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Original Article

Modulation of Doxorubicin-Induced Cardiotoxicity by *Averrhoa bilimbi* extract

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ABSTRACT

Introduction: Severe cardiotoxicity limits the use of doxorubicin in cancer treatment. Doxorubicin toxicity involves the generation of reactive oxygen species (ROS) and hence several antioxidants and plant products have been tried to minimise the cardiotoxicity. The *Averrhoa bilimbi* fruits (Family: oxalidaceae) extract had good *in vitro* free radical scavenging activity in preliminary tests, further, it is to screened for its protective activity against doxorubicin induced cardiotoxicity. **Methods:** Methanolic extract of *A. bilimbi* (BM) was tested for cardioprotective activity in cell lines and in BALB/c mice. BM-extract was tested on Vero-cells and H9c2(2-1)-cardiomyoblasts for its protection from doxorubicin induced toxicities. Extract-BM was examined in doxorubicin-induced acute cardiotoxicity in BALB/c mice. The cardioprotective efficacy was tested in Swiss mice with Ehrlich Ascites Carcinoma (EAC). **Results:** BM-extract significantly protected the doxorubicin toxicities on Vero-cells and H9c2(2-1)-cardiomyoblasts. The extract-BM pretreatment controlled doxorubicin induced toxicities in terms of reduced serum CK-MB, LDH, cardiac tissue-TBARS and elevation of other enzymatic and nonenzymatic antioxidants. Extract-BM protected the body-weight loss and DNA-fragmentation in heart tissue. Further, cardioprotection was established by histopathological examination. The combination of doxorubicin with BM-extract significantly increased the mean survival time (MST) in EAC-mice. BM-extract treatment modulated the antioxidants both in heart and liver tissues of EAC-mice. For mechanistic approach, BM-extract showed *in vitro* antioxidant activity (measured by DPPH and FRAP assay) and inhibited intracellular generation of ROS and nitrite in RAW264.7 cells. **Conclusion:** The protection from doxorubicin-induced cardiotoxicity by BM-extract was attributed to its antioxidant activity and inhibition of intracellular free radical generation.

Key words: *Averrhoa bilimbi*, Cardioprotection, Antioxidant, Doxorubicin; FRAP assay.

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INTRODUCTION

Doxorubicin is a potent and widely used broad spectrum anticancer agent. But, its long term use is limited due to the multi-organ toxicity, notably severe cardiotoxicity. In cardiac cells, doxorubicin can penetrate the outer mitochondrial membrane and reduced by the NADH-dehydrogenase result in the formation of a lipophilic semiquinone. It competes with coenzyme-Q10 diverting electrons to molecular oxygen to form superoxide radicals and damage the cardiac tissues.[1]

The early effects of doxorubicin occur after a single dose which includes pericarditis-myocarditis syndrome, acute left-ventricular dysfunction and arrhythmias. Late effects include a dose-related cardiomyopathy which may progress to congestive heart failure. As the cardiotoxicity involves a free radical mechanism several antioxidants and plant products have been tested for the cardioprotective effects, but none of them proved to be a good choice.[2] This failure could be due to a lack of understanding the mechanism behind doxorubicin-induced cardiotoxicity or flaw in the animal models used to test the agents.

Dexrazoxane, an iron chelator is a FDA-approved drug available for preventing cardiotoxicity associated with doxorubicin. Dexrazoxane is a cyclic-EDTA derivative that readily penetrates cell and convert in to a ring-opened chelating agent and interferes with the iron mediated free radical generation to protect cardiac tissue from doxorubicin-cardiotoxicity.[3] Dexrazoxane is also proven to inhibit topoisomerase-II thereby decreases the damage of cardiac-tissues from extravasation of doxorubicin.[4] Despite of this drug, the cardiotoxicity has been reported in many cases and the monitoring of doxorubicin therapy is necessary.[5]

Fruits of *Averrhoa bilimbi* are commonly known as bilimbi and locally used for inflammatory conditions. Earlier reports on this plant fruits have proved the benefits in experimental diabetes,[6] hyperlipidemia[7] and in liver toxicity.[8] As a routine testing of different plant extract for their *in vitro* toxicity on Vero cells we observed the incubation with *Averrhoa bilimbi* fruit extract there was nearly 100% survival of cells at high concentrations (1 mg/ml). This finding made us to screen for its cytoprotective activity in cardiomyocytes. The experimental results established a significant modulation of cardiotoxicity induced by doxorubicin both *in vitro* and *in vivo* system.

MATERIALS AND METHODS

Preparation of extract and phytochemical tests

The fresh fruits of *Averrhoa bilimbi* were obtained from Udupi district, cut it into small slices and dried in a hot air oven at a temperature not exceeding 50˚C. The dried material was powdered by wet grinding. The powdered material (200 g) is subjected to Soxhlet extraction using methanol as solvent. The extract solution then concentrated using a Rotavapor at 40˚C under vacuum. The extract paste was then lyophilized using a freeze drier till it becomes fine powder (BM-extract). The powder was weighed and yield was calculated. The lyophilized extract was stored in a closed air tight container till it was used for *in vitro* and *in vivo*

studies. Further, the routine qualitative chemical tests for identifying the constituents present as per standard procedures.

In vitro studies

Cell lines: All the cell lines required for the study were procured from National Centre for Cell Sciences, Pune, India. The cells procured are then sub-cultured and tested for the activity as per the standard procedures.

In vitro cytotoxicity in normal cells: Sulforhodamine B (SRB) assay was performed to study the cell viability. The Vero cells (normal monkey kidney tissue cell line) were used. The graded concentration of BM was tested for its cytotoxicity on Vero cells and doxorubicin was used as positive control for cytotoxic activity.

In vitro cytotoxicity in cancer cells: HCT-116 and MCF-7 cells were used for testing the cytotoxicity on cancer cells as per the earlier literature.[9] The anticancer values were reported as IC_{50} from the graph of % cell viability against the concentration of the extract or the standard.

