PROTECTIVE EFFECT OF A.CALAMUS EXTRACT AND ALPHA-ASARONE AGAINST DOXORUBICIN INDUCED MYOCARDIAL TOXICITY
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Doxorubicin (DOXO) (adriamycin) an anthracycline antibiotic is well established and highly efficacious drug in the fight against many kinds of cancer like solid tumors, leukemia, soft tissue sarcoma, breast cancer, small cell carcinoma of the lung and oesophageal carcinomas. (Blum and Carter, 1974; Chabner et al., 2001). But the clinical usefulness is still restricted due to its specific toxicities to cardiac tissue. (Zhon et al., 2001). Congestive heart failure, cardiomyopathy and electrocardiographic changes were demonstrated after cumulative doxorubicin administration (Lenaz and Page, 1976). The mechanism proposed for cardiotoxic effects of doxorubicin include free radical induced myocardial injury, lipidperoxidation (Myers, 1977), mitochondria damage (Bier and Jaenke, 1976), decreased activity of Na⁺K⁺ ATPase (Geetha and Devi, 1992), vasoactive amine release (Bristow et al., 1980), impairment in myocardial adrenergic signaling/regulation, increase in serum total cholesterol triglyceraride and low dencity lipoproteins (Ilskowic and Sing, 1997).

Although DOXO-induced injury appears to be multifactorial and complex (Singal et al., 1997), most of the studies support the view that an increase in oxidative stress, evidenced by an increase in free radicals and lipid peroxidation as well as a decrease in antioxidants, plays an important role in the pathogenesis of DOXO-induced cardiotoxicity (Singal et al., 1998). Liberation of free radical is central mechanism of doxorubicin induced damage to the myocardium (Potemski et al., 2006). Due to the presence of less developed antioxidant defence mechanisms, heart is particularly vulnerable to injury by anthracyclin induced reactive oxygen species (ROS). ROSs also causes damage to cellular components and consequently led to cardiomyocyte apoptosis or death (Menna et al, 2007). Doxorubicin also causes the elevation of cardio biomarker enzymes like lactate dehydrogenase (LDH) and creatinine phosphokinase (CPK). Endogenous antioxidant deficits have been suggested to play a major role in doxorubicin induced cardiomyopathy and heart failure (Hanaa et al., 2005). Because of free radical plays important roles in DOXO-induce cardiotoxicity, it is logical to consider antioxidants as primary potential therapeutic agent to prevent such toxic effect. A series of antioxidants have been studied to decrease ROS. However, many of them only have a limited cardioprotective effect or have other side-effects (Elbl et al., 2006; Wouters et al, 2005).
Acorus calamus L., (Family: Araceae) commonly known as Sweet Flag, is an important herbal drug used in the ancient system of medicine. The fragrant oils obtained by alcoholic extraction of the rhizome of A.calamus are mainly used in the pharmaceutical and oenological industries (Bertea et al., 2005). Roots and rhizomes of A.calamus have been used in the Indian and Chinese systems of the medicine for hundreds of years for its beneficial role in improved learning performance, and its anti-aging effect (Zhang et al., 1994). Our previous study showed that the ethanolic extract of A.calamus have strong antioxidant and radiopretecting property (Sandeep and Nair, 2010, Sandeep and Nair 2012.). There are two major active substances namely α-asarone and β-asarone isolated from volatile oil of A.calamus (Baxter et al., 1960). Alpha-asarone has been reported to exhibit neuroprotective action through the blockage of N-methyl-D-aspartate receptor (Cho, et al., 2002) and acetylcholinesterase inhibitory activity (Mukherjee et al., 2007). It also shows anticarcinogenic activity in human carcinoma cells (Hu and Ji, 1986). The present investigation was set to evaluate the protective effects of A.calamus extract and its pure component α-asarone against DOXO-induced cardiotoxicity in mice.

10.2. MATERIALS AND METHODS

10.2.1. Preparation of the extract
Aqueous-ethanol extract of Acorus calamus was prepared as described in section 2.2.1.

10.2.2. Animals
Female Swiss albino mice of 6 weeks old weighing 25 ± 2 g were employed for cardiotoxicity studies.

10.2.3. Determination of protection against doxorubicin induced cardiotoxicity by A.calamus and α-asarone
Animals were randomly divided into 7 groups of five each.
Group I - Untreated control.
Group II - Doxorubicin, (25mg/kg body weight ip, as single dose).
Group III- *A. calamus* extract (250 mg/kg body weight), one hour before DOXO administration

Group IV- *A. calamus* extract (250 mg/kg body weight), one hour after DOXO administration

Group V- α-asarone (50 mg/kg, body weight), one hour before DOXO administration

Group VI- α-asarone (50 mg/kg, body weight), one hour after DOXO administration

Group I treated with (vehicle) distilled water was kept as untreated control. Group II injected with Doxorubicin (25 mg/kg body weight ip, as single dose) kept as treated control. Group III and Group V were administered with aqueous-ethanolic extract of *A. calamus* extract (250 mg/kg body weight) and α-asarone (50 mg/kg, body weight), respectively one hour before DOXO injection kept as pre treatment group and Group IV and Group VI were administered with aqueous *A. calamus* extract extract (250 mg/kg body weight) and α-asarone (50 mg/kg, body weight), respectively one hour after DOXO injection (post treatment group).

