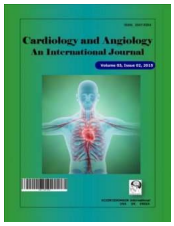

Cardiology and Angiology: An International Journal
4(3): 98-106, 2015, Article no.CA.2015.032
ISSN: 2347-520X, NLM ID: 101658392

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Antioxidant and Radical Scavenging Properties of β-Carotene on Cisplatin Induced Cardiotoxicity

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CA/2015/19092
Editor(s):
(1) Wilbert S. Aronow, University of California, College of Medicine, Irvine, USA.
Reviewers:
(1) Anonymous, Chiang Mai University, Thailand.
(2) Pratibha Ravindra Kamble, Division of Cardiac Surgery, Ohio State University Medical Centre, USA.
Complete Peer review History: http://sciencedomain.org/review-history/10338

Original Research Article

Received 25th May 2015
Accepted 3rd July 2015
Published 26th July 2015

ABSTRACT

Objective: To investigate the protective role of β-Carotene against cisplatin induced cardiotoxicity in male albino rats.
Methods: Various biochemical parameters such as Creatine kinase-MB, Lactate dehydrogenase (LDH), Alkaline phosphatase (ALP), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Triglycerides (TG) and Total cholesterol (TC) are being assessed. Also the levels of the *in vivo* antioxidants such as Reduced glutathione (GSH), Catalase (CAT), and Malondialdehyde (MDA) in the post mitochondrial supernatant of heart were measured. In addition, the histopathological studies were performed to study the protective activity of β-carotene.
Results: Cisplatin administration has shown the elevated levels of the cardiac markers and diminished the endogenous antioxidant levels when compared with the normal rats. β-carotene treatment showed the inhibitory effect on the free radicals showing decreased levels of the cardiac

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markers like CK-MB, LDH, AST, ALT and ALP. The β-carotene treated rats showed significant ($p<0.001$) decrease in lipid peroxidation in both prophylactic and curative groups when compared to the cisplatin group. Also showed a significant ($p<0.05$, $p<0.001$) increase in the levels of GSH in prophylactic and curative group respectively when compared with the cisplatin group. Both prophylactic and curative groups have shown a significant ($p<0.001$) increase in the levels of CAT. Further, the histopathological studies confirm the protective effect of β-carotene.

Conclusion: These findings justify the biological and traditional uses of β-carotene as confirmed by its promising radical scavenging activity against cisplatin induced cardiotoxicity.

Keywords: β-Carotene; cardiotoxicity; lipid peroxidation; reduced glutathione; catalase; post mitochondrial supernatant.

1. INTRODUCTION

Cisplatin (CP) is widely used and highly effective antineoplastic agent. It remains as a standard component for the treatment of head and neck tumours. It is also used to treat many solid tumours, including those of the lung, testis, ovary and breast etc. The main dose limiting side effect is nephrotoxicity which was recognized since its introduction [1].

Besides, it also causes several dose dependent adverse effects, notably neurotoxicity, hepatotoxicity, and cardiotoxicity etc [2-3]. Administration of cisplatin with other antineoplastic drugs like methotrexate, 5-fluorouracil, bleomycin and doxorubicin are associated with lethal cardiomyopathy [4].

The emergence of the cardiotoxicity which includes the changes in the cardiac events such as arrhythmias, cardiomyopathy, electro-cardiographic changes and congestive heart failure is being reported in many case reports. Though intense efforts are made over decades to find the equally potent but less toxic drug, cisplatin is widely prescribed [5-6].

Carotenoids are a group of naturally occurring fat-soluble compounds. Over 700 naturally occurring carotenoids are identified so far. β-carotene (BC) is one of the most prominent natural antioxidants, orange-coloured carbon-hydrogen carotenoid and a precursor of vitamin A [7-8]. It is an organic hydrocarbon containing compound specifically referred as a terpenoid, reflecting its derivation from isoprene units. BC is a common substance abundant in yellow-orange fruits and vegetables (eg: carrots, pumpkins and sweet potatoes etc.) and in dark green leafy vegetables and also the most widely distributed carotenoid in foods that colours them orange [9]. It is known to induce hepatic enzymes that detoxify the carcinogens in a rat model [10], has strong inhibitory effect against DMH (1,2-dimethylhydraine) induced colon tumours [11]. Moreover, BC has an ability to function as a chain breaking antioxidant in the lipid environment at partial pressures of oxygen that are more likely considered in mammalian cells [12].

