Pharmacogn. Mag.
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ORIGINAL ARTICLE

Amelioration of Doxorubicin-Induced Cardiotoxicity by Oral Lyophilized *Dunaliella salina* Supplement in a Female Wistar Rat Model with Active Estrous Cycle and Surgical Menopause

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Submitted: 29-Feb-2020 Revised: 28-Mar-2020 Accepted: 21-Apr-2020 Published: 28-Aug-2020

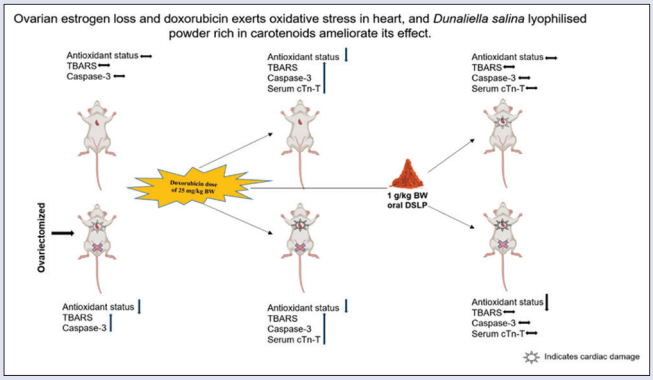
ABSTRACT

Background: Whole-cell *Dunaliella salina* is effective in preventing doxorubicin (DOX)-induced cardiotoxicity in female Wistar rats with surgical menopause. **Materials and Methods:** In our experiment, we surgically induced menopause in a group of Wistar rats and given doses of DOX and/or *D. salina* lyophilized powder and compared its effects with the group of rats having a normal estrous cycle. A cumulative dose of 25 mg/kg BW of DOX was given to induce cardiotoxicity and was countered by 1000 mg/kg BW of *D. salina* oral supplement or 10 mg/kg BW of tamoxifen. Totally two groups of rats were supplemented with *D. salina*, in one of the groups it was given either a week before DOX initiation, and in the other group, it was given alongside DOX doses. **Results:** The concomitant *D. salina* supplement has been found to reduce the cardiac damage due to DOX better than the groups that received the supplement beforehand DOX initiation or tamoxifen. Besides, the supplement was also found to maintain nitroso-redox balance, thus preventing cardiomyocyte death as compared with the rats that received DOX alone. It was evident from the histopathological examination and serum levels of cardiac troponin-T. **Conclusion:** Thus, oral carotenoid supplementation can be a promising functional food over tamoxifen, which would help in recovering from menopause and prevent anthracycline cardiotoxicity.

Key words: Cardiotoxicity, carotenoids, doxorubicin, *Dunaliella salina*, estrogen-menopause, functional food

SUMMARY

- Cardiotoxicity and surgical menopause were induced in rats by 25 mg/kg BW cumulative dose of doxorubicin and ovariectomy, respectively
- Rats were supplemented with 1 g/kg BW of *Dunaliella salina* lyophilized powder for three days a week
- *D. salina* supplementation helped to reduce cardiac damage, which was evident by cardiac troponin-T levels and histopathology. It prevented oxidative stress due to sudden estrogen loss, which was evident from various antioxidant parameters.



Abbreviations used: DSLP: *Dunaliella salina* lyophilized powder; DOX: Doxorubicin; ROS: Reactive oxygen species; cTn-T: Cardiac troponin-T; OVX: Ovariectomized; SC: Subcutaneous; IP: Intraperitoneal; H and E: Hematoxylin and eosin; ELISA: Enzyme-linked immunosorbent assay; HRP: Horseradish peroxidase; SOD: Superoxide dismutase; GSH: Glutathione; Gpx: Glutathione peroxidase; NOS: Nitric oxide synthase; TBARS: Thiobarbituric acid reactive substances; DEVD-pNA: N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide.

