Protective effect of *Azadirachta indica* A. Juss against doxorubicin-induced cardiac toxicity in tumour bearing mice

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Doxorubicin (DOX) treatment (12 µg/g body weight, once a week for 2 weeks) resulted in a significant decrease in the heart rate along with an increase in QRS, ST, and QT intervals. Histopathological studies showed cardiomyocyte degeneration, cytoplasmic vacuolation and macrophage infiltration in cardiac tissue. A marked increase in the rate of apoptosis was also observed. An increased oxidative stress was evidenced by significantly higher levels of lipid peroxidation (LPO) and depletion of reduced glutathione. A decrease in the activity of cellular antioxidant defence enzymes was also observed. The decrease in the heart rate and ECG alterations were prevented significantly by AAILE (100 µg/g body weight, po) co-treatment, started two weeks prior to DOX treatment and continued till the termination of the experiment. The cardioprotection was also evident from histopathology and decrease in the rate of apoptosis in cardiomyocytes. AAILE co-treatment also prevented DOX-induced increase in LPO and decrease in antioxidant defence enzymes. The results suggest that AAILE administration prevents DOX-induced cardiotoxicity.

Keywords: *Azadirachta indica*, Cardiac toxicity, Doxorubicin, Oxidative stress, Tumour

Doxorubicin (DOX) has been established as an effective anticancer drug for the treatment of wide range of malignant conditions. However, DOX treatment leads to severe cardiotoxicity which remains a major concern for its use in cancer patients. The risk of cardiotoxicity is dose dependent and increases with an increase in the amount of dose. Cardiotoxicity can be acute, sub-acute, or chronic. Acute cardiotoxicity develops within minutes of intravenous administration of DOX and is usually reversible but early onset of cardiac toxicity has been shown to be predictive of future heart failure. Sub-acute and chronic forms of DOX-mediated cardiotoxicity manifest as a permanent and irreversible damage to the cardiac tissue.

The exact mechanism(s) of DOX-induced cardiotoxicity is yet to be fully elucidated. A number of proposed mechanisms include: the generation of reactive oxygen species (ROS), apoptosis, DNA interference, and metabolic alterations etc. DOX on reaction with molecular oxygen generates significantly large amount of free radicals which is the major source of oxidative stress mediated cellular damage. The role of iron has also been investigated by several authors in DOX-induced cardiotoxicity and it is proposed that free intracellular iron increases oxidative stress of the cell. These events contribute to death of cardiomyocytes and are considered the primary reason of DOX-induced cardiac side effects.

Since DOX is an effective antineoplastic drug, considerable efforts have been made to explore strategies effective in reducing DOX-induced cardiac dysfunction. However, there is no effective cure against DOX-induced cardiotoxicity. Pharmaceutical agents in the form of supplements have been tested in experimental animal studies to reduce the risk of DOX-induced cardiotoxicity. Antioxidants such as mercaptopropionyl glycine (MPG), selenium, superoxide dismutase and dexrazoxane have been reported to decrease DOX-induced cardiotoxicity. The use of antioxidants, iron chelators, amino acid supplements etc. have limited success and in some cases additional side effects have been reported. Dexrazoxane, drug known for its iron-chelating effects is in clinical use but usage is limited because of its myelosuppressive side effects. Other strategies such as slow infusion of DOX and reduction in the cumulative dose have decreased the incidence of acute cardiotoxicity but do not completely eliminate the risk of chronic cardiotoxicity. Therefore, further research is needed.
required to develop interventions to prevent the cardiotoxicity arising due to DOX treatment.

_Azadirachta indica_ A. Juss is one of the most versatile medicinal plants having wide spectrum of biological activity. It has been shown to be effective against coronary artery disease, hypertension and arrhythmias. Peer et al. have reported cardioprotective potential of _A. indica_ against isoprenaline induced myocardial infarction in rats. More than 135 compounds have been isolated from different parts of the plant. The presence of wide range of biologically active molecules makes _A. indica_ effective against various health ailments. In addition, it is also known to be a strong antioxidant, iron chelator and modulator of immune response as well as antioxidant defence system of the body. Elevation of antioxidant defence enzyme levels have been reported after treatment of _A. indica_ to mice. In some cases antioxidant mediated effects of _A. indica_ are reported to have better potency in comparison to known antioxidants like vitamin E, ascorbate, and melatonin.

