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Research Article

Protective effect of black grapes on cadmium induced hepatotoxicity in rats

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ABSTRACT

Cadmium is a major environmental pollutant and is known for its wide toxic manifestations. In the present investigation cadmium (5 mg/kg) was administered orally for 4 weeks to induce hepatotoxicity in rats. Tissue damage induced by cadmium was clearly shown by increased activities of serum hepatic markers-AST, ALT, ALP, total protein along with the increased level of lipid peroxidation indices-TBARS, significantly decreased levels of enzymatic antioxidants like SOD, CAT and non-enzymatic antioxidants like GSH. Statistically significant decrease in hemoglobin, RBC and WBC was observed in cadmium treated animals when compared to normal control group. Administration of Black grapes (*Vitis vinifera*) extract 400 mg/kg given by oral route significantly reversed activities of serum hepatic markers to their near-normal levels, significantly reduced lipid peroxidation, restored the levels of antioxidant defense in liver, produced improvement in hematological parameters when compared to Cd-treated rats. The present study suggested that Black grapes may be beneficial in ameliorating the cadmium induced oxidative damage in the liver of rats.

Keywords: Cadmium; Black grapes; Antioxidants; Hepatoprotection

INTRODUCTION

Cadmium (Cd) is a relatively rare element that occurs naturally in ores together with zinc, lead and copper or is emitted into the air through the process of volcanic emission. It became commercial in the 20th century due to agricultural and industrial applications [1]. Occupational exposure to cadmium, such as working with cadmium containing pigments, plastic, glass, metal alloys and electrode material in nickel - cadmium batteries, and non-occupational exposure, such as food, water and cigarette smoke induces uptake of Cd from the environment into the body through pulmonary and enteral pathways [2]. Cadmium absorbed and accumulates mainly in the kidney and liver, and then it is bound to the apoprotein metallothionein [3]. The intracellular release of cadmium is responsible for the generation of reactive oxygen species, glutathione depletion, lipid peroxidation, protein cross-liking, DNA damage, culminating ultimately in oxidant-induced cell death [4, 5] Black grapes (*Vitis vinifera*) is one of the most widely grown fruit crops in the world. Grape juice jams and raisins are also important commodities in the market of the whole world. Numerous studies focused on the health-promoting and antioxidant effects of grapes. Interest in the health benefits of Black grapes has increased due to their high phenolics contents. Most phenolics in Black grapes are located in the seeds [6]. Gallic acid, catechin and epicatechin are the main phenolics found in Black grapes seeds, while ellagic acid and myricetin are the major ones in the skins. Black grapes well known for their high levels of antioxidants and polyphenols, have also shown promise as novel antimicrobial agents [7], anti-cancer properties [8], anti-inflammatory activity [9] and antimicrobial activity against *Escherichia coli* O157:H7 [10], antiulcerative, antiarthritic, anti-viral, prevent skin aging, scavenge free radicals and inhibit UV-radiation induced peroxidation activity [11, 12].

In recent years there has been an increased interest in the application of antioxidants to medical treatment as information is available linking the development of human diseases to oxidative stress [13]. Little information is available on protective effect of Black grapes against cadmium induced hepatotoxicity. In the present study an experimental model of rats treated with Cd during four weeks as

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a model of Cd induced hepatotoxicity was used. In this model it is aimed at studying the protective effect of concurrent administration of Black grapes on Cd induced hepatic damage was assessed and if the protective effect of Black grapes is based on its antioxidant properties.

MATERIALS AND METHODS

Collection of plant material: The Black grapes were collected from the local market in Ranga Reddy District and the botanical authentication was done by Dr. Ram Chandra Reddy, Head of Botany Department, Osmania University, Hyderabad.

Preparation of the hydroalcoholic extract: The skin, seed and pulp were separated from Black grapes and were shade dried individually. The dried skin, seed and pulp were ground to powder. This dried powder was used for soxhlet extraction. Extraction was done by using the soxhlet apparatus at a temperature below 60ºC for 24 hours. Powder was extracted with 60% water and 40% methanol. The solvent was evaporated under vacuum, which gave semisolid mass (yield: 57%w/w) with respect to the dried powder. Oral suspensions containing 400mg/ml of the hydroalcoholic extract of Black grapes were prepared using saline, were given daily in the morning as a single dose, and this suspension was used for the evaluation of oxidative stress in liver of rats.

