBE exhibit promising dose-dependent potential to alleviate the elevation of liver enzymes and main their homeostasis in the serum. We have also observed that the administration of CBE causes significant role in maintaining the optimistic antioxidant defense by regulating the levels of GSH, SOD, and CAT and reduced the level of MDA, which is a main indicator of hepatic tissue injury.

In conclusion, we demonstrated that daily dose of 200 or 400 mg/kg shows greater protective potential against CCl_4-induced hepatotoxicity and thus can be used as an alternative natural hepatoprotective agent.

Conflict of interest statement

Authors declare there is no conflict of interest in the present research work.

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