Amelioration of doxorubicin induced cardio-and hepato-toxicity by carotenoids

ABSTRACT
Aim of Study: The aim of this study is to explore the ability of the carotenoids (CARs) to offer protection against acute cardiotoxicity and hepatotoxicity induced by doxorubicin (DOX) (25 mg/kg) in tumor bearing Swiss albino mice.

Materials and Methods: Tumor bearing Swiss albino mice administered with DOX (25 mg/kg, i.p) and two doses of CARs (50 and 100 µg/kg). 24 h after administration of the drugs, histopathological evaluation of tumor, liver and heart tissues carried out. Furthermore, various antioxidant parameters in these tissues were investigated. Serum marker enzymes for tissue injury were examined.

Results: Administration of CARs prevented the depletion of antioxidants in the heart and liver, thereby protecting the tissue damage and release of marker enzymes. However, similar antioxidant depletion was not observed in the tumor tissue. CARs prevented DOX induced variation in tissue architecture in heart and liver tissues. However, CARs did not influence DOX induced alterations in the tumor.

Conclusion: Administration of CARs could prevent DOX induced acute toxicity to heart and liver.

KEY WORDS: Antioxidant status, cardiotoxicity, carotenoids, doxorubicin, hepatotoxicity

INTRODUCTION
The anthracycline drug, doxorubicin (DOX) is widely used to treat leukemias and carcinomas of breast, lungs, thyroid and ovary. However, systemic toxicity at clinically relevant doses limits its therapeutic application to some extent. The induction of toxicity is mediated by free radical generation and resultant oxidative stress through two main pathways: A non-enzymatic pathway that utilizes iron and an enzymatic mechanism using the mitochondrial respiratory chain. The deleterious consequences from oxidative stress on free radical induced damage can be mitigated by antioxidants.

Fruits and vegetables are rich sources of antioxidant compounds, which protective against a variety of diseases, particularly cardiovascular diseases and epithelial cancers. The defensive effects of natural antioxidants in fruits and vegetables are related to three major groups: Vitamins, phenolics and carotenoids (CARs). Ascorbic acid and phenolics are hydrophilic antioxidants while CARs are lipophilic antioxidants. CARs constitute a large group of naturally-occurring pigments found in plants, algae and various microorganisms and have polyisoprenoid structure with a long conjugated double bond system forming the backbone of the molecule, which may be terminated by cyclic end groups that contain oxygen-bearing substitutes.

The electron-rich conjugated system of the polyene could be responsible for the antioxidant activities of the CARs.

The present study aims to examine the ability of CARs to mitigate DOX-induced cardio and hepatotoxicity in tumor bearing Swiss albino mice.

MATERIALS AND METHODS

Chemicals
CARs was obtained as a gift for research to Cherupally Krishnan Krishnan Nair by Parry India. 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium, 2,4,6-tripyridyl-s-triazine (TPTZ), riboflavin, reduced glutathione (GSH), 5,5’ dithiobis-2-nitro benzoic acid were purchased from Sigma Chemical Company Inc., St. Louis, MO, USA. DOX of a reputed manufacturer was purchased from the local chemist. All other chemicals and reagents used in this study were of analytical grade procured from reputed Indian manufacturers.

Animals
Male Swiss albino mice, 6-8-week-old (body weight 25-30 g), were kept under standard conditions of temperature (25 ± 5°C) and humidity in the Centre’s Animal House Facility. The animals were provided with standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water ad libitum. All
experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee and were conducted strictly adhering to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals constituted by the Animal Welfare Division of Government of India.

Determination of antioxidant activity
The DPPH radical scavenging\(^{[13]}\) and ferric reducing antioxidant power (FRAP) assay\(^{[14]}\) were used to evaluate the antioxidant properties of CARs.

Administration of drugs
DOX was administered intraperitoneally as a single dose of 25 mg/kg. CARs dissolved in 0.5% dimethyl sulfoxide was orally administered to animals at doses of 50 µg/kg and 100 µg/kg.