In vitro cytoprotective activity in Vero cells and H9c2(2-1) myocytes: Experiments were carried out as per the standard protocol.[10] Doxorubicin (1 μM) was used as agent for cytotoxicity. The cells were incubated with graded doses of BM-extract for 1 h prior to doxorubicin (1 μM) exposure. The cell viability was assessed by SRB-assay after 24 and 48 h of incubation with doxorubicin.

Effect on doxorubicin-efficacy in cancer cells (HCT-116 and MCF-7): BM was tested for its efficacy in combination with doxorubicin on cancer cells (HCT-116 and MCF-7). Experiments were carried out as per the standard protocol.[11] The cancer cells were incubated with different concentrations of BM, 1 h prior to doxorubicin (1 μM) exposure. The cell viability was assessed by SRB-assay after 48 h.

In vivo studies

Animals

Swiss albino mice and BALB/c mice were procured and acclimatized to the experimental room conditions for a period of 15 days prior to the experiments. The animals were maintained in a room with controlled humidity and temperature 23±3°C and 12/12 h dark and light cycles. The mice were fed with standard food pellets and water *ad libitum*. All the animal studies were conducted after obtaining the ethical clearance from IAEC Manipal University (IAEC/KMC/91/2012).

Acute oral toxicity study

Acute oral toxicity study was conducted as per OECD guidelines (OECD 425). Swiss albino mice were used for the study. One animal was dosed at a time and observed for a period of 48 h before dosing the next animal and a total of 5 animals were dosed. The 4 h fasted animals were dosed with 2 g/kg BM extract orally. The animals were observed intensively for the first 30, 60 min then for each 2 h. The animals were observed for any death for a period of 2 days. Four more animals were dosed and observed similarly for symptoms and lethality.

Cardioprotective effect in doxorubicin induced acute cardiotoxicity in BALB/c mice

Male BALB/c mice (30-35 g) were used for doxorubicin induced acute cardiotoxicity model. Animals were divided into 4 groups of 8 animals each group. Group-1 remains as normal control (NC) without any treatment. Group-2 (BM-control group) animals were treated with BM once a day orally (200 mg/kg) for a period of total 13 days. Group-3 mice (DOXO group) received single i.p. injection of doxorubicin 20 mg/kg considered as cardiotoxic control group. The group-4 (DOXO+BM group) animals received 9 days pre-treatment of BM (200 mg/kg, p.o.) followed by a single i.p. injection of doxorubicin (20 mg/kg) on 10th day. Extract treatment was continued till day-13. The body weight was noted every day, before and after doxorubicin injection and difference in body weight is calculated. The body weight changes documented were compared among the different treatment groups. All the animals were sacrificed 72 h after doxorubicin injection (13th day). Blood serum was separated and estimated the cardiac biochemical markers such as creatinine kinase (CK-MB) and lactate dehydrogenase (LDH) commercial autoanalyser kits.

Immediately after sacrifice heart was perfused with PBS and excised out and rinsed with PBS to make it free from blood. The excised heart was dried between the blotting papers and weighed to calculate relative heart weights.

Relative heart weight = [heart weight (g) /body weight (g)] x 100

One animal-heart from each group was transferred to 10% formalin for histopathological investigations. The heart of other animals was kept in PBS at -20°C till they processed for biochemical parameters in tissue homogenate. The antioxidant markers such as GSH, catalase and lipid peroxidation (TBARS) were determined in cardiac tissue homogenate by standard methods.[12] Further, DNA fragmentation assay was performed to study the extent of cardiac tissue damage.

Cardioprotective effect in Ehrlich Ascites Carcinoma (EAC) model

The EAC-cells originally obtained from Amala Cancer Research Center, Kerala, India, and were maintained in Swiss albino mice by serial i.p. transplantation. The ascites fluid was withdrawn and transplanted in a healthy mouse, after 15 days again the ascites fluid was withdrawn using 18 gauge needle to sterile tube. The cell count was determined by trypan blue dye exclusion assay. The cell suspension thus obtained was then diluted with sterile saline to attain a cell count of 1x 10^7 cells /ml. From the stock, 0.25 ml of cell suspension was injected in to the peritoneal cavity of animals to induce cancer.[9]

Healthy Swiss albino mice (25-30 g) divided into 5 groups of 10 animals each. The group-1 animals were not induced cancer and represented the normal-control. Group-2 mice (EAC-mice) were intraperitoneally injected 2.5x10^6 EAC-cells/ mouse. The day of injection of cells by i.p. was considered as Day-0. Group-3 animals were treated with BM (200 mg/kg p.o.) for a period of 28 days prior to EAC-injection and treatment continued further for 14 days. Group-4, EAC induced animals were injected 8 mg/kg doxorubicin i.p. in four divided doses starting from day-1. Mice in group-5 treated with BM (200 mg/kg p.o) for a period of 28 days prior to EAC-injection and treatment continued for further 14 days after EAC injection along with 8 mg/kg of doxorubicin 8 mg/kg i.p in four divided doses starting from day-1.

Each group contained a total of 10 animals of both sex in equal number and four animals were sacrificed on 14th day for blood parameters and cardiac marker CK-MB. Further, heart and liver tissue were isolated for estimation of antioxidant parameters. The other parameters assessed were total WBC count, body weight, % increase in body weight, heart weight and relative heart weight, antioxidant markers [such as GSH, catalase, nitrites, lipid peroxidation (TBARS)] and mean survival time (MST).

Free radical scavenging activity

As per the literature free radical scavenging and iron chelation are the probable mechanisms proposed for cardioprotective activity of many substances.[13] Hence the radical scavenging activity was tested for the BM-extract. Diphenyl-picryl-hydrazyl (DPPH) radical scavenging assay and ferric reducing antioxidant power (FRAP) assays were performed to determine the antioxidant activity and iron chelating ability of extract-BM.[14] Similarly, ROS and NO generation are well known marker for intracellular oxidative stress and inflammation. Hence the inhibition of in-

tracellular ROS-generation and nitrite release was studied in RAW264.7 cells as per the literature.[15]

Statistical analysis

All the data were presented as Mean ± SEM, and statistical analysis was carried out by one way ANNOVA followed by Tukey post hoc test using GraphPad Prism 6.0 (Demo Version). A value of $p<0.05$ was considered statistically significant.