Considering the multifaceted etiology of DOX-induced cardiotoxicity and cardioprotective potential of _A. indica_ in experimental animal models, the present study has been designed to investigate the possibility of use of _A. indica_ to eliminate DOX-induced cardiac toxicity.

### Materials and Methods

**Chemicals and reagents**—7,12-dimethylbenz(a)anthracene (DMBA), reduced glutathione (GSH), oxidised glutathione (GSSH), nitroblue tetrazolium (NBT) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Company, St. Louis, USA. Doxorubicin (DOX), bovine serum albumin (BSA), 2-thiobarbituric acid (TBA), 5-5’–dithiobis (2-thiobarbitiolic acid) (DTNB), hydrogen peroxide (H2O2) and glutathione reductase (GR) were purchased from local Indian firms and were of highest purity grade. TUNEL Assay kit was purchased from Trevigen, Inc, 8405 Helgerman Ct. Gaithersburg.

**Preparation of aqueous Azadirachta indica leaf extract**—_A. Indica_ leaves were collected after proper identification by botanist from Panjab University campus, Chandigarh, India. The leaf extract was prepared as describe earlier. Briefly, fresh leaves of _A. indica_ were obtained and washed properly with distilled water. The aqueous extract was obtained by grinding the leaves in a mixer and filtering the aqueous portion of mixture. The fraction obtained was then centrifuged and supernatant was collected. The supernatant was then freeze dried to obtain aqueous _A. indica_ leaf extract (AAILE).

**Treatment of animals**—Male Balb/c mice in body weight range 25-30 g were procured from Central Animal House, Panjab University, Chandigarh (India) and housed in the animal room of Department of Biophysics, Panjab University, Chandigarh (India). The animal room was well-ventilated and maintained at 21±2 °C, and 50-60 % RH.

The animals had free access to drinking water and fed with standard pellet diet. The experimental animals were acclimatized to the experimental conditions for one week before starting the experiment. All experimental procedures were approved by institutional ethics committee and conducted according to the guidelines of Indian National Science Academy for the use and care of experimental animals.

The skin cancer model was developed as described earlier. Briefly, animals received topical application of DMBA (500 nmol/100 µL of acetone) for two weeks, twice a week followed by TPA (1.7 nmol/100 µL of acetone) for 18 weeks, twice a week.

After 22 weeks of first DMBA application, the animals were segregated into following five groups of 9-10 animals each: (I) normal, (II) tumour, (III) tumour+AAILE, (IV) tumour+DOX and (V) tumour+DOX+AAILE. DOX was administered intravenously to group IV and group V animals twice at an interval of two weeks (cumulative dose 12 µg/g body weight). AAILE was administered by oral route to group III and group V animals thrice a week on alternate days for four weeks (100 µg/g body weight). The treatment of AAILE was started 2 weeks prior to the administration of DOX. Group I and group II animals did not receive any special treatment.

**Electrocardiographic assessment**—ECG was recorded in animals at different time intervals as described previously. Elastic cotton jacket containing two circular platinum leads (radius: 3 mm) was wrapped around the thoracic cavity of mouse. Lead II electrodes were placed below right clavicle and 11th intercostal space left to sternum and right lower abdomen respectively. Prior to ECG, the ventral thoracic region of each animal was carefully shaved and a conductive ECG gel was applied over each electrode. For each ECG record, the most stable 3 min
continuous segment was chosen for study. The heart segment analysis of ECG was done with the help of computer software as described previously.

Histopathological investigations—After 24 h of second DOX administration, animals were sacrificed by cervical dislocation under mild ether anesthesia and left ventricle portions of cardiac tissue of mouse from each group were fixed in neutral formalin for 24 h. Tissues were then dehydrated in grades of alcohol and embedded in paraffin wax. Section (5 µm thick) were cut and placed on slides and stained with Haematoxylin and Eosin stains. The stained sections were visualized under light microscope (LEICA DM 3000).

Cell death assay (TUNEL assay)—TUNEL assay was performed on deparaffinised and rehydrated liver sections for the detection of DNA fragmentation with in situ apoptosis detection kit following the manufacturer’s specifications with minor modifications. The DNA fragmentation in apoptotic cells was visualized by the detection of brominated nucleotide (BrdU), incorporated by terminal deoxynucleotidyltransferase (TdT) onto the free 3’OH residue of the DNA fragment. To make DNA accessible to the labelling enzymes, the membranes were permeabilized by incubating with proteinase K for 30 min. Quenching of endogenous peroxidase activity was done by incubating in H₂O₂. The TdT incorporated brominated nucleotides were revealed by incubation with biotin labelled anti-BrdU antibody. Biotin labelled fragments were visualized by incubation with streptavidin horse radish peroxidase and subsequently with 3’,3-diamino benzidine (DAB). The slides were counter stained with methyl-green for better background details and observed under light microscope.