After 24 hours of DOXO injection, the blood was collected by direct cardiac puncture under light ether anesthesia and serum was separated for the biochemical estimations. The heart was dissected quickly, weighed and one heart from each group was taken for histopathological examination. The remaining heart homogenates (10% w/v) was analyzed for antioxidant status.

10.2.4. Assessment of cardiotoxicity

Assessment of cardiac biomarkers such as creatinine kinase-MB (CK-MB) (Section 2.2.18.), lactate dehydrogenase (LDH) (section 2.2.17) and other serum biochemical parameter i.e., glutamate oxaloacetate transaminase (GOT) (section 2.2.9), were analysed.

10.2.5. Determination of antioxidant status in heart tissue

The heart was excised after sacrificing the animals and washed with ice-cold PBS and 10% homogenate was prepared in PBS (pH 7) as described in section 2.2.2. A part of this homogenate was used for the determination of reduced glutathione (GSH) (section 2.2.3). Rest of the homogenate was centrifuged at 10,00 rpm for 10 minute
for removing the cell debris, unbroken cells, nuclei, erythrocytes and mitochondria. The supernatant was used for the estimation of superoxide dismutase (SOD) (section 2.2.4), catalase (CAT) (section 2.2.5), glutathione peroxidase (GPx) (section 2.2.6), and malondialdehyde (MDA) section 2.2.3. The protein was estimated by Lowry’s method of (section 2.2.8).

**10.2.6. Histopathological studies**
For histopathological studies, heart was fixed immediately in 10% formalin for a period of at least 24 hour and embedded in paraffin wax. Sections of 5 micron thickness were made using a microtome and stained with haematoxylin-eosin. The histopathological examinations were carried out at Sudharma Metropolis Pathological Laboratory, Thrissur, Kerala, India.

**10.3. RESULTS**

**10.3.1. CARDIOPROTECTION BY A.CALAMUS AND ALPHA-ASARONE**

**10.3.1.1. Serum biochemical parameters**
It can be seen that single i.p injection of DOXO significantly elevated serum LDH and CK-MB levels to $4730.29\pm278.525$ U/L and $711.03\pm29.41$ U/L respectively, as compared with the untreated control group, indicative of cardio toxicity. Both pre and post treatments with *A.calamus* extract or α-asarone significantly ($p<0.001$) ameliorated CK-MB and LDH enzyme activities [$A.calamus$ (pre treated)- LDH- $3076.87\pm39.32$ and CK-MB- $272.14\pm21.78$; α-asarone (pre treated)- LDH- $2046.57\pm697.02$ and CK-MB- $316.06\pm30.87$; *A.calamus* (post treated)- LDH- $3400.33\pm527.24$ and CK-MB- $349.44\pm74.99$ and α-asarone (post treated)- LDH- $3273.72\pm319.31$ and CK-MB- $303.68\pm48.77$ respectively] (Fig 10.1 and Fig 10.2). The levels of serum GOT and was also elevated in DOXO alone treated animals and were found to be reduced in *A.calamus* extract or α-asarone administered mice (*A.calamus* from $255.2\pm57.63$ to $118.88\pm49.39$ (pre) and $100.92\pm7.8$ (post); α-asarone, from $255.2\pm57.63$ to $92.58\pm9.5$ (pre) and $100.93\pm7.9$ (post) in pre and post treated mice respectively (Fig.10.3).
**Fig. 10.1.** Effect of *A.calamus* extract and α-asarone on creatinine kinase-MB (CK-MB) in doxorubicin induced cardio toxicity in mice. Values are expressed as mean ± SD (n = 5). a-P < 0.001 Vs. DOXO

**Fig. 10.2.** Effect of *A.calamus* extract and α-asarone on lactate dehydrogenase (LDH) in doxorubicin induced cardio toxicity in mice. Values are expressed as mean ± SD (n = 5).

a-P < 0.001 Vs. DOXO
Fig. 10.3. Effect of *A.calamus* extract and α-asarone on serum glutamate oxaloacetate transaminase (SGOT) enzyme in doxorubicin induced cardio toxicity in mice. Values are expressed as mean ± SD (n = 5). a-P< 0.001 Vs. DOXO; c- P< 0.05 Vs. DOXO