The BC molecule reacts with the free radical, resulting in the formation of a new much more stable radical possibly due to the presence of a conjugated double bond system which facilitates a resonance condition [13]. It is known that β-carotene can quench singlet oxygen with a multiple higher efficiency than α-tocopherol. The *in vitro* studies have shown the potent antioxidant activity of BC [7].

Nutrition has a prominent role in the prevention of many chronic disease such as cancers, degenerative brain diseases and cardiovascular diseases. The consumption of food based antioxidants including BC seems to be useful for the prevention of cataracts and macular degeneration. Several studies have revealed the protective effect of food based BC, along with diet rich in fruits and vegetables, on liver carcinogenesis as well the lung disease [7].

Earlier literature suggests the beneficial effects of use of antioxidants such as silymarin, acetyl-L-carnitine & DL-α-lipoic acid which are proven to be potential against the cardiotoxicity associated with cisplatin in experimental animals [4]. Most endogenous compounds exhibit antioxidant functions which often act synergistically with the antioxidants supplied through the dietary origin [14].

The role of BC in the prevention of cardiotoxicity, especially by CP has not been established yet; the focus on the most important non enzymatic antioxidant activity of BC in scavenging the

oxygen free radicals released by the administration of cisplatin is being assessed. The protective effect of β-carotene on heart is studied by estimating various biochemical parameters, antioxidant parameters (on the post mitochondrial supernatant of the organ) and the histopathological findings. The rationale behind this is to study the cessation of adverse effects caused by ROS generated by the overdose of CP by using a natural antioxidant-carotenoid, β-carotene.

2. MATERIALS AND METHODS

2.1 Chemicals and Drugs

Cisplatin injection available as Cytoplatin 50 mg/50 ml manufactured by cipla is used. β-carotene was obtained from sigma aldich. The biochemical kits used are obtained from Coral, Excel and Span diagnostics. All the chemicals used were of analytical grade.

2.2 Animals and Experimental Design

Male albino Wistar rats were purchased from Teena labs, Hyderabad. The animals are subjected to acclimatization for one week provided with food and water *ad libitum*. The animals were maintained at a controlled temperature under 12hrs dark/light cycle in the animal house at CMR College of pharmacy, Hyderabad approved by CPCSEA (657/PO/a/12/CPCSEA -June, 2012).

The experimental animals weighing 200-250 g were used in the studies which were divided into five groups containing six in each. The treatment is followed according to the details mentioned in Table 1. Blood samples are collected after 2 hrs of the treatment on the last day. The blood was withdrawn by retro-orbital puncture and the animals were sacrificed by spinal dislocation [15]. The changes in body weights are noted. Olive oil is used as vehicle for administration of BC.

2.3 Biochemical Estimations

The blood samples obtained are centrifuged at 3000 g for 10 mins at 4℃ and the serum obtained from the blood was used for the evaluation of biochemical parameters such as CK-MB, LDH, AST, ALT, ALP, TG and TC.

Hearts were rapidly excised, trimmed of connective tissue and weighted, subjected to washing with ice-cold normal saline to make free from blood and were used for the preparation of post mitochondrial supernatant for *in vivo* antioxidant studies and histopathological studies. The hearts of the animals were dissected out soaked in 10% formalin solution, stained with eosin and haematoxylin. 5 µm thick sections were observed under light microscope with magnification 100x.

2.3.1 *In vivo* antioxidant studies

The tissue homogenates (10% w/v) were prepared with a Teflon homogenizer using ice-cold 1.15% KCl [16]. The homogenates were centrifuged at 800 g for 5 min at 4℃ (REMI C-24) to separate the molecular debris. The Post mitochondrial supernatant is collected after centrifuging at 10000 g for 20 mins which was used for *in vivo* antioxidant parameters like lipid peroxidation (LPO), Catalase (CAT) and reduced glutathione (GSH).