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DOI: 10.4103/pm.pm_52_20

Access this article online
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INTRODUCTION

Chronic heart failure is one of the critical clinical side effects of anthracycline chemotherapy, especially in cases of solid tumors. Female sex could be one of the risk factors for anthracycline cardiotoxicity;[1] thus, in turn, the risk of chronic heart failure in breast cancer survivors becomes higher, which might affect the quality of life after surviving a deadly disease.[2] Estrogen, a prominent female sex hormone, has pleiotropic effects on overall physiology. It is believed to have antioxidant function through indirect up-regulation of antioxidant gene expression and decreasing superoxide production. Higher estrogen level is associated with higher risk of breast cancer and fall in estrogen level has negative effects like osteoporosis, inflammation, increased risk of cardiac damage etc., Breast cancers by large are characterized by estrogen receptor-positive types. Along with hormone replacement therapy agents, in chemotherapy treatment cycle anthracycline class drugs and alkylating agents such as doxorubicin (DOX) and cisplatin are also included. Higher estrogen levels are associated with a higher risk of breast cancer, and a fall in estrogen level has negative effects such as osteoporosis, inflammation, and increased risk of cardiac damage. DOX, an anthracycline class drug, is still an effective chemotherapy to treat solid tumors such as breast cancers irrespective of its estrogen receptor

Cite this article as: Gomathinayagam S, Gothandam KM. Amelioration of doxorubicin-induced cardiotoxicity by oral lyophilized *Dunaliella salina* supplement in a female Wistar rat model with active estrous cycle and surgical menopause. Phcog Mag 2020;16:S284-9.

status, but it comes with side effects such as cardiotoxicity and alopecia. The mechanism by which DOX is toxic to cancer cells is different from its mechanism in affecting the cardiomyocytes. The widely accepted mechanism of DOX cardiotoxicity is due to increased oxidative stress and lipid peroxidation in cardiac cells. After all, there is menopause which is an unavoidable condition in women after a certain age. If a patient undergoing chemotherapy will by chance suffer this estrogen loss, the effect of DOX cardiotoxicity might be exacerbated.[3] Tamoxifen, which is a hormone-based therapy agent in estrogen receptor-positive breast cancer, also has anti-estrogenic effects in mammary tumors to limit cancer progression and is believed to have pro-estrogenic effects in cardiomyocytes. In a modest number of research studies, there are observations of the protective effects of tamoxifen in DOX cardiotoxicity both in laboratory and retrospective meta-analyses involving clinical studies.[4,5] Deriving on previous reports, it is natural to hypothesize that tamoxifen combined with DOX might positively affect the endogenous antioxidant mechanism and prevent cardiac damage during chemotherapy. However, dexrazoxane, a cytoprotective iron-chelating agent, is believed to limit the generation of oxygen-free radicals from DOX metabolism and is one of the clinically suggested cardioprotective agents that are administered prophylactically.[4] The search for potential candidates like dexrazoxane to prevent DOX cardiac damage is continuing, and many studies have focused on detoxifying reactive oxygen species (ROS) using active compounds with antioxidant properties and enzymes.

Besides, plant-sourced natural products have been shown to be effective in maintaining this antioxidant status. Carotenoids are the most potent antioxidants and have been reported to play protective effects against several oxidative stress-induced ailments. Micro and macro algae consumption is increasing globally owing to their functional and nutritional benefits. Furthermore, the cultivation of algae is relatively easy and cost-effective. They produce a variety of compounds that are beneficial to humans such as fibers, proteins, and carbohydrates. Notably, microalgae produce some high concentrations of some carotenoids.[6] Attenuation of DOX cardiotoxicity by intravenous administration of carotenoids like lycopene and tomato extract has been reported to be cardioprotective in DOX-induced cardiotoxicity in mice.[7] A previous study in our laboratory has shown that oral lyophilized *Dunaliella salina* lyophilized powder (DSLP), a marine unicellular alga rich in carotenoids, can be used as a functional food that has protective effects against mammary tumors in rats.[8] *D. salina* has been commercialized as a nutraceutical supplement and is available in various markets from Asia-Pacific to Europe. Thus, in the present study, we compared the protective effects of tamoxifen and DSLP supplementation *in vivo,* mimicking the hormonal changes that occur during menopause.

MATERIALS AND METHODS

Chemicals

Adriamycin, the brand name of DOX and tamoxifen was purchased from Apollo Pharmaceuticals, Chennai. The cardiac troponin-T (cTn-T) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Kinesis, USA. All chemicals used were from Sigma Aldrich (Merck), Germany. Spectrophotometric caspase-3 and nitric oxide synthase (NOS) kits were purchased from Biovision, USA.