10.3.1.2. Antioxidant status
Generation of malondialdehyde (MDA) was measured as a marker of lipid peroxidation and an indicator of oxidative injury. The MDA level in heart tissue was increased significantly in the DOXO -treated group compared with the untreated control group. The increase in MDA by DOXO was significantly attenuated by *A.calamus* extract and α-asarone (Fig.10.4).
Fig. 10.4. Effect of *A. calamus* extract and α-asarone on lipid peroxidation levels in doxorubicin induced cardiotoxicity in mice. Values are expressed as mean ± SD (n = 5). b- P< 0.01 Vs. DOX; c-P< 0.05 Vs. DOX

The total antioxidant activity, as a measure of antioxidant status, was significantly decreased in the heart tissue of the DOXO-treated group (Table 10.1.). In the *A. calamus* + DOXO and α-asarone + DOXO group, the total antioxidant activity was significantly increased compared with the DOXO- treated groups. Different antioxidant enzymes were examined in the heart tissue from all the groups and the data are shown in Table 10.1. The DOXO-treated mice showed a significant decrease in SOD and glutathione peroxidase (GPx) levels compared with the untreated controls. The DOXO-induced decrease in SOD and GPx levels was attenuated significantly by *A. calamus* extract and α-asarone administration. The group of animals administered with *A. calamus* extract prior to DOXO treatment (pre-treatment group) had higher antioxidant levels than the group of animals treated with DOXO and then given *A. calamus* extract (post-treatment group). Similarly the pre-treatment of α-asarone resulted in significant increase of SOD, catalase, GPx and GSH in a significant manner than that of the post treated group (Table 10.1).
Table 10.1. Effect of *A. calamus* extract (ACE) and α-asarone on antioxidants level in doxorubicin induced cardiotoxicity in mice. Values are expressed as mean ± SD (n = 5).

<table>
<thead>
<tr>
<th></th>
<th>GSH (nmoles/mg protein)</th>
<th>GPx (Unit/mg protein)</th>
<th>SOD (Unit/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37.23±2.74</td>
<td>22.83±3.13</td>
<td>10.83±3.03</td>
</tr>
<tr>
<td>DOXO (25mg/ kg b.w)</td>
<td>11.23±2.74</td>
<td>11.92±0.54</td>
<td>5.33±3.01</td>
</tr>
<tr>
<td>ACE+DOXO (250mg/ kg b.w)</td>
<td>31.35±3.32a</td>
<td>21.62±1.12b</td>
<td>7.93±2.033d</td>
</tr>
<tr>
<td>DOXO+ACE</td>
<td>25.87±1.23a</td>
<td>16.21±3.1c</td>
<td>8.29±1.19d</td>
</tr>
<tr>
<td>α-asarone+DOXO (50mg/ kg b.w)</td>
<td>36.55±5.63a</td>
<td>23.89±1.77a</td>
<td>14.19±4.12b</td>
</tr>
<tr>
<td>DOXO+α-asarone</td>
<td>26.68±3.45a</td>
<td>20.48±5.98b</td>
<td>9.45±1.3d</td>
</tr>
</tbody>
</table>

a- P< 0.001 Vs. DOXO
b- P< 0.01 Vs. DOXO
c- P< 0.05 Vs. DOXO
d- non significant Vs. DOXO

10.3.2. Morphological study

Light microscopic examination of hearts stained with hematoxylin and eosin of the untreated control and *A. calamus* or α-asarone treated animals displayed almost a normal morphological appearance, whereas the hearts of the DOXO-treated animals showed myocardial degeneration including the loss of myofibrils and focal cytoplasmic vacuolization (Fig. 10.5.B). In mice pre-treated with *A. calamus* extract and then given DOXO, there was a significant reduction in the severity of myocardial degeneration (Fig.10.5.C).
**Fig. 10.5.** Effect of *A. calamus* extract and α-asarone on DOX induced cardiac tissue damage in mice (X 40).

[A] Heart tissue from untreated control mice shows normal cellular architecture. [B] Heart from mice treated with DOXO (25 mg/kg b.w, ip) exhibited severe myocardial degeneration including the loss of myofibrils and focal cytoplasmic vacuolization. [C & D] Heart tissue pre-treated with *A. calamus* extract (250 mg/kg b.w) and α-asarone (50 mg/kg b.w) along with DOXO (150 mg/kg b.w) shows normal architecture with mild myocardial degeneration. [E & F] Heart tissue administered with *A. calamus* extracts (250 mg/kg b.w) and α-asarone (50 mg/kg b.w) after DOXO (150 mg/kg b.w) treatment also shows near normal architecture.
The mice pre-treated with α-asarone also showed highly-preserved appearance of cardiac muscle fibers with slight degeneration of the heart tissue as can be seen in Fig. 10.5. D. Almost similar result was observed in the post-treatment groups ie, DOXO+ A. calamus and DOXO+ α-asarone (Fig. 10.5 E & F). Thus the hearts of both the treated groups showed only minimal changes compared with the untreated mice.