Chemicals: Cadmium chloride (CdCl2) was purchased from ICN pharmaceutical company (USA).1, 1-diphenyl, 2- picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, Mumbai. All chemicals for sensitive biochemical assays were obtained from Sigma Chemicals Co. India and Hi media Chemicals, Mumbai, India. Distilled water was used for biochemical assays. All kits were obtained from Span Diagnostics Ltd., Surat, India.

Animals: Adult male Sprague–Dawley rats (150 ± 10 g body weight) were obtained from the departmental animal facility where they were housed under standard husbandry conditions (25 ± 2 °C temp., 60–70% relative humidity and 12 h photoperiod) with standard rat feed and water *ad libitum*. Experiments were conducted in accordance with the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and experimental protocols were approved by the Institutional Animal Ethics Committee (CPCSEA/1217/2008/a).

Experimental design: The albino rats were divided into four groups of 6 rats each. Rats receiving cadmium chloride were given 5 mg/kg body wt in distilled water and given by oral route. 1/5th of the LD_{50} dose i.e. 400mg/kg of the extract was selected for evaluation of cadmium induced hepatic damage based on our previous stidies.

- Group 1: Normal control group received 0.9 % normal saline by oral route
- Group 2: Black grape extract group received 400 mg/kg by oral gavage daily for 30 days.
- Group3: Cadmium control group received cadmium chloride - 5 mg/kg, p.o. daily for 30days.
- Group 4: BGE +Cadmium group received Black grape extract 400mg/kg by oral gavage daily followed by cadmium chloride 5 mg/kg, p.o., daily for 30 days.

On completion of the experimental period, animals from all groups (group 1, 2, 3and 4) were killed on day 31 after initiation of the experiment by cervical decapitation. Blood was collected in heparinized tubes; serum was isolated to assess various biochemical variables. Tissue samples (liver) of each animal were immediately processed for the biochemical analysis. All assays were performed with freshly isolated samples. The samples were maintained at −20 °C before performing assays (not longer than 7 days).

Blood biochemical analysis: Blood samples were allowed to stand at room temperature for 30 min and serum was isolated by centrifugation at 1000 × *g* for 15 min and used for estimation of serum glutamate pyruvate transaminase, serum glutamate oxaloacetate transaminase [14], ALP [15], total protein [16].

Biochemical assays: Liver was minced and homogenized (10% w/v) in ice-cold 0.1 M sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 rpm for 15–20 min at 4°C twice to get the enzyme fraction. The supernatant was used for biochemical assays.

Lipid peroxidation (LPO): LPO was estimated colorimetrically by measuring malondialdehyde (MDA) formation as described by Nwanjo and Ojiako, 2005 [17]. In brief, 0.1 ml of homogenate was treated with 2 ml of a 1:1:1 ratio of TBA–TCA–HCl (TBA 0.37%, TCA 15%, HCl 0.25 N) and placed in water bath at 65°C for 15 min, cooled, and centrifuged at 5,000 rpm for 10 min at room temperature. The optical density of the clear supernatant was measured at 535 nm against a reference blank. The MDA formed was calculated by using the molar extinction coefficient of thiobarbituric acid reactants (TBARS;

1.56×10^5l/mole cm^{-1}). The product of LPO was expressed as nmol of MDA formed per g of tissue.

Superoxide dismutase (SOD): Hepatic SOD activity was assayed according to the method of Marklund and Marklund, 1974 [18]. For the control, 0.1 ml of 20 mM pyrogallol solution was added to 2.9 ml of Tris buffer and mixed, and reading was taken at 420 nm after 1.5 and 3.5 mins. The absorbance difference for 2 min was recorded and the concentration of pyrogallol was adjusted in such a way that the rate in change of absorbance per 2 min was approximately 0.020–0.023 optical density units. Liver extract (200 µl) was treated with 10 µl of 25% triton X-100 and kept at 4°C for 30 min. To 2.8 ml of Tris buffer, 0.1 ml of treated sample was added and mixed, and the reaction was started by adding 0.1 ml of adjusted pyrogallol solution (as for control). Reading was taken at 420 nm after 1.5 and 3.5 mins and the difference in absorbance was recorded. The enzyme activity was expressed as U/ml of extract and 1 U of enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyrogallol by 50%.

Catalase (CAT): Catalase (CAT) activity was estimated following the method of Aebi, 1993 [19]. The homogenate (100 µl) was treated with ethanol (10 µl) and placed on an ice bath for 30 min. To this, 10 µl of 25% triton X-100 was added and again kept for 30 min on ice. To 200 µl phosphate buffer (0.1 M), 50 µl of treated liver homogenate and 250 µl of 0.066 M H_2O_2 (prepared in 0.1 M phosphate buffer, pH 7.0) were added in a cuvette. The decrease in optical density was measured at 240 nm for 60 s. The molar extinction coefficient of 43.6 cm^{-1} was used to determine CAT activity. One unit of activity is equal to the moles of H_2O_2degraded/min/mg protein.