RESULTS

Extraction yield and phytochemistry of BM-extract

The yield of BM-extract was 10% of dry powder weight. The preliminary phytochemical investigated for the presence of phenols, tannins and flavonoids in BM-extract.

Cytotoxicity of BM-extract

The BM-extract did not produce any toxicity on Vero cells when tested by SRB-assay. On cancer cell lines the BM-extract was cytotoxic at high IC_{50} values (871.0 ± 51.15 and 960.6 ± 49.40 µg/ml, respectively on HCT-116 and MCF-7 cells). Thus the anticancer activity of the extract was not significant compared to doxorubicin on cancer cells tested.

Protective effect of BM-extract on doxorubicin induced cytotoxicity *in vitro*

Protection in Vero cells

The activity was expressed as % cell viability. The data represented in table 1. Doxorubicin at 1 µM concentration had 63.92 ± 0.63% and to 44.28 ± 1.38% cell viability after 24 and 48 h respectively. The pre-treatment with every concentrations of BM-extract produced a significant increase in cell viability when compared to DOXO alone ($p<0.05$). The protection was dose depended but 400 µg/ml of BM-extract did not produce any significant increase in cell viability (Table 1).

Protection in H9c2(2-1)

The effect of different concentration of extract-BM on H9c2(2-1) cells in presence and absence of doxorubicin (1 µM) was determined after 24 h of incubation. The results were expressed as % cell viability and the data represented graphically (Figure 1).

When the cells are exposed to various concentration of BM for 24 h, the higher concentrations such as 250 and 500 µg/ml produced low cytotoxicity but lower concentrations did not produce any cell toxicity. The pretreatment with various concentrations of extract-BM prior to doxorubicin treatment increased the cell viability. The 62.5 and 125 µg/ml concentrations of BM showed a significant increase ($p<0.05$) in cell viability compared to DOXO. Whereas, the higher concentrations of BM also increased the viability but, they were statistically insignificant.

Effect of BM-Extract on the efficiency of doxorubicin in cancer cell lines

To test whether the extract compromising the efficiency of doxorubicin in cancer cell lines a 48 h SRB-assay was performed on the cancer cells treated with various concentrations of extract 1 h prior to exposure of 1 µM doxorubicin. The data represented in Figure 2.

The extract-BM did not show any significant increase or decrease in the cytotoxicity of doxorubicin in HCT-116 and MCF-7 cell line. But, at concentration 400 µg/ml, BM showed a significant decrease in cell viability compared to doxorubicin (Figure 2A) inferring an increase in efficiency of doxorubicin by BM.

Acute oral toxicity of BM-extract

As per OECD guideline, a limit test of single dose (2 g/kg) administered to Swiss albino mice did not produce any lethality and did not show any mortality for 14 days. Hence the extracts were confirmed to be no-lethal up to 2 g/kg dose. On the basis of promising-protecting results in cell lines and innocuous property to rodents the *in vivo* dose was arbitrarily selected as 200 mg/kg in both acute doxorubicin induced cardiotoxicity mouse model and EAC-mouse models.

Effects of BM in doxorubicin induced acute cardiotoxicity in BALB/c mice

Effect of extract BM on body weight and relative heart weight

There was a significant reduction ($p<0.05$) in the weight of mice treated with doxorubicin and pretreatment with the extract BM did not protect the body weight loss compared to normal control. Also, there was no significant difference in the weight of animals treated with extract alone when compared to normal control (Figure 3A).

The treatment with BM-extract alone did not produce any significant difference in relative heart weight compared to the normal control. Whereas, the doxorubicin significantly reduced the relative heart weight compared to normal control (Figure 3). Pretreatment with the extract BM significantly prevented the decrease in relative heart weight by the doxorubicin treatment ($P<0.05$).

Effect of BM-extract on serum CK-MB and LDH

The mice treated with doxorubicin produced significant increase in serum CK-MB when compared to normal control mice ($p<0.05$), indicating myocardial damage (Figure 4). Pretreatment with BM prevents the mice from myocardial damage by doxorubicin significantly ($p<0.05$) as indicated by decreased in serum CK-MB concentrations to half compared to DOXO control mice ($p<0.05$).

The doxorubicin significantly increased the serum LDH concentration compared to the normal mice (1006±43.2 Vs 283.9±15.39 IU/l). Upon pretreatment with the BM-extract to doxorubicin induced mice significantly reduced the concentrations of LDH in serum ($p<0.05$) indicating the protection against myocardial damage by doxorubicin.

Effect of BM-extract on cardiac tissue antioxidant system

The BM-extract did not have any deleterious effect on cardiomyocytes as indicted by the normal concentrations of tissue GSH. Though doxorubicin is a cardiotoxic substance did not produce any significant depletion of GSH, rather it was increased (Figure 5A). The extract pretreatment to doxorubicin intoxicated mice produced a significant normalization of tissue GSH ($p<0.05$).

The cardiac catalase significantly decreased upon treatment with the BM-extract and doxorubicin treatment (Figure 5B). BM-extract protected the depletion of catalase by doxorubicin which was insignificant compared to normal mice.

There was an increase in cardiac nitrites in all treatment groups when compared to normal control and especially it was significantly increased ($p<0.05$) in doxorubicin treated and BM pretreated mice (Figure 5C).

The cardiac tissue TBARS significantly increased in animals of all the groups compared to normal control ($p<0.05$). The pretreatment with extract BM could not normalize the TBARS in cardiac tissue (Figure 5D).

Effect of BM-extract on cardiac tissue DNA fragmentation

The Figure 6, represent the fragmentation pattern of DNA extracted from heart. In normal control as well as in BM-extract treated animals DNA fragmentation was comparatively less. In doxorubicin treated animal there was a significant fragmentation of DNA as indicated by the length of movement and the BM-pretreatment reduced the doxorubicin induced apoptosis and DNA fragmentation.

Histopathology of mice heart tissue

The histology of heart tissue from normal and BM-extract treated animals were found to retain the well oriented myocardial fibers (Figure 7).

Heart tissue of doxorubicin treated animals were showing wide spread myofibrillar disorganization and extensive myocardial fiber necrosis as evident from the Figure 7C. Myofibrillar disorganization was significantly protected in extract pretreated animals (Figure 7D).