Solid tumor in animals
Solid tumor was developed in hind limbs of mice by transplanting Dalton’s Lymphoma Ascites (DLA) cells (1 × 10\(^6\) cells/animal) through subcutaneous injection. The animals were provided with standard mouse chow and water ad libitum. The treatments were started when the tumor size reached approximately 1.0 cm\(^3\) on the 13\(^{th}\) day of transplantation of DLA cells. The animals were randomly divided into five groups of five each and treated as follows.

Group-I: Untreated control group (no treatments)
Group-II: DOX control, DOX 25 mg/kg
Group-III: CARs 100 µg/kg
Group-IV: DOX 25 mg/kg + CARs 50 µg/kg
Group-V: DOX 25 mg/kg + CARs 100 µg/kg

CARs was administered 1 h after DOX administration.

After 24 h of treatment, the animals were sacrificed, blood was collected and serum was separated for the biochemical investigations. Heart, liver and tumor tissue was removed for investigations on oxidative stress (antioxidant profiles) and histopathological alterations.

Assessment of cardiac and hepatic marker enzymes and antioxidants
Biochemical parameters such as creatine kinase (CK-MB)\(^{[15]}\), lactate dehydrogenase (LDH)\(^{[16]}\), glutamate oxaloacetate transaminase (GOT)\(^{[17]}\), glutamate pyruvate transaminase (GPT)\(^{[17]}\) and alkaline phosphatase (ALP)\(^{[18]}\) were carried out.

The tissue homogenates (10% w/v) prepared in phosphate-buffered saline (containing 137 mM NaCl, 2.68 mM KCl, 10.14 mM Na\(_2\)HPO\(_4\) and 1.76 mM KH\(_2\)PO\(_4\)) were used for assessing levels of membrane lipid peroxidation\(^{[19]}\) and antioxidant status of superoxide dismutase (SOD)\(^{[20]}\), glutathione peroxidase (GPx)\(^{[21]}\), GSH levels\(^{[22]}\) and catalase (CAT)\(^{[23]}\).

Histopathological studies
For histopathological studies, tissues were fixed in 10% formalin and embedded in paraffin wax. Sections of 5 micron thickness were made using a microtome and stained with H and E and observed under the microscope. Photographs of each of the slides were taken at ×40 magnification.

Statistical analysis
The results were presented as mean ± standard deviation of the studied group. Statistical analysis of the results was performed using ANOVA with Tukey-Kramer multiple comparisons test.

RESULTS
Data presented in Figure 1a show that CARs reduce DPPH in a concentration dependent manner. FRAP estimate, the power to reduce the TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. From Figure 1b showed CARs reduces TPTZ-Fe (III) complex in a concentration dependent manner.

As evidence in data in Table 1, the antioxidant levels in cardiac and hepatic tissues were significantly lowered
upon administration of DOX. The marker enzymes for cardiac injury CK-MB, LDH and those for hepatic damage - GOT, GPT, ALP - were considerably elevated as can be realized from Table 2. Malondialdehyde (MDA) levels in heart and liver tissues of animals administered with DOX were found to be significantly higher [Figure 2a and b]. All these parameters showed tissue damage in heart and liver that were verified with histopathological slides showed in Figure 3a and b.

Treatments of animals with CARs following administration of DOX effectively protected the level of antioxidant from DOX induced depletion [Table 1]. CARs prevented the elevation of tissue injury marker enzymes in the hepatic and cardiac tissue following insult by DOX [Table 2]. In addition to these, it was observed that DOX induced peroxidative damage to membrane lipids in cardiac and hepatic tissue were also prevented by the CARs [Figure 2a and b], that can be proved by histopathological examinations in Figure 3a and b.