2.3.2 Estimation of lipid perioxidation (LPO)

The procedure was followed according to the modified method of [17]. The PMS of volume 0.5 ml was allowed to react with 0.5ml tris HCl buffer (pH 7.4) and incubated at 37℃ for 2 hrs followed by addition of 1ml of 10% ice cold trichloroacetic acid and centrifuged at 1000rpm for 10 mins and 1 ml supernatant was added to 1ml of 0.67% w/v thiobarbituric acid and boiled for 10 mins. After cooling 1 ml distilled water is added and the absorbance was measured at 532 nm against the blank without tissue homogenate.

Table 1. Treatment schedule

Group	Drug Treatment	Dose	Duration in days	Day of withdrawal of blood/ sacrifice	Purpose
I	Vehicle	1 ml/kg	1-7	7^{th}	Normal control
II	Cisplatin	7 mg/kg	1^{st}	5^{th}	Disease control
III	β-carotene	10 mg/kg	1-7	7^{th}	BC control
IV	β-carotene	10 mg/kg	1-7	7^{th}	Prophylactic
	Cisplatin	7 mg/kg	3^{rd}		
V	Cisplatin	7 mg/kg	1^{st}	11^{th}	Curative
	β-carotene	10mg/kg	5-11		

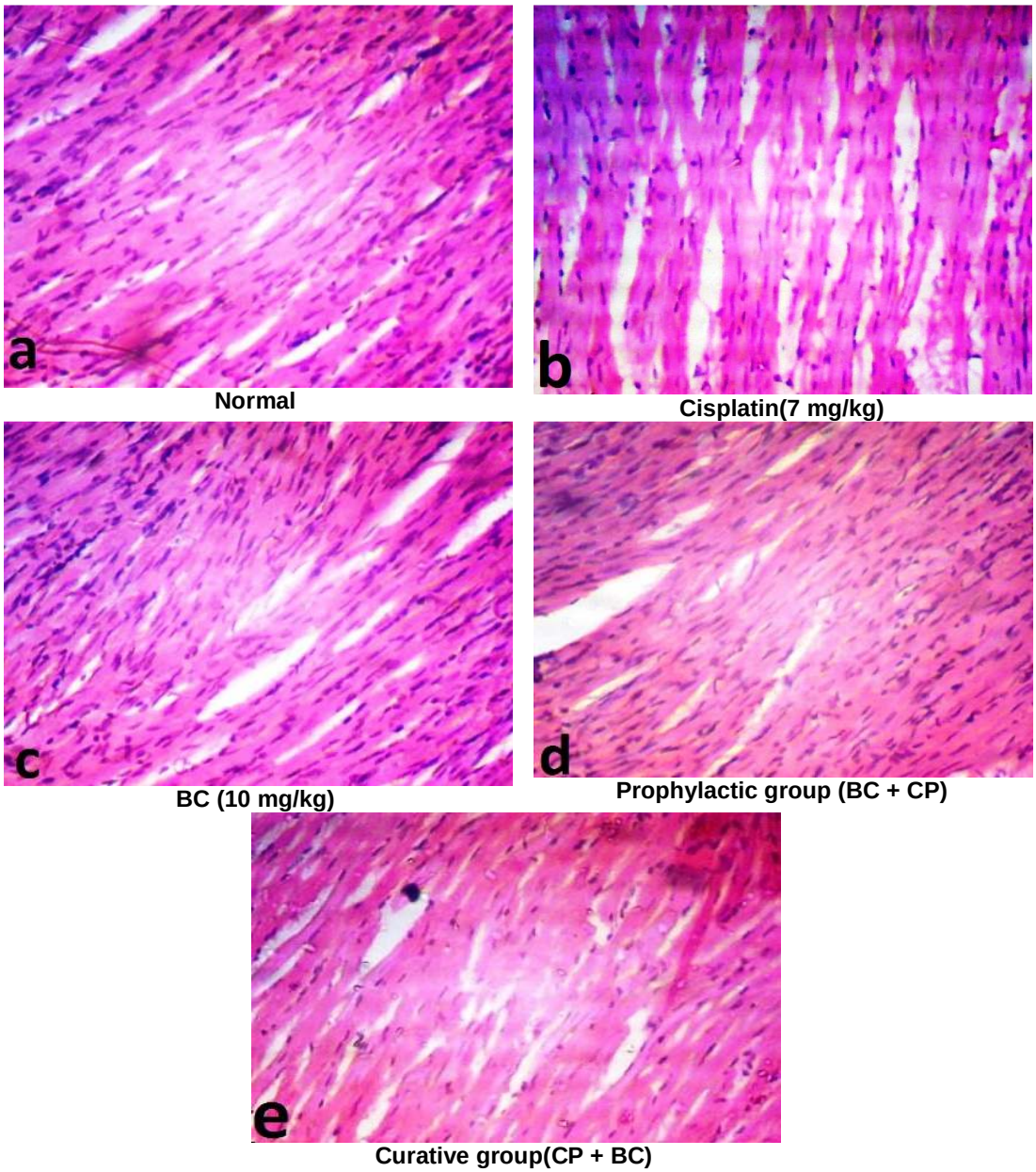

Fig. 1. Photomicrographs of histological changes of cardiac tissue under light microscopy with magnification 100X

The Cardiac damage produced by cisplatin showing the degenerative changes and necrosis of cardiac muscle fibre cells. Prophylactic (d) and curative (e) groups showing decreased myocardial necrosis.

The malondialdehyde (MDA) content, a measure of lipid peroxidation was assayed in the form of TBARS.

2.3.3 Estimation of reduced glutathione (GSH)

Modified method of [18], the PMS of volume 0.75 ml is mixed with equal volume of 4% sulfosalicylic acid and centrifuged at 1200 g for 5 mins at 4℃. 0.5 ml of supernatant was added with 4.5 ml of 0.01 M DTNB and the absorbance was measured at 412 nm against the blank without tissue homogenate.

2.3.4 Estimation of catalase (CAT)

According to the modified method of [19], the tissue homogenate in volume 0.05 ml was diluted

with 1.95 ml of 0.05 M phosphate buffer (pH 7.0) and for 2 ml of the diluted homogenate 1 ml of 0.019 M hydrogen peroxide (prepared using 0.05 M phosphate buffer) was added and immediately the absorbance was measured at 240 nm against the blank without tissue homogenate for 2 minutes with 60 seconds intervals and the average is considered.

2.3.5 Statistical analysis