Animals

Female Wistar albino rats, 7–8 weeks old, weighing 120–125 g, were used for the study. The animals were maintained in an experimental area in Vellore Institute of Technology (VIT) animal house, VIT, Vellore, India, and were housed in polypropylene cages under optimal conditions. The local institutional animal ethics committee of VIT, Vellore, India, approved the experimental design (proposal number: VIT/IAEC/15/Sep1/14). The animals were provided with a standard pellet diet (4.1% fat, 22.2% protein, and 4.0% carbohydrates, as a percentage of total kcal) and water *ad libitum*. The surgical procedure and post-operative care were absolutely taken as per the guidelines.

Dunaliella salina lyophilized powder preparation

DSLP is given as a supplement to rats in specific groups according to the protocol approved by the Institutional Animal Ethical Committee. A mixture of carotenoids dominated by β-carotene is the active ingredient in the whole-cell *D. salina* supplement. The preparation of DSLP, the total carotenoid and individual carotenoid contents (lycopene (28.55 ± 2.01 μg/100 mg), β-carotene (158.34 ± 13.76 μg/100 mg), and lutein (53.81 ± 3.98 μg/100 mg) have been estimated and reported in our previous study.[8]

In vivo experimental design

Ten days after the surgical ovariectomy in 15 rats out of thirty is done, the rats were caged with three ovariectomized (OVX) and three rats with active menstrual cycle in each group (n = 3), where rats receiving only saline served as a control, and a group receiving a cumulative dose of 25 mg/kg BW DOX alone subcutaneous (SC) weekly over 5 weeks served as a cardiotoxicity control. The other groups received oral tamoxifen (1 mg/mL) or an oral dose of DSLP (1000 mg/kg BW) for 3 days a week along with SC DOX like the positive control group. In one of the DSLP-supplemented groups (preinitiation DSLP), the DSLP was given for 5 weeks before until a week before the first DOX injection and the other group received DOX and DSLP jointly for 5 weeks. Rats with active ovarian cycle and OVX in each group were distinguished by ear punches. The followings are the different groups that included OVX and normal rats.

Control

Saline for 5 weeks.

Doxorubicin alone

Cumulative dose of 25 mg/kg BW DOX alone SC over 5 weeks.

Doxorubicin and tamoxifen

Cumulative dose of 25 mg/kg BW DOX for 5 weeks with 1 mg/mL tamoxifen 3 days a week.

Preinitiation Dunaliella salina lyophilized powder

1000 mg/kg BW of DSLP for 1 week followed by 25 mg/kg BW DOX SC weekly once for 5 weeks.

Concomitant Dunaliella salina lyophilized powder

1000 mg/kg BW of DSLP and a cumulative dose of 25 mg/kg BW DOX SC for 5 weeks concomitantly.

Ovariectomy in rats

Wei Zou's method was adopted to do bilateral ovariectomy in rats under isoflurane anesthesia. After inducing anesthesia, the animal was made to lie on its back over a warm plate (37°C) with its nose inserted into the anesthesia mask. A small incision was made in the lower abdomen to find periovarian fat with the ovary on both sides. The ovaries were removed by cutting the fallopian tube, and the flanking uterine horn was returned into the coelom.[9] The wound was closed with sterile clips followed by surgical adhesive. Prophylactic intravenous cefazolin antibiotic dose was given post-operatively to prevent infection.

Histopathological staining

The hearts of all animals were excised rapidly after sacrifice. The left ventricular tissue was immersed in a 10% formalin solution for fixation, dehydrated with different grades of ethanol from 50% to 100%, and then embedded in wax paraffin. Sections of 3–5 μm thickness were cut and stained with hematoxylin and eosin and then studied under a light microscope.

Enzyme-linked immunosorbent assay for cardiac troponin-T

Rat serum levels of cTn-T were estimated using the KinesisDx Rat Cardiac Tn-T ELISA kit.[10] Serum was extracted from the coagulated rat blood by centrifugation. Samples were added to the anti-cTn-T-coated microtiter wells, followed by biotin conjugate and streptavidin-horseradish peroxidase. The respective substrate was added to colorimetrically estimate cTn-T levels against the 4-parameter cTn-T standard curve. The estimated cTn-T levels are expressed as picogram per milliliter of serum in the bar graph [Figure 1].