Cardioprotective efficacy of BM-extract in EAC-mouse model

Effect of extract BM-extract on body weight and relative heart weight in EAC-model

The weight gain of EAC-inoculated mice was progressive during the treatment period. There was 8.25% weight gain on 7th day upon EAC-inoculation. The BM-extract treatment produced 4.33% weight gain in animals on 7th day. Treatment with doxorubicin (total dose 8 mg/kg) decreased body weight significantly on 7th day (Figure 8). The doxorubicin treated animals had a negative weight gain indicating toxicity of drug. Further, pretreatment with extract BM could not prevent the weight loss by doxorubicin in EAC-mice.

The weight gain in EAC-inoculated mice on day 14 was 28.04% and BM treated group was 16.27%. The weight gain upon treatment with BM-extract was significantly less compared to EAC-control. The difference in weight gain upon treatment with doxorubicin (0.19%) was statistically significant compared to EAC-control. This result was similar in the animals of group DOXO+BM (3.44%). But, upon treatment with BM along with doxorubicin did not produce any statistical difference when compared to doxorubicin alone (Figure 8).

The relative heart is the ratio of heart weight with body weight of the same mice. Doxorubicin treatment caused a significant reduction in relative heart weight. In EAC-mice there was a significant decrease in the relative heart weight compared to normal animal and this could be due to the increased body weight rather than cardiotoxicity (Figure 8). There was no significant difference in relative heart weight between EAC-mice and BM-extract pretreated mice. The reduction in relative heart weight of EAC-mice treated with DOXO was significant compared to EAC control. The treatment with DOXO+BM to EAC mice significantly normalised relative heart weight compared to EAC control (Figure 8).

Effect of BM-extract on blood parameters in EAC model

There was a significant increase in the WBC count in EAC-control mice and extract treated EAC-mice when compare to normal mice. The decrease in WBC count in BM treated EAC-animal was significant ($p<0.05$) compared to EAC-control. The DOXO treated as well as DOXO+BM treated EAC-mice, the WBC count remained near normal. Treatment with BM, DOXO and DOXO+BM significantly ($p<0.05$) decreased WBC count compared to EAC-control. Pretreatment with BM and the treatment with doxorubicin did not show any significant change in WBC-count (Figure 9A).

The DOXO treatment to EAC-mice significantly increased ($p<0.05$) the serum CK-MB compared to EAC-control. Upon treatment with DOXO+BM normalised the serum CK-MB significantly (Figure 9B).

Effect of BM-extract on heart antioxidants in EAC-model

The cardiac GSH significantly ($p<0.05$) increased in EAC-mice compared to normal untreated mice. Further, the GSH in BM, DOXO as well as DOXO+BM treated mice were insignificant low compare to EAC-mice (Figure 10A).

The catalase levels were found to be very low in EAC control along with all the treatment groups compared to normal control mice. The treatments, could not replenish to normal catalase in heart tissue. There was no significant difference in BM and DOXO treatment but DOXO+BM treatment significantly increased the catalase compared to EAC control (Figure 10B).

The EAC inoculation could not significantly increase nitrite in heart tissue compared to normal mice. There was a significant increase in cardiac nitrite concentration upon DOXO treatment compared to EAC-control. The DOXO+BM treatment significantly lowered the doxorubicin induced nitrite generation compared to DOXO treated mice (Figure 10C).

The growth of EAC in mice increased TBARS in heart tissue compared to normal mice. Extract-BM treatment normalised the TBARS compared to EAC-mice. The DOXO and DOXO+BM treatments also protected the lipid peroxidation (MDA) but it was not statistically significant (Figure 10D).

Effect of extract BM on liver antioxidant parameters in EAC model

The EAC-growth in mice significant decrease the liver GSH compared to normal mice. None of the treatment group has produced elevation in decreased liver-GSH (Figure 11A).

The EAC-inoculation in mice significantly decreased ($p<0.05$) liver catalase compared to normal mice. Extract-BM treatment increased liver catalase however treatment with DOXO and DOXO+BM significantly increased ($p<0.05$) liver catalase compared to EAC-control. There was no statistical difference between DOXO and DOXO+BM treatment groups.

In the liver of EAC-mice the nitrite concentration was increased with respect to the normal control and the extract-BM decreased liver nitrite concentration in compare to EAC-control. Administration of DOXO to EAC-mice increased the nitrite level significantly ($p<0.05$) compared to EAC-control and there was a significant reduction ($p<0.05$) of DOXO-induced nitrite levels upon BM treatment (Figure 11C).

The EAC-growth in mice significantly increased ($p<0.05$) lipid peroxidation in liver indicated by increased TBARS concentration. Doxorubicin treatment significantly increased the TBARS levels and the extract BM treatment and DOXO+BM treatment significantly decrease ($p<0.05$) the liver-TBARS compared to EAC-control mice. Whereas the DOXO+BM prevented lipid peroxidation and brought it to almost normal.

Effect of BM-extract on MST in EA bearing mice

The survival rate of mice after EAC-inoculation is depicted by Kaplan-Meier survival plot (Figure 12). The EAC-control group animals had MST of 19 days. The EAC-mice treated with of DOXO (8 mg/ kg) increased the MST to 24 and it is significant compared to EAC-control. Further, BM-extract pretreatment (DOXO+BM) significantly ($p<0.05$) increased the MST of EAC-mice to 39 days.

Free radical scavenging activity

DPPH radical scavenging activity by BM extract

The DPPH free radical scavenging activity of the extract-BM was determined at absorption maxima of 490 nm and the antioxidant activity was reported as IC_{50} obtaining from the graph of % radical scavenging against concentration of the extracts/standard (Figure 13).

FRAP values of BM-extract

The ferric ion reducing ability of extract was determined by FRAP assay and the values were expressed as µM of ferrous equivalent by extrapolating the absorbance in a standard plot of $FeSO_4.7H_2O$. The FRAP-values are presented in Table 2. FRAP-assay measures the total antioxidant activity of the extract. The extract-BM had dose dependent ferric reducing ability.