Biochemical parameters—

Myocardial lipid peroxidation: Tissue homogenates were precipitated using cold TCA. The non-protein sulfhydryl groups were assayed with DTNB solution to yield yellow coloured complex which was read at 412 nm. GSH was used as a standard and GSH content of the sample was expressed as nanomole GSH/mg protein.

Myocardial superoxide dismutase: The superoxide scavenging activity of superoxide dismutase (SOD) was assessed by measuring the inhibition of nitroblue tetrazolium reduction to blue colour formazan at 560 nm by superoxide anions generated by photo-oxidation of hydroxylamine hydrochloride. The activity was expressed as IU/mg protein, where 1 IU is defined as the amount of enzyme inhibiting the increase in optical density by 50%.

Myocardial catalase: The catalase activity was measured as the rate of reduction in intensity of H₂O₂ at 240 nm. The rate of change in OD was obtained and the activity of enzyme was expressed as IU/mg protein.

Myocardial glutathione peroxidase: The activity was assessed by measuring the rate of oxidation of NADPH at 340 nm in the presence of reduced glutathione and H₂O₂. Enzyme activity was expressed as nanomole of NADPH consumed/min/mg protein.

Myocardial glutathione reductase: The activity of glutathione reductase was assessed as rate of oxidation of NADPH by GR in presence of GSSG at 340 nm. Enzyme activity was expressed as nanomole of NADPH oxidized/min/mg protein.

Protein estimation: Protein content of samples was estimated by the method of Lowry et al. using bovine serum albumin (BSA) as a standard.

Statistical analysis—The data were expressed as mean±SD and analysed by one-way ANOVA followed by post hoc test. P value less than or equal to 0.05 was considered to be statistically significant.

Results

Electrocardiographic assessment—A significant decrease in the heart rate (bradycardia) was noticed after 2 weeks of DOX treatment to tumour bearing mice (Gr. IV) (Fig. 1). However, administration of AAILE to tumour bearing-DOX treated animals (Gr. V) had significantly improved heart rate...
compared to tumour bearing-DOX treated animals (Gr. IV). ST segment, QT interval and QRS complex which were significantly increased in Gr. IV animals compared to Gr. I and Gr. II got normalized after AAILE co-administration in Gr. V animals (Table 1). Heart rate and duration of cardiac intervals were not affected in Gr. II and III animals compared to normal animals (Gr. I).

**Histopathology**—Histopathological analysis of cardiac tissue was conducted at the end of the treatment period in all groups. Normal myocardial histoarchitecture was observed in Gr. I, II and III animals (Fig. 2a, 2b). The administration of DOX to tumour bearing mice (Gr. IV) resulted in myocardial degradation, which is characterized by partial or total loss of cytoplasm. In some myocytes the myofibrils were completely disorganised by large, clear space indicative of intracellular edema (Fig. 2c, 2d). In addition to cardiomyocytes injury, DOX treatment (Gr. IV) produced vacuolation and induction of fibrosis. A significant improvement was observed upon AAILE co-administration to DOX treated animals (Gr. V). There was a reduction in the overall vacuolation and degree of myofibrillar loss (Fig. 2e, 2f).

**Cell death analysis**—Figure 3 showed left ventricular TUNEL stained sections of mice heart from different treatment groups. Apoptotic cells in addition for being TUNEL positive (i.e. nucleus stained brown) also displayed certain features such as; chromatin material was confined to nucleus, chromatin condensation and chromatin marginalization etc. The overall percentage of apoptotic cells in the myocardium of normal, tumour bearing and AAILE treated animals (Gr. I, II and III) were very low (Fig. 4). DOX treatment (Gr. IV) significantly increased number of apoptotic cells in myocardium compared to normal animals (Gr. I). A significant reduction was noted upon AAILE co-treatment in group V compared to group IV mice. However, the percentage of apoptotic cells remained significantly higher compared to normal animals (Gr. I).