Biochemical assays

Catalase test

The assay determined cardiac catalase activity based on the catalase activity on H_2O_2 in the presence of dichromate and acetic acid to give chromic acetate, a colored end product with an absorbance maximum at 570 nm. It is expressed as μmol of H_2O_2 consumed per milligram of protein.

Superoxide dismutase

Superoxide dismutase (SOD) was determined in heart tissue homogenized in phosphate-buffered saline (PBS) at pH 7.4, following the method published by Kakkar *et al.*[11] Absorbance was measured at 560 nm. The calculated activity is in micromoles of enzyme per milligram of tissue taken for the assay.

Glutathione and glutathione peroxidase

Glutathione (GSH) in heart tissue was measured using the method given by Moron *et al.*[12] Reduced GSH forms a colored complex in the presence of 5, 5' dithio 2-nitro benzoic acid with an absorbance maximum at 412 nm. Thus, it was estimated spectrophotometrically and expressed in nmol per gram of tissue. Glutathione peroxidase (GPx) was determined in heart tissue homogenized in PBS at pH 7.4, according to the method described by Hafeman *et al.* (1974).[13]

Nitric oxide synthases

Nitric oxide (NO) production in the heart was evaluated using the BioVision's NOS Activity Assay Kit, USA). As an index of NO production, this assay employs the reduction of nitrate to nitrite that reacts with the chromogenic Griess reagents 1 and 2.

Lipid peroxidation assay

Cardiac lipid peroxidation was measured by the malondialdehyde-thiobarbituric acid reactive method using the Himedia EZassay thiobarbituric acid reactive substances kit, India. The manufacturer-provided color developing solution was added to standard or sample tubes and incubated in a boiling water bath for 1 h. After boiling, the tubes were transferred to an ice bath, centrifuged, and absorbance was measured at 540 nm.

Caspase-3 assay

Cardiac caspase-3 activity was assayed using Cayman's colorimetric assay according to the manufacturer's protocol. The cleavage of labeled N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide by caspase-3 released a chromogenic substance, which was read in a microtiter plate reader (BioRad) with an absorbance maximum at 405 nm.

RESULTS

Histopathological analysis

Histopathological analysis revealed typical features such as cylindrical cardiomyocytes and oval-shaped nuclei (black arrow) in both control rats and OVX control rats [Figure 1]. In contrast, DOX-treated rats showed high distortion of cells with interstitial fibrosis (blue arrow) with pyknotic nuclei (yellow arrow), indicating cells undergoing necrosis.[14] The rats that received DSLP along with DOX dose showed cardiac damage to a significantly lesser magnitude when compared with rats that received DSLP before DOX dosage and also tamoxifen.