Inhibition of intracellular ROS and NO generation in RAW264.7 cells

Inhibition of intracellular ROS and nitrite generation was studied using RAW 264.7 cell line. These cell lines are challenged with lipopolysaccharide (LPS) which can stimulate the generation of ROS and nitrite in RAW264.7 cells. The extract BM inhibits significantly the generation of intracellular ROS and nitrite. L-NAME and curcumin were used as

Table 1: Protection of Vero cells from doxorubicin (1 µM) toxicity by BM-extract

Extract Conc. (µg/ml)	% Cell viability when exposed to doxorubicin (1 µM)*	
	BM (24 h)	BM (48 h)
0	63.92±0.63	44.28±1.39
50	75.74±3.49[a]	52.39±0.62[a]
100	77.64±1.3[a]	52.21±1.26[a]
200	77.53±2.43[a]	58.51±0.36[a]
400	74.96±3.53[a]	45.90±1.22

*All values are expressed as Mean ±SEM, [a] = p<0.05 compared without extract BM.

Table 2: FRAP-values of extract-BM and ascorbic acid (All values are Mean±SEM)

Concentration (µg/ml)	FRAP in µM of ferrous equivalent	
	Ascorbic acid	BM
2	76.59±0.59	-
3.91	150.51±0.98	1.69±0.52
7.81	287.57±3.08	3.45±0.78
15.63	546.59±8.24	5.80±0.52
31.25	851.1±6.62	13.45±0.52
62.5	-	23.45±0.52
125	-	42.47±4.42
250	-	72.67±0.85
500	-	147.57±2.97
1000	-	211.69±0.78

Table 3: Inhibition of intracellular nitrite and ROS generation by BM-extract in RAW 264.7 cells

Assay	Extract/ Standard	IC_{50} (µg/ml) Mean values
Nitrite assay	L-NAME	39.11
	BM-extract	53.71
ROS assay	Curcumin	99.35
	BM-extract	55.07

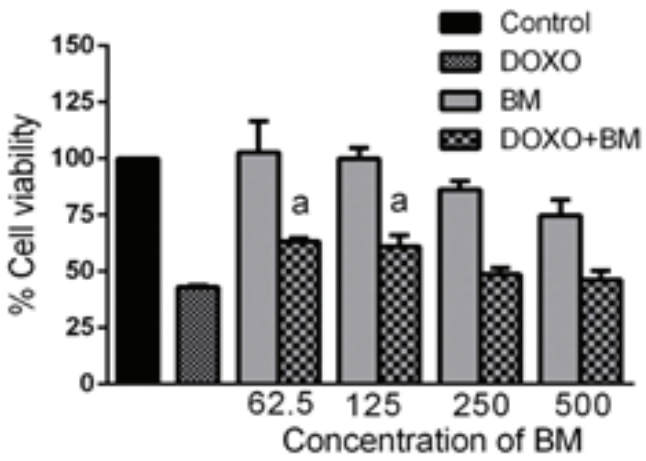

Figure 1: Protection by BM-extract on doxorubicin (1 µM) induced H9c2(2-1) cell cytotoxicity; a = p<0.05 compared to DOXO.

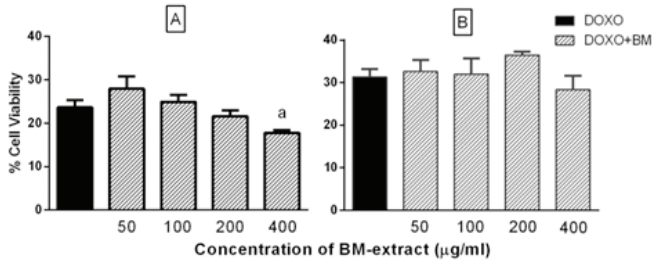

Figure 2: Effect of BM-extract on doxorubicin efficiency on [A] HCT-116, [B] MCF-7 cells; a = p<0.05 compared to DOXO.

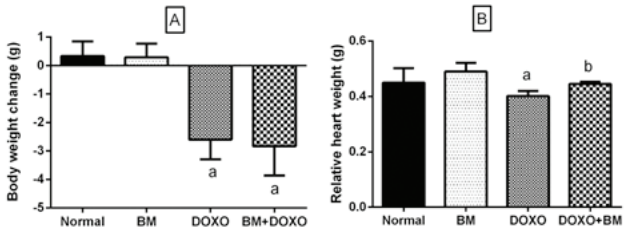

Figure 3: Effect of BM-extract on body weight (A) and relative heart weight (B); a: p<0.05 compared to normal control; b: p<0.05 compared to DOXO control.

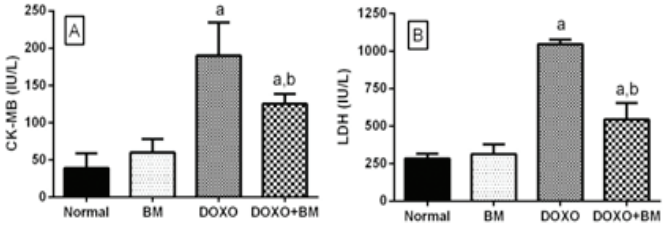

Figure 4: Effect of BM-extract on serum CK-MB (A) and LDH (B) in Doxorubicin induced acute cardiotoxicity; a :p<0.05 When compared to normal control ; b: p<0.05 compared to DOXO.

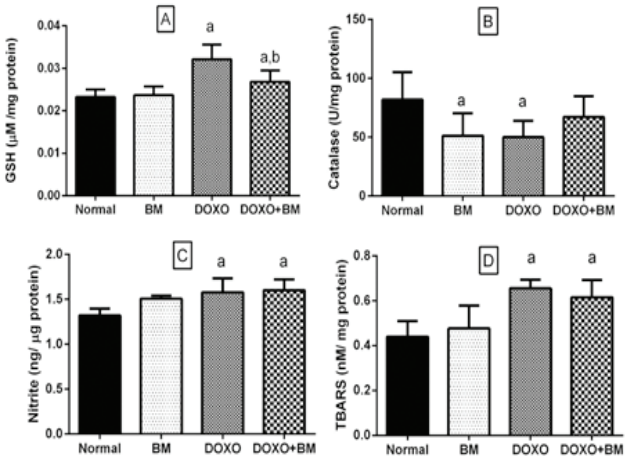

Figure 5: Effect of BM-extract on heart tissue antioxidants; a: p<0.05 compared to normal control; b: p<0.05 compared to DOXO.