10.4. DISCUSSION

The anthracycline derivative doxorubicin is widely used to treat various types of cancer; however, its clinical use is limited by dose-dependent cardiotoxicity, leading to severe congestive heart failure, often termed “doxorubicin cardiomyopathy.” The present work was designed to investigate the potential cardioprotective effect of the extract of A. calamus and its pure component α-asarone.

The involvement of oxygen free radical, superoxide radical and oxidative stress has been strongly accepted as crucial factors in the pathogenesis of DOXO-induced cardiac damage (Mimnaugh et al., 1985; Sarvazyan et al., 1995) Heart tissue is particularly vulnerable to free radical injury, because it contain low levels of free radical detoxifying enzymes such as SOD, GSH and CAT. Furthermore DOXO has a high affinity for the phospholipid component of the mitochondrial membrane in cardiac myocyte, leading to accumulation of DOX in the heart tissue (Takacs et al., 1992). From the present study it is clear that administration of DOXO in a single dose of 25 mg /kg, i.p. induced oxidative stress in cardiac tissues as manifested elevated serum levels of LDH and CK-MB. Actually, these enzymes are considered important markers of early and late cardiac injury especially during clinical follow-up of doxorubicin therapy (Fadillioglu and Erdogan, 2003). The increased levels LDH in serum suggest an increased leakage of this enzyme from mitochondria as a result of toxicity induced by DOXO treatment. Many previous studies have demonstrated similar elevations in cardiac enzymes activities in rats following challenge with a single cumulative dose of doxorubicin (Yagmurca et al., 2003; Nagi and Mansour, 2000). Administration of A. calamus extract or α-asarone, prior to or after DOXO significantly protects mice from DOXO-induced elevated LDH and CK-MB levels. The levels of serum GOT and GPT were elevated in DOXO treated animals and administration of A. calamus extract or α-asarone significantly reversed the levels of GOT and GPT when compared to DOXO alone treated control animals. When
compared to the effect of A. calamus extract with that of its pure component α-asarone on cardiac serum parameters, pre-treatment with α-asarone gave better protection than that of its crude extract and almost similar effect was observed in the post treated group except in the case of SGOT.

Doxorubicin administration induced oxidative stress in cardiac tissues as manifested by the alterations observed in cardiac antioxidant defense systems both enzymatic and nonenzymatic. From the present study it is clear that DOXO reduced significantly the cardiac GSH content, besides it notably lowered the cardiac enzymatic activities of SOD and GPx associated with a marked increase in cardiac lipid peroxidation as manifested by increased MDA level. Both ACE and α-asarone inhibited the DOXO induced consequence and significantly increase the antioxidant enzymes SOD and glutathione peroxidase. In addition both A. calamus extract and α-asarone normalized oxidative stress markers such as MDA, and also cardiac GSH status, indicating a significant reduction in the extent of oxidative myocardial damage by restoring the total antioxidant capacity. This DOXO-induced myocardial damage was also confirmed by myocardial histopathological changes and the histopathological reports suggest that pre and post treatment of A. calamus extract and α-asarone attenuate the DOXO induced changes in cardiac tissue supporting the protective action of ACE and α-asarone against DOXO induced cardiotoxicity. The studies revealed that the pre treatment with A. calamus extract and α-asarone offered enhanced protection than that with the post treatment, which may be due to the pre activation of the antioxidant enzymes in the animal system.

Though the exact mechanisms whereby doxorubicin would induce cardiac toxicity is not fully explored, the principal mechanism could possibly be through free radical generation by the “redox-cycling” of the anthracycline molecule and/or by the formation of anthracycline-iron complexes (Hrdina et al., 2000). This concept of oxidative damage has been well documented in a plethora of previous reports (Li et al., 2000; Morishima et al., 1998). Hence administration of A. calamus extract or α-asarone ahead and after doxorubicin challenge to mice ameliorated all the biochemical parameters altered by the cytotoxic anticancer drug and thus offered cardioprotection possibly through antioxidant and anti-radical effects.

To conclude formation of free radicals from doxorubicin recycling is a crucial factor in the pathogenesis of doxorubicin cardiotoxicity. Administration of A. calamus
extract or α-asarone before and after DOXO treatment in Swiss albino mice ameliorated all the biochemical parameters altered by this cytotoxic anticancer drug. A. calamus extract or α-asarone prevented the DOX induced myocardial toxicity by boosting the endogenous antioxidant activity, restoring the cardiac biomarker enzymes like CK-MB and LDH and preventing the degeneration of cardiac tissue. Thus, both A. calamus extract and α-asarone could possibly improve the therapeutic benefits of doxorubicin.