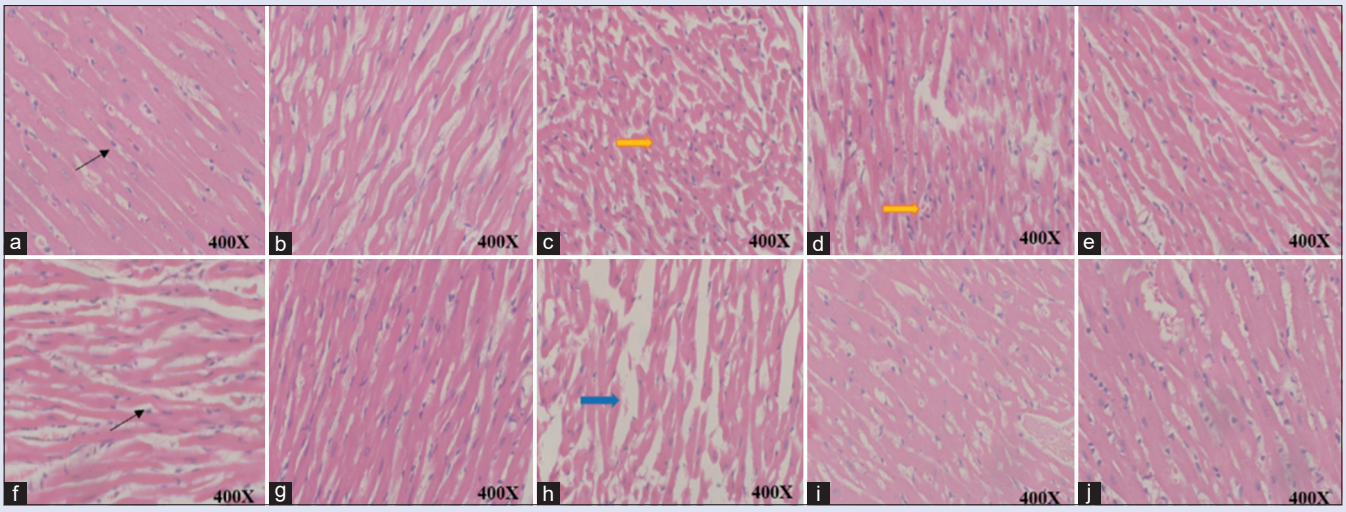


Figure 1: Hematoxylin and eosin stained histo-pathological photomicrograph of rat left ventricle in ×400. (a and b) Control and control ovariectomized, (c and d) doxorubicin and doxorubicin ovariectomized, (e and f) doxorubicin + tamoxifen and doxorubicin + tamoxifen ovariectomized, (g and h) preinitiation *Dunaliella salina* lyophilized powder and preinitiation *Dunaliella salina* lyophilized powder ovariectomized, (i and j) concomitant *Dunaliella salina* lyophilized powder and concomitant *Dunaliella salina* lyophilized powder ovariectomized respectively

Rat cardiac troponin-T by enzyme-linked immunosorbent assay

Cardiac tissue-specific troponin T levels were estimated. An increase in the cTn-T levels in the serum indicates the extent of cardiac tissue damage [Figure 2]. The tamoxifen dose did not prevent cardiac damage effectively; however, the serum levels of cTn-T in the tamoxifen group were lower than the group that received DOX alone. Near comparable levels of cTn-T in the control and concomitant DSLP groups indicate that DSLP was functionally effective in preventing cardiac damage due to DOX ($P < 0.05$).

Antioxidant status of the heart

Catalase and superoxide dismutase

The activity of these disproportionating enzymes in the heart tissue in the event of DOX, tamoxifen, and oral DSLP treatment has been measured and graphically represented in Figure 3. Sudden loss of estrogen was found to have a significant effect on the activity of these enzymes in comparison to the estrogen available groups ($P < 0.0001$).

Glutathione and glutathione peroxidase

Another system to neutralize the free radical attack involving GSH and GPx was also evaluated. Although circulatory GSH and GPX activities have been reported to be not correlated with estrogen, in the heart tissue there is a considerable effect of estrogen absence related to the GSH and GPx activities [Figure 4a and b]. However, concomitant DSLP supplementation was found to maintain this optimal in comparison to other groups ($P < 0.0001$).

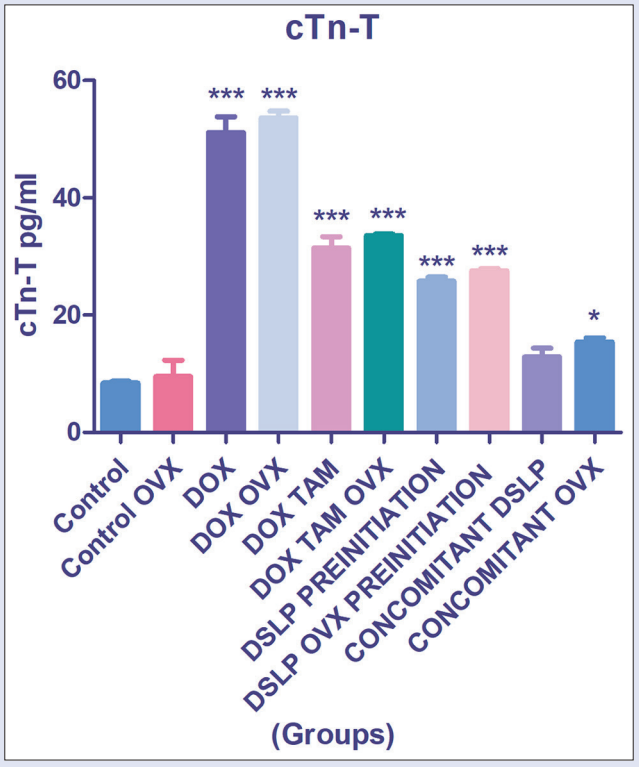


Figure 2: Bar graph showing cardiac troponin-T levels in blood serum of different groups