Glutathione (GSH): Reduced glutathione (GSH) was determined by the method of Ellman, 1959 [20]. In brief, 1 ml of supernatant was taken after precipitating 0.5 ml of liver homogenate with 2 ml of 5% TCA. To this, 0.5 ml of Ellman's reagent (0.0198% DTNB in 1% sodium citrate) and 3 ml of phosphate buffer (1 M, pH 8.0) was added. The color developed was read at 412 nm. Reduced GSH concentration is measured by using a drawn standard curve and was expressed as mg/g of tissue.

Hematological parameters: Hemoglobin content (Hb %), Red blood cell count and Total leukocyte count were studied for hematological investigation. Immediately after collection of blood, blood was transferred to sterile test tube containing anticoagulant at a ratio of 1: 10. The collected blood was used for different hematological parameters within two hours of collection. Blood samples were analyzed for Hemoglobin (Hb) by acid heamatin (Sahali's hemoglobinometer) method. The result was then expressed in g %. Red blood cell (RBC) and white blood cell (WBC) counts were determined using an improved Neubauer counting chamber following the procedure documented by Cheesbrough and McArthur (1976) [21].

Statistical Analysis: The experimental results were expressed as the Mean ± SEM with six rats in each group. The variation between various groups were analyzed statistically using one-way analysis of variance (ANOVA) using the Graph Pad Prism version 5.0, followed by Dunnett's multiple comparison test (DMCT). Results were considered statistically significant when $P < 0.05$.

RESULTS

Assessment of blood biochemical variables: Figure 1 displays the results of enzymatic activities of SGPT, SGOT, ALP and total protein levels in control, Black grape extract treated group, cadmium chloride-exposed group and BGE+cadmium chloride treated groups. These enzymes are normally embedded in the hepatocyte plasma membrane, mainly in the canalicular domain. The alteration in these enzymes indicates the damage to the cell. Figure 1 shows the toxicological profile of cadmium chloride on various blood biochemical variables. The activities of SGPT, SGOT and ALP were significantly increased and total protein levels were significantly decreased in cadmium chloride-exposed group when compared with the control group ($P \leq 0.001$). Treatment with Black grape extract reversed cadmium induced alterations ($P \leq 0.01$) in different blood variables significantly towards control.

Organ weight changes: Figure 2 shows the changes in the liver weight of cadmium treated rats and control. As shown in the figure, cadmium chloride induced a significant ($p < 0.01$) reduction on the average organs weight (liver). Pretreatment with Black grapes improved the organ weight significantly ($P<0.05$). However there was no significant decrease in the liver weight in black grapes extract treated group.

Determination of lipid peroxidation, Catalase, SOD and reduced glutathione contents: Table 1 shows the results of lipid peroxidation and reduced glutathione, SOD and catalase levels in all groups. Administration of cadmium chloride to rats induced an increase in lipid peroxidation with a concomitant decline in reduced glutathione, SOD and catalase levels, in liver ($P \leq 0.01$). This

confirms that cadmium chloride accentuate lipid peroxidation an indicator of tissue damage and GSH, SOD and Catalase are presumed to be an important endogenous defenses against peroxidative destruction of cellular membranes. Thus alterations in both the parameters were significantly recouped by the treatment with Black grape extract.

Effect of Black grapes on Hematological Parameters on Cadmium chloride induced hepatotoxicity : The results of the effect of Black grapes in cadmium chloride induced toxicity in rats on some hematological parameters are demonstrated in Table 2. In cadmium chloride treated group Hb content ($P \leq 0.01$), the red blood cell count ($P \leq 0.01$) and total leukocyte count ($P \leq 0.01$) was significantly decreased. While pretreatment with Black grapes produced a reversed effect on Hb content, RBC and TLC count ($P < 0.05$) when compared to Cd treated rats.

DISCUSSION

Available literature indicates that no previous studies have been done to evaluate the antioxidant capacity of Black grapes and its protective effect against cadmium intoxication. The mechanisms of cadmium-induced damage include the production of free radicals that alter mitochondrial activity and genetic information [22]. Therefore, some authors have postulated that antioxidants should be one of the important components of an effective treatment of cadmium poisoning [23].