The statistical analysis was carried out using one way ANOVA followed by tukey's multiple comparison test and the values were expressed as mean ± SEM. Values at $P<0.05$ are considered statistically significant.

3. RESULTS

Protective effects of BC against CP induced cardiotoxicity was established by observing the parameters such body weight, heart weight, cardiac biomarkers such as CK MB and endogenous antioxidants.

3.1 Body Weight and Heart Weight

The animals treated with CP significantly ($p<0.001$) decreased the body weight when compared to the normal rats. Also, significant ($p<0.001$) decrease in the weight of the heart when compared with the normal rats as mentioned in the Table 2.

3.2 Biochemical Estimation

The animals treated with CP have shown the elevated levels of the enzymatic markers such as CK MB, LDH, AST, ALT and ALP when compared to normal rats. It also has shown the elevated levels of Triglycerides (TG) and Total cholesterol (TC). The treatment groups both prophylactic and curative has shown significant reduction of the markers in serum as represented in the Tables no. 2 and 3.

3.3 Antioxidant Biomarkers

The elevated levels of MDA clearly indicate the myocardial damage with a significant reduction in the non-enzymatic antioxidants such as GSH and CAT. Upon treatment, both groups (prophylactic and curative) showed a significant decrease in lipid peroxidation levels and would have successfully defended in the depletion of GSH and CAT as the details mentioned in Table 4.

3.4 Histopathological Observations

The histopathological studies according to [6] [20], have shown the cardiac damage including, degenerative changes and necrosis of cardiac muscle fibre cells with fibrous tissue reaction in cisplatin group (Fig. 1). The hearts of the Prophylactic and curative groups showed decreased myocardial necrosis.

4. DISCUSSION

Chemotherapy, especially using CP declines the normal homeostasis of the body [21]. Cisplatin is still a frequently used chemotherapeutic agent due to its potential activity on carcinoma cells, despite of its frequent adverse effects. There are various outcomes to attenuate its toxicity which includes dose optimization, excess hydration therapy, use of osmotic diuretics etc though of limited usefulness [22].