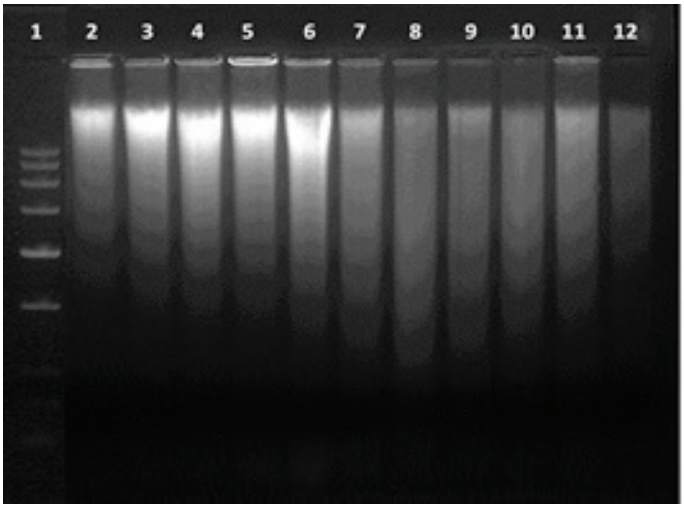

Figure 6: Effect of BM-extract on cardiac tissue DNA fragmentation; Lane 1: low range molecular weight DNA marker; Lane 2 & 3 represent Normal; 4, 5& 6, represent BM; 7, 8 & 9 represent DOXO; 10, 11 & 12 represent DOXO+BM.

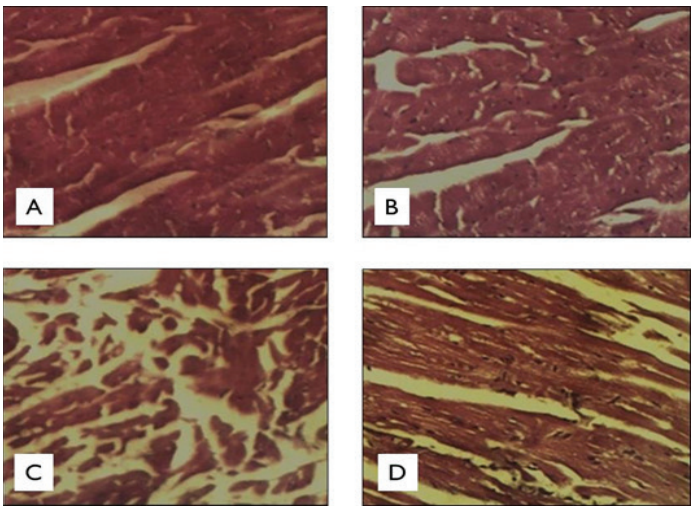

Figure 7: Sections of heart tissue showing changes in myocardium (40x); H and E staining; A: Normal control; B: BM-extract control; C: Doxorubicin treated; D: BM-extract pretreated.

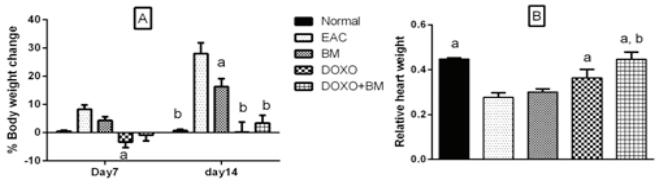

Figure 8: Effect of extract BM on body weight and relative heart weight of EAC- mice; Values are Mean± SEM; a: p<0.05 compared to EAC control on 7th day; b: p<0.05 compared to EAC control on 14th day.

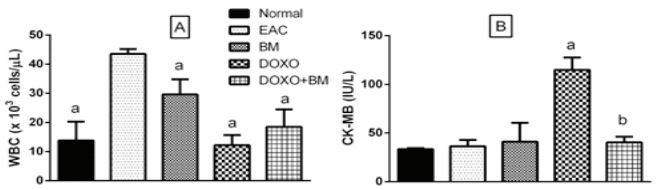

Figure 9: The blood parameters in EAC-model; [A] Total WBC count [B] Serum CK-MB; Values are Mean±SEM; a: p<0.05 compared to EAC-control; b: p<0.05 compared to DOXO.

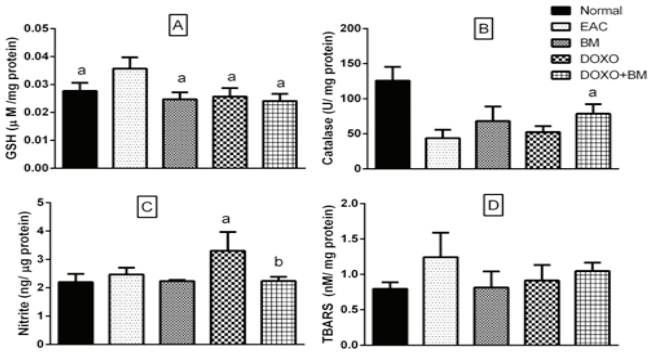

Figure 10: Effect of BM-extract on cardiac antioxidant system in EAC-model; [A] GSH; [B] Catalase; [C] Nitrite; [D] TBARS in heart tissue homogenate; a: p<0.05 compared to EAC-control; b: p<0.05 compared to DOXO.

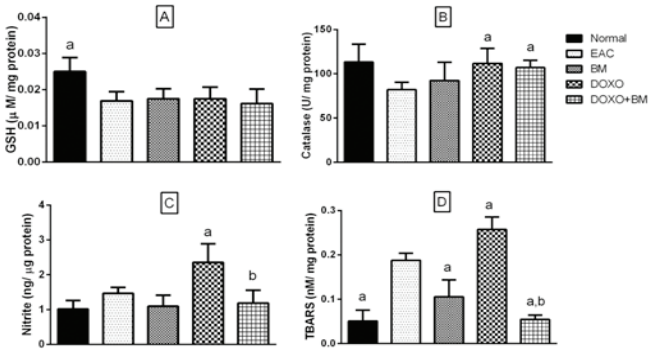

Figure 11: Effect of BM-extract on liver antioxidant system in EAC-model; [A] GSH; [B] Catalase; [C] Nitrite; [D] TBARS in liver tissue homogenate; a: p<0.05 compared to EAC-control; b: p<0.05 compared to DOXO.