Nitric oxide synthase

NOS-dependent ROS formation[14] could be having a role in DOX-induced cardiotoxicity. There is evidence that NOS directly acts on DOX molecule to form DOX semiquinone,[15] which down-the-stream produces oxygen-free radicals. Table 1 represents the NOS activity of heart tissue in the different groups.

Lipid peroxidation

Figure 5 shows the effect of DSLP in reducing lipid peroxidation levels in heart tissue against the DOX and tamoxifen groups. Estrogen loss due to surgical menopause increased the lipid peroxidation level significantly in all the groups ($P < 0.0001$).

Caspase-3 assay

Caspase-3 in cardiac cells seems to be activated by DOX administration, as it is evident from Table 1. Caspase-3 activation leads to apoptosis in cardiomyocytes and DSLP supplementation reduces caspase activation due to DOX ($P < 0.05$).

DISCUSSION

Estrogen loss exerts oxidative stress on the heart tissue, and DOX is toxic to the heart by the ROS it can release. Very potent antioxidants like carotenoids are abundantly present in marine algae, which have been reported to reduce the ROS and oxidative burden on various organs during diverse pathological conditions, including cancer.[7,15,16] As far as, the nexus of estrogen loss-DOX cardiotoxicity-cardiovascular disease is concerned, the requirement or timing of prophylactics like dexrazoxane or hormone replacement therapy is crucial. In our experiment, oral DSLP when given along with a cumulative DOX dose of 25 mg per kg bodyweight of rat, ameliorated the effect of estrogen loss and modestly prevented the damage due to DOX. On histopathological examination, the concomitant administration of oral DSLP prevented karyolysis to a reasonable extent when compared to the DOX alone and DOX + tamoxifen groups. The heart section of the OVX rats showed increased cytoplasmic vacuolation [yellow arrows in Figure 1]. Cardiac damage was additionally assessed by the serum levels of rat cTn-T. DOX-treated rats had a roughly fivefold increase in cTn-T from the base levels, where DSLP supplementation significantly prevented cardiac damage. In addition, the loss of estrogen also had effects on the cTn-T level, whereas tamoxifen did not show any positive effect on cTn-T levels. There were changes in the status of innate antioxidant machinery in different groups. The antioxidant machinery includes catalase, SOD, GSH, and GPx. SOD converts free radical oxygen into hydrogen peroxide, and catalase neutralizes the toxic hydrogen peroxide. GSH and GPx reduce the lipid peroxides into its alcohols. Higher levels of lipid