During the physiological process the electrons continuously escape from respiratory chain partially reduces the molecular oxygen generating the superoxide anions, precursors of ROS which are continuously produced in mitochondria. The efficient mitochondrial antioxidant defense system maintains the balance between the generation of ROS and detoxification.

Table 2. Effect of BC on CP induced changes in body weights, heart weights and various biochemical markers

Group	Treatment	Changes in body weight (gms)	Heart Weight (mg)	Body weight/Heart weight ratio	CK MB (U/L)	LDH (U/L)
I	Normal	12.3 ±1.59	739.2±17.19	1/0.0035	667.4±29.03	408.9±17.47
II	CP	-23.67±2.51^{x}	559.3±19.23^{x}	1/0.0032	2560.0±110.00^{x}	1760.0±57.19^{x}
III	BC	19.33±2.06^{a}	774.3±10.82^{a}	1/0.0035	635.4±53.26^{a}	368.7±12.66^{a}
IV	BC + CP	-2.00±2.78^{a}	658.2±9.20^{a}	1/0.0033	705.9±59.18^{a}	586.7±36.16^{a}
V	CP + BC	-13.8±1.22^{c}	604.3±19.00^{a}	1/0.0033	1147.0±71.07^{a}	724±44.64^{a}

Values are expressed as (Mean ± SEM). ^{x} $p<0.001$, ^{y} $p<0.01$ when compared to normal group
^{a} $p<0.001$, ^{b} $p<0.01$, ^{c} $p<0.05$ when compared to cisplatin control group

Table 3. Effect of BC on CP induced changes in various biochemical markers

Group	Treatment	TG (mg/dl)	TC (mg/dl)	ALP (U/L)	AST (U/L)	ALT (U/L)
I	Normal	48.7±3.75	49.6±3.45	308.2±8.45	151.4±12.67	26.7±1.17
II	CP	68.7±3.05^{y}	71.1±3.26^{x}	650.4±12.72^{x}	271.9±15.98^{x}	78.5±5.01^{x}
III	BC	46.3±3.00^{a}	42.3±3.54^{a}	292.5±15.90^{a}	149.1±7.70^{a}	24.4±1.90^{a}
IV	BC + CP	54.6±3.53^{c}	51.3±1.63^{a}	329.0±17.89^{a}	163.8±4.14^{a}	29.3±2.28^{a}
V	CP + BC	56.5±2.36^{ns}	58.6±3.14^{c}	441.0±14.07^{a}	199.6±5.04^{a}	33.1±1.10^{a}

Values are expressed as (Mean ± SEM). $^{x}p<0.001$, $^{y}p<0.05$ compared to normal group.
$^{a}p<0.001$, $^{b}p<0.01$, $^{c}p<0.05$ when compared to cisplatin control group

Table 4. Effect of BC on CP induced changes in various antioxidant biomarkers

Group	Treatment	MDA (nmol/g)	GSH (nmol/g)	CAT (nmol/g)
I	Normal	1.1±0.05	4.2±036	16.7±0.64
II	CP	9.7±0.41^{x}	1.6 ±0.20^{x}	3.4±0.23^{x}
III	BC	1.4±0.36^{a}	4.3 ±0.36^{a}	17.3±1.52^{a}
IV	BC + CP	2.2±0.58^{a}	3.9±0.45^{a}	16.0±1.40^{a}
V	CP + BC	3.5±0.49^{a}	3.7±0.23^{b}	18.0±1.97^{a}

Values are expressed as (Mean ± SEM). ^{x} p<0.001 compared to normal group ^{a} p<0.001, ^{b} p<0.01, ^{c} p<0.05 when compared to cisplatin control group

GSH is low molecular weight endogenous radical scavenger in the cytoplasm and is one of the important inhibitors of free radical generation mediated by lipid peroxidation [20]. The reduction in the GSH and other related endogenous antioxidants by cisplatin, shifts the cellular redox status leading to the accumulation of endogenous ROS within the cells enhancing the lipid peroxidation which is another pathway for the cardiac cells damage [23,24]. The oxidative stress reflects a shift towards the imbalance of the antioxidant defense system leading to the damage of the cellular components, notably proteins, membrane lipids and nucleic acids, leading to the leakage of cardiac markers which indicate the cardiac injury [25,26].