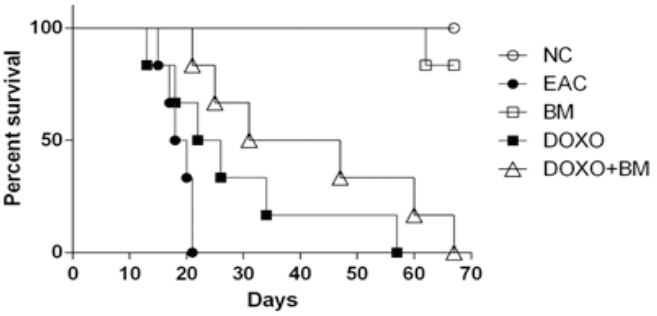

Figure 12: Kaplan-Meier survival plot of EAC- mice and treatment with BM-extract; Mean survival time (MST) was obtained by column table analysis using GraphPad Prism.

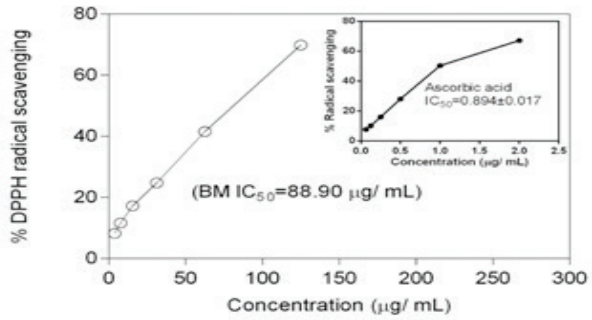

Figure 13: Percentage DPPH-radical scavenging by BM-extract and Ascorbic acid (inset picture)

internal standard for nitrite and ROS inhibition assays respectively. BM-extract showed better activity than curcumin in inhibition of intracellular ROS (Table 3).

DISCUSSION

The extract-BM was tested in Vero cell line to see its effect on normal cells which was safe up to 1000 µg/ml. The extract was safe up to the concentrations 1000 µg/ml. In Vero cells the lower concentrations of extract had 100% cell viability. This property together with its antioxidant activity makes it an ideal cytoprotective agent. Hence it was decided to analyse further for its cytoprotection in presence of doxorubicin. Followed by cardioprotective property test in cardiomyocytes H9c2(2-1) cells. The BM-extract was innocuous to H9c2(2-1) cell at 62.5 and 125 µg/ml.

BM (400 µg/ml) significantly increased the efficiency of doxorubicin on cancer cells HCT-116. This indicates that the extract BM not only protects the myocytes but also enhances the anticancer activity of doxorubicin on cancer cells. This property is an additive effect which is necessary for any cancer protective agent.

The doxorubicin administration (20 mg/kg) resulted in a significant decline in body weight of BALB/c mice. The body weight parameter was considered only for 3 days after doxorubicin administration hence, the body weight gain might not be significant upon pretreatment. The relative heart weight is an excellent indicator of the doxorubicin toxicity on heart.[16] In present study the decline in relative heart weight in doxorubicin group may be due to a loss of myocytes in response to toxicity which was protected by pretreatment of MB.

During cardiac tissue damage the CK-MB concentration releases in to blood which was observed in serum of doxorubicin treatment group[17] indicating the induction of cardiotoxicity in mice. Serum CK-MB in BM-extract treated group implies it's per say safety profile on cardiac tissue. DOXO+BM pretreated mice controlled the unwanted increase in serum CK-MB levels by doxorubicin, indicating a defence mechanism towards cardiac tissue damage by doxorubicin. Moreover, the serum LDH levels profoundly increased in doxorubicin treatment which was reversed significantly by DOXO+BM group specifies the hepato-protective effect along with cardio-protective property. In the literature, the bilimbi was proven to be a hepatoprotective in CCl_4 induced hepatotoxicity.[8] Hence, our findings support the earlier findings.

It was observed that the doxorubicin administration resulted in an up-regulation of GSH in mice indicated by an increase in cardiac GSH concentration. This might be due to an immediate response of the tissue to the toxic substance such as doxorubicin.[18] The BM pretreated animals had cardiac GSH similar to normal animal.

The doxorubicin treatment decreased the catalase levels and DOXO+BM treatment brought it towards normal. Pretreatment with BM is protecting the cardiac tissue from doxorubicin-induced oxidative-stress however BM alone fail to maintain the catalase levels after 10 days of dosing.

Nitrite levels elevate with elevation of noxious stimuli and inflammation which can be regulated by antioxidant molecules in particular cardiac tissue.[3] Doxorubicin and DOXO+BM treatment has significantly increased the nitrite level in heart tissue indicates null effect against nitrite release by BM-pretreatment may be due to minimal duration of dosing.

Doxorubicin possesses many reasons for cardiotoxicity and free radical generated toxicity may be one mechanism among them. In our previous study BM is proved to be a good inhibitor of lipid peroxidation *in vitro*[19] but, pretreatment in mice did not reflect this activity. As the doxorubicin is a highly potent cardiotoxic agent, the pretreatment with extract might not be significant to consider the factors such as dose, duration and intra species variation.

In agarose gel, the DNA fragmentation of cardiomyocytes was significantly more in doxorubicin treatment suggests its apoptotic property which was minimised by BM-pretreatment. Preventive effect of BM on cardiac tissue apoptosis by doxorubicin was supported by the heart-tissue histopathology report. Histopathological examination by H and E staining confirms the protection by BM-extract against doxorubicin induced cardiac toxicity as there was less myofibrillar damage in extract pretreated group compared to doxorubicin control.