### Table 1: Effect of administration of CAR on doxorubicin induced depletion of, SOD, GSH, GPx and CAT levels in heart, liver and tumor tissues of mice. Values are expressed as mean±SD (n=5)

| Tissue | Treatment               | SOD (Unit/mg prn) | GSH (n mole/prn) | GPx (Unit/mg prn) | CAT (Unit/mg prn) |
|--------|-------------------------|-------------------|-----------------|------------------|------------------|
| Heart  | Untreated               | 10.83±1.06        | 37.23±0.98      | 22.83±5.23       | 10.64±0.44       |
|        | CARs (100 µg/kg)        | 10.28±1.54        | 38.49±1.3       | 21.28±4.37       | 9.86±0.95        |
|        | DOX (25 mg/kg)          | 3.19±0.41         | 27.62±0.96      | 14.61±4.31       | 6.63±0.11        |
|        | DOX (25 mg/kg)+CARs 50 µg/kg | 7.66±0.60*** | 31.37±1.18***  | 16.94±3.32*      | 9.06±0.12***     |
|        | DOX (25 mg/kg)+CARs 100 µg/kg | 9.92±0.52*** | 31.81±1.24***  | 18.58±3.12***    | 9.55±0.47***     |
| Liver  | Untreated               | 13.01±1.34        | 25.89±1.47      | 23.48±1.68       | 2.49±0.35        |
|        | CARs (100 µg/kg)        | 12.47±1.12        | 27.15±2.21      | 23.94±1.45       | 2.37±0.27        |
|        | DOX (25 mg/kg)          | 4.75±0.39         | 21.01±0.50      | 13.55±1.06       | 0.56±0.09        |
|        | DOX (25 mg/kg)+CARs 50 µg/kg | 4.92±0.34         | 25.20±0.73***  | 21.11±2.36***    | 1.12±0.12**      |
|        | DOX (25 mg/kg)+CARs 100 µg/kg | 7.24±0.69*** | 25.59±0.56***  | 22.71±2.02***    | 1.52±0.17***     |
| Tumor  | Untreated               | 4.01±0.33         | 19.73±0.72      | 27.83±6.70       | 10.64±0.45       |
|        | CARs (100 µg/kg)        | 3.99±0.15         | 19.46±0.49      | 28.26±5.43       | 10.71±0.32       |
|        | DOX (25 mg/kg)          | 2.78±0.54         | 17.46±0.40      | 22.23±5.63       | 6.36±0.97        |
|        | DOX (25 mg/kg)+CARs 50 µg/kg | 1.68±0.36*** | 15.14±0.55***  | 17.73±4.75**     | 5.53±0.23***     |
|        | DOX (25 mg/kg)+CARs 100 µg/kg | 1.51±0.33*** | 14.82±0.54***  | 15.11±6.69***    | 4.66±0.71***     |

*P<0.05, **P<0.01 and ***P<0.001 when compared with respective controls. CAR=Carotenoids, DOX=Doxorubicin, SOD=Superoxide dismutase, GSH=Glutathione, GPx=Glutathione peroxidase, CAT=Catalase

### Table 2: Effect of CAR on serum marker enzymes in DOX induced toxicity in heart and liver of mice. Values are expressed as mean±SD (n=5)

| Treatments               | LDH (U/dL) | CKMB (U/L) | SGOT (U/L) | SGPT (U/L) | ALP (U/L) |
|--------------------------|------------|------------|------------|------------|-----------|
| Untreated                | 1998±10    | 96±3.5     | 152.1±23.2 | 38.32±7.34 | 65.77±7.35 |
| CARs (100 µg/kg)         | 1975±28    | 113±4.2    | 149.8±17.3 | 37.67±6.27 | 52.64±8.50 |
| DOX (25 mg/kg)           | 2929±36    | 183±6.2    | 204.3±12.2 | 75.94±7.27 | 105.7±11.13 |
| DOX (25 mg/kg)+CARs 50 µg/kg | 2633±46*** | 163±6.2**  | 184.6±11.6 | 44.25±8.01*** | 92.95±4.1  |
| DOX (25 mg/kg)+CARs 100 µg/kg | 2432±40*** | 124±13.8*** | 163.9±18.02** | 39.43±5.44*** | 80.8±3.2*** |