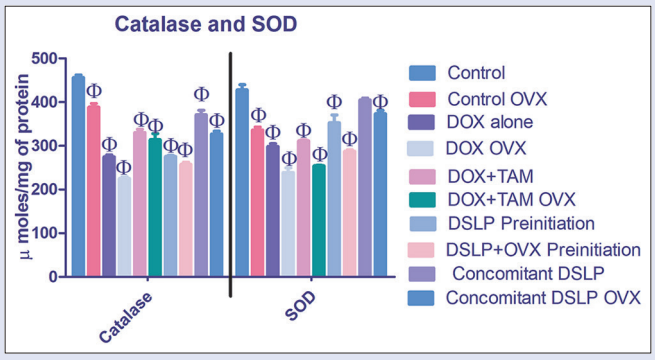
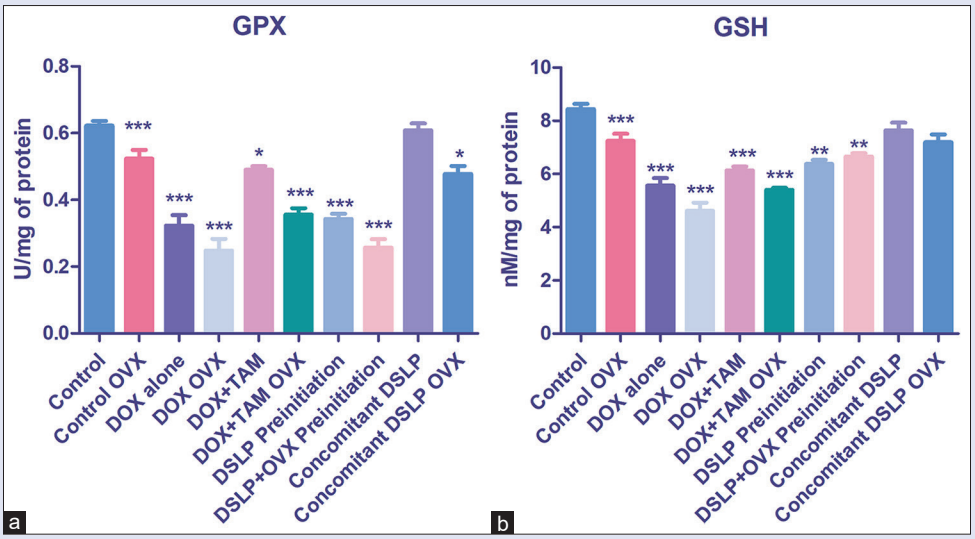


Figure 3: Bar graph showing catalase and superoxide dismutase levels in rat heart tissue normalized with total protein (ΦStatistical significance)

Table 1: Nitric oxide synthase and caspase-3 activity in heart tissue homogenate

	Control		Dox Alone		Dox + TAM		Preinitiation DSLP		Concomitant DSLP	
OVX	−	+	−	+	−	+	−	+	−	+
NOS[a]	6.9±0.37	7.3±0.79	15±0.73	13±1.10	9.7±0.51	7.8±0.97	10±0.34	9.4±0.78	8.8±0.42	7.8±0.97
Caspase-3	0.59±0.03	0.58±0.03	1.32±0.04	1.62±0.07	1.22±0.05	1.33±0.06	0.91±0.016	0.94±0.06	0.75±0.04	0.72±0.02

n=3 mean±SEM. [a]U/mg of protein; caspase-3 measured as absorbance maximum at 405 nm. SEM: Standard error of mean; NOS: Nitric oxide synthase; OVX: Ovariectomized; DSLP: *Dunaliella salina* lyophilized powder; DOX: Doxorubicin; TAM: Tamoxifen. OVX: + indicates intact ovaries;- indicates ovariectomised



Figures 4: (a and b) Bar graph showing glutathione peroxidase and glutathione status of rat heart tissue (*Statistical significance $P < 0.0001$)

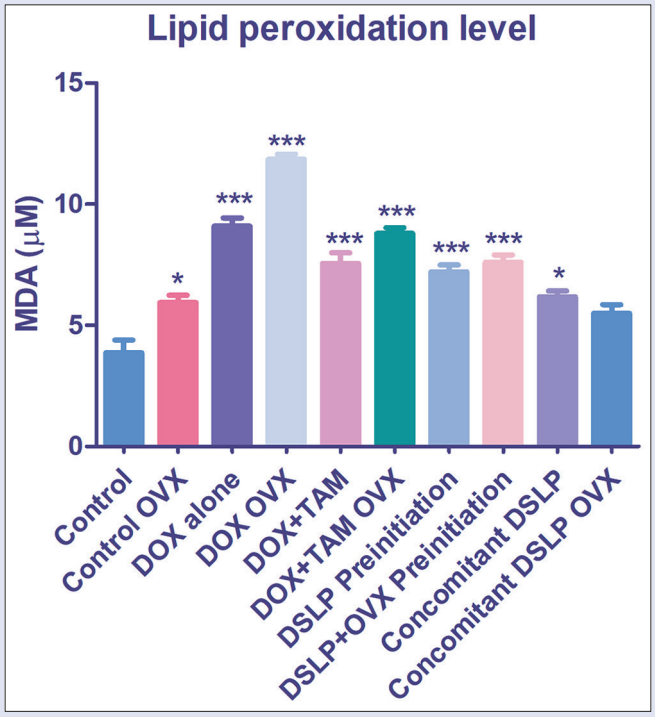


Figure 5: Bar graph showing malondialdehyde levels (thiobarbituric acid reactive substances) in rat heart tissue