The extract BM had shown considerable cardioprotective effect in doxorubicin induced BALB/c mouse model by comparing the serum CK-MB, LDH, GSH and relative heart weights. Further, to support its benefit in doxorubicin induced cardiotoxicity in cancer mouse model.

The body weight gain is an important parameter in EAC-mouse model which indicate the cancer growth in mice. In present study it is seen that the weight gain was gradual upon EAC inoculation as well as extract pretreated EAC-mice. The doxorubicin treated EAC-mice a rapid decline in weight in EAC-mice. This decline of body weight could also be due to toxicity of the doxorubicin. The extract-BM along with doxorubicin treatment in mice normalizes the weigh to near normal. The EAC inoculation in mice disturbs the different organ's physiology and changes the blood parameters which can be used as an indicator/ biomarker of the cancer induction. Following the EAC inoculation body tries to reject the cell by activation of immune system and there will be an increase in total WBC count.[9] In the present study the EAC control mice showed a 4 folds increase in the total WBC count and the extract treatment prevented the rise in total WBC count significantly. The doxorubicin treatment to EAC mice did not increase the total WBC indicating the prevention of cancer development. Similarly, the extract-BM treatment in concurrent with doxorubicin did not affect the total WBC count.

There was an increase in the serum CK-MB levels in doxorubicin treated EAC mice due to cardiac damage. The extract treatment normalizes CK-MB indicating the protection offered by the extract against doxorubicin induced cardiotoxicity. The mice showed a body weight decline after the doxorubicin treatment and similarly there lowering of relative heart weight because of toxicity. The relative heart weights were similar to normal animals in extract treatment along with doxorubicin in EAC mice indicating the normalising effect.

The increased heart GSH levels in EAC-mice was maintained towards normal by DOXO+BM BM and DOXO alone shows its complete antioxidant property. The huge decline in catalase by EAC-induction was not recovered by any of the treatment but DOXO+BM was less inefficient than DOXO treatment.

Doxorubicin treatment increased the nitrite levels in cardiac tissue of EAC model may be due to its noxious property whereas EAC itself failed.

DOXO+BM and BM maintained the normal nitrite levels proving its protective property against doxorubicin induced oxidative stress. The heart lipid peroxidation levels increased followed by EAC inoculation. The extract treatment meaningfully reduced the increase in lipid peroxidation suggesting the safety property against lipid peroxidation.

Further, studies are carried out to determine the benefits of BM on liver biomarkers/ parameters in EAC mouse models as the doxorubicin also produces toxicity to other organs such as liver. The catalase, nitrite and TBARS levels in liver tissue of EAC-mice was brought to normal level by DOXO+BM compare to DOXO treatment suggesting the antioxidant property better than doxorubicin. The rejuvenating effects and the absence of doxorubicin-induced nocuous effects in BM as well as DOXO+BM group justifies the defensive quality in cancer and normal mice.

The liver witnessed an increase in lipid peroxidation after EAC inoculation and the extract pre and post treatment prevented this increase. The doxorubicin treatment also increased the lipid peroxidation in liver of EAC mice could be because of it free radical mediated liver toxicity. The extract pretreatment prevented the doxorubicin as well as EAC associated lipid peroxidation as evident from near normal values of TBARS in liver.

The mean survival time (MST) is the most important parameter in EAC mouse cancer model. After 42 days of extract treatment to the EAC mice did not increase the mean survival time though there was a decreased in weight gain and WBC count. The extract BM treatment also improved EAC associated decline in antioxidant levels. These effects can be correlated to the free radical scavenging activity of BM. The extract BM along with doxorubicin treatment increased the MST almost 2 fold. This increase is obviously due to prevention of doxorubicin associated toxicity rather than the anticancer effect of the extract.

Earlier this plant fruits extract was reported for its beneficial use in experimental diabetes[6] and dislipidemia.[7] Both diabetes and dislipidemia are the cause for metabolic syndrome.[20] In this aspect if bilimbi fruits protect cardiomyocytes it could be beneficial in diabetic related cardiomyopathy and dislipidemia.

Presently there is only one drug dexrazoxane, approved by FDA for cardioprotective activity in doxorubicin chemotherapy. The mechanism of action of this drug is not clearly understood, but it was reported to act via chelation of iron and thereby preventing the oxidative damage in myocytes. The BM-extract also had iron chelating action determined by FRAP assay. The FRAP values of plant extract have been compared to the cardioprotection activity in literature. The BM-extract (250 mg) had FRAP values 72.67 µM equivalent to ferrous-iron which is significant for cardioprotection compare to the literature data.[13] Also it is a good antioxidant when tested by DPPH assay. In RAW264.7 cells, BM-extract could inhibit the generation of intracellular ROS at much lesser dose than curcumin. This might be because of high cell penetration by BM-extract compared to curcumin as curcumin is highly insoluble substance and hence low penetration.[21] Further, this probable mechanism could generate new area of investigation in future.

CONCLUSION

The fruits of *A. bilimbi* have potential cardioprotective activity both *in vitro* and *in vivo* models. The strong antioxidant and iron chelating activity could be one of the mechanisms as evident from our *in vitro* studies could be the mechanism of cardio-protection. Further studies are necessary to identify the active constituents present in the extract which offering the antioxidant and cardioprotection activity.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

ABBREVIATIONS USED

BM-extract: Methanolic extract of Averrhoa bilimbi; **ROS:** Reactive oxygen species; **NO:** Nitric oxide; **EAC:** Ehrlich Ascites Carcinoma; **MST:** mean survival time; **CK-MB:** creatinine kinase isoenzyme, **LDH:** lactate dehydrogenase; **GSH:** Glutathione; **TBARS:** Thiobarbituric acid reactive substances; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **FRAP:** ferric reducing antioxidant power; **FDA:** Food and Drug Administration; **EDTA:** Ethylenediaminetetraacetic acid; **SRB:** Sulforhodamine B; **PBS:** Phosphate buffer saline; **DOXO:** Doxorubicin; **OECD:** Organisation of economic cooperation and development; **LPS:** lipopolysaccharide; **L-NAME:** Nω-Nitro-L-arginine methyl ester; **CCl4:** Carbon tetrachloride.

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