**Lipid peroxidation (LPO)**—The lipid peroxidation level as assessed in terms of Thiobarbituric acid reactive substances (TBARS) was significantly increased after DOX treatment to tumour bearing mice (Gr. IV) compared to other treatment groups. A significant decrease was observed in LPO levels upon AAILE co-administration to tumour bearing-DOX treated mice (Gr. V) compared to tumour bearing-DOX treated animals (Gr. IV) (Table 2). Gr. III mice had significantly decreased LPO compared to Gr. II. Statistically non-significant alterations were observed in other treatment groups.

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### Table 1—Effects of AAILE on various ECG parameters after DOX treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal (Gr. I)</th>
<th>Tumour (Gr. II)</th>
<th>Tumour+AAILE (Gr. III)</th>
<th>Tumour+DOX (Gr. IV)</th>
<th>Tumour+DOX+AAILE (Gr. V)</th>
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</thead>
<tbody>
<tr>
<td>QRS (ms)</td>
<td>34.6±3.28</td>
<td>35.6±3.78</td>
<td>37.2±5.25</td>
<td>49.2±1.64 &lt;sup&gt;ab&lt;/sup&gt;</td>
<td>38.3±2.86 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>QT (ms)</td>
<td>68.6±5.57</td>
<td>69.8±4.54</td>
<td>70.8±3.24</td>
<td>83.6±6.83 &lt;sup&gt;ab&lt;/sup&gt;</td>
<td>75.6±4.21 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ST (ms)</td>
<td>32.3±2.77</td>
<td>32.0±2.43</td>
<td>33.1±2.23</td>
<td>48.2±4.82 &lt;sup&gt;ab&lt;/sup&gt;</td>
<td>37.3±3.94 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>R-R (ms)</td>
<td>93.0±3.40</td>
<td>90.9±4.04</td>
<td>91.8±5.97</td>
<td>116.4±7.59 &lt;sup&gt;ab&lt;/sup&gt;</td>
<td>101.3±4.0 &lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
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One way ANOVA followed by post hoc test. P values: ≤0.05 with reference to <sup>a</sup>Group I, <sup>b</sup>Group II, <sup>c</sup>Group III, <sup>d</sup>Group IV
Reduced glutathione (GSH)—A significant depletion of GSH was observed in tumour bearing-DOX treated animals (Gr. IV) compared to Gr. I and II. AAILE co-administration (Gr. V) increased the levels of GSH compared to tumour bearing-DOX treated mice (Gr. IV). No significant change was observed in myocardial GSH level of tumour bearing animals (Gr. II) compared to normal animals (Gr. I). However, AAILE administration in tumour bearing mice (Gr. II) had significantly increased level of GSH compared to other treatment groups. (Table 2).

Superoxide dismutase (SOD)—Cardiac SOD activity decreased significantly after DOX treatment to tumour bearing mice (Gr. IV) compared to other treatment groups. AAILE administration to tumour bearing-DOX treated mice (Gr. V) elevated the SOD activity compared to tumour bearing-DOX treated
mice (Gr. IV). Tumour bearing mice (Gr. II) showed significant depression in cardiac SOD activity compared to normal mice (Table 2).

Catalase—A significant increase in myocardial catalase was observed in tumour bearing-AAILE treated (Gr. III), tumour bearing-DOX treated (Gr. IV) and tumour bearing-DOX + AAILE co-treated (Gr. V) mice compared to normal (Gr. I) and tumour bearing (Gr. II) mice. However, no significant change was noticed in tumour bearing-DOX + AAILE co-treated (Gr. V) mice compared to tumour bearing-DOX treated (Gr. IV) mice (Table 2).

Glutathione peroxidase (GPx)—DOX treatment to tumour bearing mice (Gr. IV) significantly decreased myocardial GPx activity compared to normal (Gr. I) and tumour bearing mice (Gr. II). AAILE supplementation to tumour bearing-DOX treated mice (Gr. V) significantly increased the activity of GPx.

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Fig. 3—TUNEL assay of cardiac tissue in various treatment groups (a) showing TUNEL negative, normal cardiomyocytes (400X) b) DOX plus AAILE treated (Group V) (400X) c) DOX treated (Group IV) showing brown coloured TUNEL positive apoptotic cells brown colour confined to nucleus, with mild chromatin condensation (400X). Arrows indicate TUNEL positive cells.