***P<0.01 and ****P<0.001 when compared with respective controls. CAR=Carotenoids, DOX=Doxorubicin, LDH=Lactate dehydrogenase, CKMB=Creatine kinase MB, SGOT=Serum Glutamate oxaloacetate transaminase, SGPT=Serum Glutamate pyruvate transaminase, ALP=Alkaline phosphatase
In DOX alone treated tumor tissues, antioxidant enzymes such as GPx, SOD, CAT and GSH activities were reduced [Table 1] and the MDA levels were increased [Figure 2c]. Post-administration of CARs along with DOX in tumor tissue significantly reduced the antioxidant enzymes such as GPx, SOD, CAT and GSH activities and also effectively potentiated the formation of MDA in a significant manner [Figure 2c]. Histopathological slides [Figure 3c] of tumor tissues showed CARs potentiates the cytotoxic action of DOX in tumor cells.

DISCUSSION

The antineoplastic agent DOX, causes severe dose dependent cardio and hepatotoxicity mediated through oxidative injury. Fruits and vegetables are good sources of antioxidant phytochemicals like CARs, which can mitigate oxidative stress induced tissue injury. Antioxidant phytochemicals reported to prevent the risk of human diseases. Use of antioxidant during chemotherapy could be advantageous to normal tissue. Here we proved that oral administration of CARs along with DOX prevented hepato and cardio toxicity induced by drug, without affecting tumoricidal activity of the drug.

Administration of DOX significantly reduced the liver, cardiac GSH level besides lowering activities of SOD, CAT, GPx. There was increased MDA in the liver and cardiac tissues in DOX treated group compared with the untreated control. Increased reactive oxygen species leads to a lipid radical chain, which could cause peroxidative damage to the cell membrane, manifested in increased MDA levels. Oral administration of CARs at doses of 50 µg/kg and 100 µg/kg inhibited DOX induced lipid peroxidation as measured as by MDA levels in tissues and also significantly increase levels of antioxidant enzymes SOD, CAT, GPx in addition there was the increase in GSH status.

Markers for cardiac and hepatic tissue injury were elevated in DOX treated group of animals. Administration of CARs following DOX treatment decreased levels of LDH in a dose dependent manner. Hepatic function was assessed in terms of serum enzymes levels, serum GPT, serum GOT, ALP. During liver damage or hepatopathy, these enzymes were increased or elevated in serum. These enzymes leaked to bloodstream due to peroxidative damage to the cell membrane of liver. Administration of CARs prevented DOX induced hepatic dysfunction by protecting the cell membrane from peroxidative damage. Histopathological evidences also confirmed protection of cardiac and liver tissues from DOX induced injury by CARs. Prevention of DOX induced toxicity to cardiac and liver tissue by CARs, correlated with the scavenging activity of the free radicals induced DOX by CARs.

CARs increase MDA levels of tumor tissues treated with CARs and DOX apart from significantly depleting the
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antioxidant levels. Histopathological examination also reveals cytotoxic action of DOX in tumor tissue was not interfered by CARs.

The present study revealed mitigation of DOX induced cardio and hepatotoxicity by CARs without affecting its tumoricidal activity. Elucidation of the mechanisms underlying preferential protection of normal tissues, while sparing the tumor by CARs needs further investigations.

ACKNOWLEDGMENT

We thank BRNS, Department of Atomic Energy, Government of India for the research Grant to CKK and JRF fellowship to IR.

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Cite this article as: Indu R, Azhar TS, Nair A, Nair CK. Amelioration of doxorubicin induced cardio-and hepato-toxicity by carotenoids. J Can Res Ther 2014;10:62-7.

Source of Support: Nil, Conflict of Interest: None declared.