peroxidation activate apoptotic markers like caspase-3. Figure 3 shows that estrogen loss impacted catalase and SOD levels even in control rats, and DSLP supplementation improved these two parameters, except that the catalase level was not statistically significant. Estrogen loss had similar effects on GSH and GPx levels, but DSLP supplementation significantly improved their levels. All these parameters together impacted the lipid peroxidation level. The OVX rat had a slightly higher level of lipid peroxidation, and DSLP supplementation prevented the extent of lipid peroxidation. NOS, apart from releasing NO, reduces DOX to the semiquinone radical. The peroxynitrite formed from NO and superoxides increased lipid peroxidation, as shown in Figure 5.[17,18] The levels of caspase-3 death protease were higher in the groups that had greater oxidative damage in the heart tissue due to DOX and estrogen loss than in the control and DSLP groups. Hence, collectively, it is inferred that DSLP supplementation helps maintain the intrinsic antioxidant mechanism in the event of oxidative stress.

Estrogen is a steroid hormone present at a higher level in females than in males for a certain period in their lifetime. This hormone has diverse effects on an individual's health from mental well-being to cancer. In every female, there would be a period called menopause, which results because of the sudden plunge in estrogen secretion.[3,13] During this period, adequate care should be taken to prevent ailments that could affect one's quality of life. Ovarian estrogen loss in female cancer patients undergoing anthracycline class medication can have severe pathophysiological implications in the heart. Pieces of evidence suggest that DOX treatment can increase the chances of cardiovascular disease; an ill-timed estrogen loss during this chemotherapy in a breast cancer patient can augment the risk.[19] Several plant-based compounds have been investigated for their potential to prevent DOX-induced cardiotoxicity. In a similar study, the steroid saponin dioscin was found to prevent DOX-induced cardiotoxicity by effectively mitigating oxidative stress.[20] When carotenoids such as lycopene and tomato extract were given to rats through IP, it resulted in minimal necrosis due to DOX in the rat heart.[7] In a study evaluating the preventive properties

of beta-carotene alone in a cisplatin-induced cardiotoxicity rat model, it was observed that beta-carotene could be a potential supplement to prevent cardiotoxicity.[21] It should also be noted that the consumption of carotenoids, if not orally, might do more harm than good. We had fixed the dosage of DSLP supplement based on our previous study as mentioned earlier and it was not found to have any adverse effect when supplemented throughout the study. A review work by Khosrow-Khavar *et al.* concluded that tamoxifen might have a cardioprotective effect in patients administered aromatase inhibitors and tamoxifen.[4] In our animal experiment, when tamoxifen was administered alongside DOX, its cardioprotective effects were less in magnitude than the DSLP-supplemented groups. There was also a significant difference in the amelioration effect between both the DSLP supplement groups. The group that received DSLP concomitantly with DOX dose had very little damage to the cardiac tissue.[22,23] Nevertheless, the rats in the group that received DSLP until 1 week before the first DOX had higher oxidative stress due to estrogen loss and also the damage due to DOX. Our study was limited by a lack of facilities to do Masson's trichrome staining to estimate collagen deposition between cardiomyocytes and to estimate total oxidative status and oxidative stress index.

CONCLUSION

Thus, the lyophilized form of oral *D. salina* could be taken if not as a regular supplement, but during cancer chemotherapy cycles to prevent anthracycline cardiac toxicity. In cases of menopause overlapping with the chemotherapy cycle, carotenoid supplementation could help maintain the nitrosoredox balance by up keeping intrinsic antioxidant mechanisms in addition to preventing damage due to DOX toxicity.

Acknowledgements

The authors would like to acknowledge Dr. Ramajayaraj Charles Darwin University, Australia**,** Ms. Ajitha Gomathi, Ms. Jayasindu, and Ms. Shreyangi Chakroborty for their help with this project.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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