The present study concentrates on the possible protective effect of Black grapes on oxidative damage generated by cadmium induced hepatotoxicity. Liver function tests were done for different studied groups to assess their status. Biochemical analysis was done for oxidative stress indices such as lipid peroxidase level. The activity of antioxidants was measured e.g. reduced glutathione, (GSH), superoxide dismutase (SOD) and catalase (CAT) because these antioxidants are the commonest to be affected by cadmium toxicity [24].

In this work the liver enzymes SGOT, SGPT and ALP in the Cd treated group were significantly elevated, total protein levels were significantly reduced as compared with the control group, denoting the presence of liver dysfunction [25]. In concurrent administration of Black grapes and Cd, the levels of liver enzymes activity were significantly reduced and total protein levels increased as compared with the Cd-group. This finding indicates the protective effects of Black grapes in ameliorating the hepatotoxic effect of Cd.

In the current study, lipid peroxidation level was significantly elevated in plasma, liver tissues of rats treated with cadmium compared to control group thus suggesting increased oxidative stress. These results were supported by Manca et al., (1991) [26] who reported that LPO is an early and sensitive consequence of Cd exposure. Also, Hassoun and Stohs (1996) [27] demonstrated that oxidative stress was induced following oral administration of cadmium chloride to rats. A similar data had been reported by Jurezuk et al., (2004). In addition, Elizabeth et al., (2003) [28] reported that cadmium is thought to induce lipid peroxidation and this has often been considered to be the main cause of its deleterious influence on membrane-dependent function.

In the present study the elevation in the free radicals (LPO) induced by cadmium alone was very significantly decreased in the presence of Black grapes. This means that Black grapes minimized the toxic effect of cadmium via its antioxidant activity. These results are in line with the view held by Yassa et al., (2008) [29] who confirmed the role of Black grapes as an antioxidant agent in blood.

In agreement with a previous study, the level of GSH was significantly decreased in the liver extracts of cadmium treated group compared to the control group. This decrease in GSH levels may be due to its consumption in the prevention of free radical-mediated lipid peroxidation [30, 31]. Also, GSH may be consumed in the detoxification of heavy metals [32]. Furthermore, it has been suggested that the decrease in GSH levels upon cadmium exposure might impair the degradation of lipid peroxides, thereby leading to its accumulation in the target organs [33]. In controversy to the current results, Kamiyama et al., (1995) [34] reported an increase in GSH level in liver tissue after Cd injection which could be explained as a protective mechanism.

In the present study CdCl2 exposure increased LPO levels in liver with alterations in antioxidant defenses (SOD, CAT and GSH). Thus it may be possible that oxidative stress and disturbance in antioxidant defenses were the causes for liver damage induced by CdCl2 in this experimental model.

Hematological indices were significantly reduced in occupational workers. Our results correlated with these findings, showing decreases in the RBC, WBC and hemoglobin concentrations in cadmium exposed animals. These reductions can be attributed to the combined effect of the inhibition of hemoglobin synthesis and shortened life-span of

circulating erythrocytes. On hypothesis to explain the beneficial effects of Black grapes in ameliorating biochemical parameters and hematological changes is that Black grapes may contain flavonoids, catalase and phenolic compounds including resveratrol, anthocyanins. The hepatoprotective activity may be due to these phytoconstituents present in the extract.

CONCLUSION

The present study demonstrated that Black grapes administered in combination with cadmium minimized its hazards. Black grapes can protect against oxidative stress induced by cadmium, by lowering the free radicals and increased the levels of antioxidants. Further studies are required to recommend the use of Black grapes and its therapeutic potential in human.

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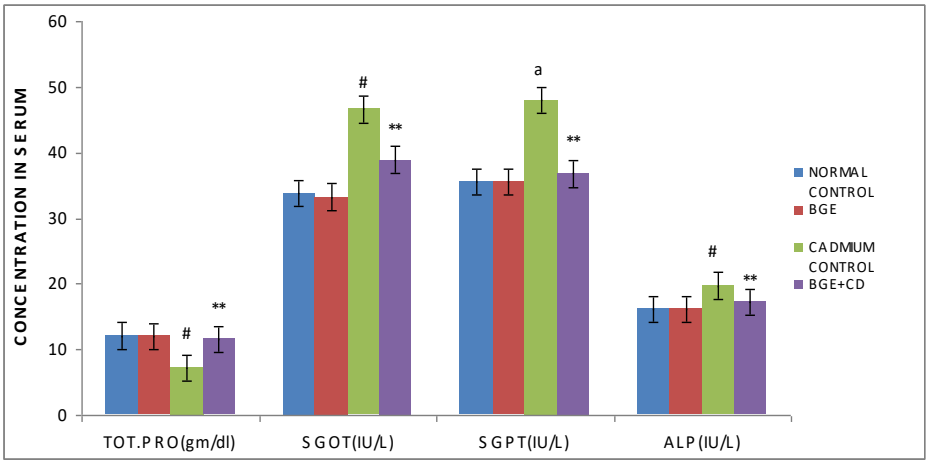