The loss of weight is might be due to the gastrointestinal toxicity and by reduced ingestion of food [27]. The body weight/heart weight ratio is decreased in Cisplatin treated group which has increased in the BC treatment groups when compared with the toxic group which might be due to the prevention of oxidative stress induced cell death.

In the present study the administration of single intraperitoneal dose of CP (7 mg/kg) [28] imbalanced the ratio of ROS and detoxifiction by enhancing the ROS generation and depletion of the endogenous enzymatic antioxidants such as GSH, and CAT [29]. The ROS generated by cisplatin during this process triggers the mitochondrial permeability transition pore opening which permits the release of cytochrome c from mitochondria to cytosol activating the mitochondrial pathway leading to apoptosis. Also, cisplatin is converted to highly reactive form, which reacts with thiol containing compounds such as glutathione and causes its depletion [4]. In the present has shown the significant elevation of the serum cardiac markers such as CK MB, LDH, AST, ALT and ALP when compared to the normal rats [20]. CK-MB is the main marker indicating cardiac damage, the other enzymes also represent the extent of damage. Although, the endogenous enzymes are also present in various other tissues, the elevated levels of *in vivo* antioxidants such as MDA and the decline of GSH and CAT clearly indicate the cardiac damage. The ROS generated due to the administration of CP (7 mg/kg) might have caused the membrane injury indicated by the lipid peroxidation causing the loss of function and integrity of myocardial membrane leading to the leakage of the cardiac markers into the circulation. The administration of BC protected the myocytes against CP by decreasing their susceptibility to the free radicals.

BC is one the most prominent natural compound with lipophilic nature which contains an extended system of conjugated double bonds which are responsible for their antioxidant activity. The mechanisms by which BC protect biological systems, against cisplatin induced damage is associated with lowered DNA damage, decreased lipid peroxidation against oxidative damage tends to depend largely on the polyene chain in the center of the molecule which is responsible for physical quenching [30,31]. BC undergoes oxidation by the ROS, which involves

the interruption of conjugated double bond system either by cleavage or by addition to one of the double bonds of BC molecule. The possible mechanisms by which BC scavenge free radicals include either radical addition, electron transfer to the radical or the allylic hydrogen abstraction leads to the formation of stable metabolites [32].

Singlet oxygen, though it is short lived, can be intercepted by reactions occurring near diffusion control, the process of physical quenching which is the main domain for carotenoids [14]. The phenolic antioxidants like Flavanoids, phenolic acids (cinnamic acid and hydroxylated benzoic acid), tocopherols and ascorbic acid exerts their radical scavenging activity by their hydroxyl groups (combined with a conjugated double bond system) at the outer part of the molecule reacting with the radicals to form a resonance-stabilized radical [31-34].

The biological defense developed can be classified as prevention, interception and repair. The defense against the highly reactive hydroxyl radical can only be by prevention or repair because any agent capable of interception would have to be present at very high concentrations, which would be biologically intolerable simply for osmotic reasons [14]. Generally, the repair occurs via enzymatic mechanisms, which are important for the repair of DNA damage before it is fixed as mutation. Also, the reacylation is the main pathway by which the repair of phospholipids and repair or synthesis of proteins occur [14].

The increase in the levels of LPO and the decrease in the levels of GSH and CAT in the present study clearly indicate the oxidative stress induced by CP. The increased lipid peroxidation leads to the increased utilisation of GSH in the heart, as observed in our study. The prophylactic and curative groups have shown the significant reduction in lipid peroxidation levels which is indicated by the recovered levels of GSH and CAT. The leakage of the markers such as CK MB, LDH, AST, ALT and ALP from the cardiac myocytes significantly reduced possibly due to the protective effect of BC against free radicals when compared to CP group.

5. CONCLUSION

In conclusion, the results of the study suggest that the suppression of the oxidative stress by BC could be an effective strategy for the treatment of CP induced cardiomyopathy. However, further studies have to be performed to confirm the therapeutic activity of BC in the patients receiving CP chemotherapy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/10338