Fig. 4—Effect of AAILE on apoptotic index in cardiac tissue after DOX treatment [Values are mean±SD (n=9-10). P values: ≤0.05 with reference to a Group I, b Group II, c Group III, d Group IV].
compared to tumour bearing-DOX treated mice (Gr. IV). GPx activity did not differ significantly in normal (Gr. I) and tumour bearing mice (Gr. II). AAILE treatment to tumour bearing mice (Gr. III) significantly increased the activity of GPx compared to normal mice (Gr. II) (Table 2).

Glutathione reductase (GR)—GR activity was significantly decreased after DOX treatment to tumour bearing mice (Gr. IV) compared to normal (Gr. I) and tumour bearing mice (Gr. II). The activity was significantly increased in tumour bearing-DOX + AAILE co-treated group (Gr. V) compared to tumour bearing-DOX treated animals (Gr. IV). No significant alterations were observed in other treatment groups (Table 2).

Discussion

Azadirachta indica has tremendous medicinal potential. The extracts of various parts of the plant have been used against variety of ailments and have been demonstrated to be effective against various types of cancers. The plant not only offers protection against cancer but also reduces the side effects of certain chemotherapeutic drugs when given as an adjunct to standard chemotherapy. Pre-treatment of Swiss mice with A. indica reduced leucopenia and neutropenia induced by cyclophosphamide and cisplatin plus 5-Flourouracil. Therefore, it is legitimate to explore cardioprotective potential of A. indica against DOX-induced cardiotoxicity.

The development of cancer model prior to the induction of DOX-induced cardiotoxicity was considered important because studies suggested that during carcinogenesis physiology of the whole body gets affected. In the present study, treatment of DOX to skin tumour bearing mice resulted in significant cardiotoxicity as revealed by changes in ECG and myocardium morphology. The ECG alterations such as elongation of QT interval, widening of ST segment, and bradycardia observed in ECG of DOX treated animals are considered as one of the most reliable predictor of DOX-induced cardiotoxicity. The QRS, ST elongation and PR widening after DOX treatment has also been demonstrated. QT elongation is attributed to the disturbance in the ion flux across the membrane of myocytes. This is further related to the structural damage incurred to these cells by DOX. The histopathological studies revealed that left ventricular tissue of animals treated with DOX had significant structural and organisational alterations in terms of presence of myocardial degeneration, cytoplasmic vacuolization, and intracellular edema which correlated well with ECG changes in DOX-induced cardiotoxicity. However, the QT segment elongation was significantly prevented by AAILE and histopathology of animals co-treated with AAILE was improved signifying the cardioprotection.

The bradycardia observed after DOX administration could be correlated to the free radical accumulation and cellular damage. AAILE co-treatment returned heart rate of animals to normal, indicating that it has reduced the deteriorating effect of DOX on cardiac tissue. The DOX-induced cardiac cell death and cardiac failure are the fields of major concern and extensive research. The apoptotic index assessed in accordance to the morphological criteria of apoptosis along with TUNEL staining characteristics demonstrated a decrease in apoptosis after pre-treatment of AAILE to DOX administered.
mice. This observation indicated that AAILE played an important role in reducing the extent of damage produced by DOX treatment to cardiomyocytes.

The damage produced by free radicals manifests as lipid peroxidation (LPO) at the cell membrane. LPO is not just an indicator of free radical damage but also induces further tissue damage by production of wide range of other free radicals which are far more potent and damaging. The cardiac tissue is susceptible to DOX-induced damage due to the higher number of mitochondria which are source of extensive ROS generation. The profound amount of oxygen present in the cardiac tissue makes it a major site for the metabolism of DOX. DOX metabolism is mediated through the use of molecular oxygen, whose metabolism products act as important contributor to toxicity. The superoxide free radicals produced in the process are converted to less reactive H₂O₂ by action of SOD. H₂O₂ is further decomposed to water by the action of catalase or GPx. The activity of myocardial catalase is very low and the conversion of H₂O₂ or related reactive molecules is carried out primarily by myocardial GPx. Therefore, activity of myocardial GPx is thought to play a significant role in DOX-induced cardiotoxicity. The increased activity of SOD and GPx in AAILE co-treated group might indicate the protective role played by AAILE in DOX-induced cardiotoxicity. The results were further supported by decreased levels of LPO in cardiac tissue of this group.

Conclusion
It may be concluded that AAILE pre-treatment to tumour bearing mice has provided protection against cardiotoxicity induced by DOX. The modulatory effect of AAILE on antioxidant defence system might have played a significant role in the reduction of DOX-induced cardiotoxicity.

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