Figure 1: Effect of Black Grapes on Serum Parameters in Cadmium Induced Hepatotoxicity. Values are expressed as mean ± SEM, n=6. The intergroup variation between various groups was analyzed statistically using Dunnett's multiple comparison test. #P<0.01 vs normal control; aP<0.001 vs cadmium control; **P<0.01 vs cadmium control

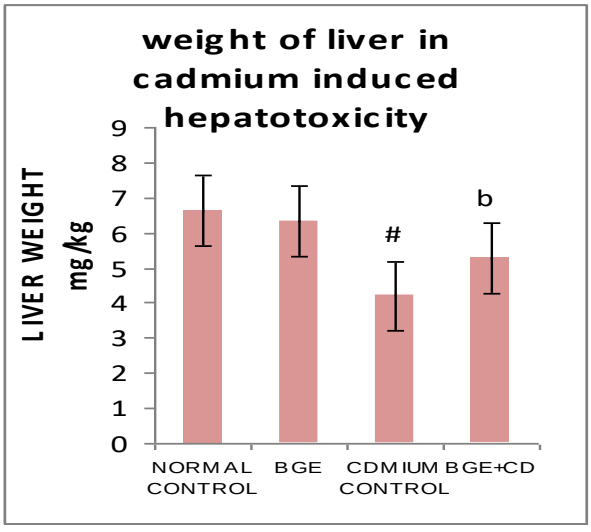

Figure 2: Effect of Black Grapes on Liver Weight in Cadmium Induced Hepatotoxicity.
Values are expressed as mean ± SEM, n=6. The intergroup variation between various groups was analyzed statistically using Dunnett's multiple comparison test. # P<0.01 vs normal control; a P<0.001 vs cadmium control; **P<0.01 vs cadmium control

Table 1: Effect of Black grapes on antioxidant parameters on cadmium induced hepatotoxicity

GROUPS	SOD IU/L	CATALASE k/min	GSH µg/ml	MDA nm/g tissue
Normal Control	16.1 ±2.03	16.16 ±1.66	15.61 ±2.41	12.49 ±1.50
BGE	16.1 ±2.22	16.1 ±2.94	15.5 ±2.56	12.41 ±2.81
Cadmium Control	12.9 # ±1.5	13.2 # ±2.97	13.4 # ±2.86	12.8 ±3.10
BGE+CD	15.1** ±2.03	15.4 ** ±3.32	15.2[a] ±3.09	11.6** ±2.44

Values are expressed as mean ± SEM, n=6. The intergroup variation between various groups was analyzed statistically using Dunnett's multiple comparison test.

# P<0.01 vs. normal control

[a] P<0.001 vs. cadmium control

** P<0.01 vs. cadmium control

Table 2: Effect of Black Grapes on Hematological Parameters on Cadmium Induced hepatotoxicity

Groups	Hemoglobin (g/dl)	RBC count (million /cmm)	WBC count (thousand/dl)
NormalControl	10.8	9.4	4,900
BGE	10.6	9.3	4,700
Cadmium control	8.4#	3.1#	2,300#
BGE+CD	9.6*	3.5*	4,700*

Values are expressed as mean ± SEM, n=6. The intergroup variation between various groups was analyzed statistically using Dunnett's multiple comparison test.

# P<0.01 vs. normal control

* P<0.05 vs. cadmium control

Abbreviations:

Fig. - Figure
i.p. - intra peritoneal
kg - kilogram
mg - milligram
ml - millilitre
p.o. - Per oral
w/w - weight/ weight
w/v - weight/ volume
BGE - Black grape extract
LD_{50} - Lethal dose
rpm - rotations per minute
GSH - Glutathione
CAT - Catalase
LPO - Lipid peroxidation.
SOD - Superoxide dismutase
MDA - malondialdehyde
SGPT – serum glutamate pyruvate transaminase
SGOT – serum glutamate oxaloacetate transaminase
ALP - alkaline phosphatase
TBARS - thiobarbituric acid reactive substances

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