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PROTECTIVE EFFECT OF *SANTALUM ALBUM* ON DOXORUBICIN INDUCED CARDIOTOXICITY IN RATS

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ABSTRACT

Doxorubicin (DOX), the use of (a potent antitumor antibiotic) has been limited due to generation of ROS followed by the development of life-threatening cardiomyopathy. The aim of this study was to determine the protective effect of aqueous extract of *Santalum album* in acute doxorubicin induced cardiotoxicity. Since it is the part of several unani and ayurvedic formulations used for cardioprotection since long. Thirty male wistar rats used in study were divided in five groups v.i.z. normal control, doxorubicin control, standard control, test sample Dose 1 and Dose 2. The study was carried out up to 31 days and animals were sacrificed on 32nd day. It was found that doxorubicin treatment increased MDA, LDH, CK-MB levels significantly ($P < 0.01$), which were decreased by administration of *S. album* aqueous extract (SAAE) and desferrioxamine, significantly. The level of IL-6 and TNF-α in serum whereas caspase-3 in cardiac tissue were also reversed significantly on administration of SAAE. The histopathological studies showed that administration of *S. album* attenuated the sign of vacuolization and myocardium structure appears to be restored compared with DOX only group. There is a marked reduction of vacuolization and myocardium appears to be normal in desferrioxamine treated group. Antioxidant effect of SAAE has been shown to decrease doxorubicin induced cardiotoxicity.

KEYWORDS: *Santalum album*, doxorubicin, *in-vitro*, cardiotoxicity.

INTRODUCTION

Doxorubicin is a well-known drugs used in chemotherapy having a large activity against solid tumors and malignancies in humans. The use of doxorubicin is limited due to its

cardiotoxicity [1] Lipid peroxidation and inhibition of long chain fatty acid oxidation in cardiac tissues is the primary pathway of induction of cardiotoxicity by doxorubicin.[2] The other reports showed the production of reactive oxygen species (ROS) as the chemical structure of DOX is prone to generation of free radicals. It is associated with the reduction in the level of cardiac tissues antioxidants which causes scavenging of free radicals. [3] This oxidative stress led to the damage to the heart by damaging the heart tissues.

Santalum album family Santalaceae is the drug which is used in many herbal unani and ayurvedic preparations which are used as cardiotonics. Khamira abresham hakim arshadwala and khamira marwareed etc. Many scientific studies have proven the cardioprotective effects of some unani drugs.[4] Several phytochemical investigations on *S. album* have led to the isolation of Antiinflammatory, Anti-pyretic, Antifungal, Antibacterial compounds, hypotensive agent or blood pressure depressant.[5] The free radical scavenging effects of *S. album* on nitric oxide was measured *in-vitro*.[6]

Furthermore, the use of *S. album* as a cardioprotective agent is emphasized because of the low toxicity and anticancer activity of α santalol a sesquiterpene alcohol. In view of this, since DOX-induced cardiotoxicity is linked to oxidative stress, The iron chelator, desferrioxamine (DFX), is used in the treatment of iron overload status and prevents interaction of DOX with iron, thus preventing DOX-induced damage in cultured heart cells.[7] Desferrioxamine acts as an antioxidant through its ability to decrease the amount of free iron available for the ROS production through the formation of DOX-iron complex, [8] pretreatment of DFX can prevent the cardiotoxicity induced by doxorubicin[9] therefore it is selected as a standard drug in present study.

Here, we examine the potential protective role of *S. album* in doxorubicin-induced heart damage in rats. Our results show that pre-treatment with *S. album* significantly suppresses cardiomyopathy induced by doxorubicin in wistar rats.

2. MATERIALS AND METHODS

2.1. Plant material and preparation of extract

Santalum album wood was procured form the local market khari baoli, Delhi and identified by Dr. S. Ahmad, Department of Pharmacognosy and Phytochemistry, Hamdard University, New Delhi, a sample specimen was deposited in the herbarium of the Bioactive Natural Product Laboratory. Specimen no-54/SA/BNPL/2012. 250 grams of powdered drug soaked

in water and extracted on a heating mantle. finally extract was filtered through a muslin cloth and concentrated in a rotary evaporator under reduced pressure to obtain a thick semisolid brown paste. The final yield obtained was found to be 2 g (w/w).

2.2. *In vitro* antioxidant activity

In vitro antioxidant activity was determined by different methods such as Reducing power method, Hydrogen peroxide scavenging activity and Superoxide anion radicals scavenging activity.[10] Control sample was prepared containing the same volume without any extract and reference was ascorbic acid.

2.2. In Vivo Study

2.2.1. Animals

In-vivo study was carried out in Adult male Wistar rats 10-12 weeks old weighing 150-200g. Animals were issued from central animal house facility of Jamia Hamdard. Study protocol was approved by Jamia Hamdard Animal Ethics Committee (JHAEC) with registration number 173/CPCSEA. All animals were housed at the temperature of 20-25°C under a 12 h light/dark cycle throughout the experiment and fed a standard diet *ad libitum*. All animals received humane treatment and study was conducted in accordance with the strict guidelines of Institutional ethics committee of Jamia Hamdard.

2.2.2. Experiment design

Thirty animals were randomly divided into five groups of six animals each. Group I (NS) served as normal control and received 0.5% Carboxy methyl cellulose (CMC; p.o.) for 28 days and normal saline (s.c.) on days 29 and 30. Group II (DOX) served as positive control and received 0.5 CMC (p.o.) for 28 days and doxorubicin (20 mg/kg body weight, s.c.) on 30th day while, the remaining groups [GroupIII (DOX + SA30), Group IV (DOX + SA60) received respectively 30 and 60 mg/kg body weight of SAAE daily for 30 days and group V (DOX + DFX)] received 50mg/kg body weight of desferrioxamine 30 days (i.p.) and DOX (20 mg/kg, i.p.) on 31st day.

The protocol for DOX treatment schedule was as per the previous works from Sharma H. et al. 2011 [11]. At the end of the experimental period (i.e. 32nd day), animals were fasted overnight (12 h) and blood samples were collected from retro-orbital sinus under mild ether anesthesia. Plasma was obtained by cold centrifugation of samples at 3000 rpm for 10 min.

Later, animals were sacrificed by cervical dislocation under mild anesthesia and heart was excised and stored at 80 ^{o}C for further evaluations.

2.2.3. Biochemical estimations

Extent of lipid peroxidation was estimated by the method reported by Iqbal *et al* (2008) [12]. Serum CK-MB and serum LDH activity were estimated by spectrophotometrically using commercially available kits (Reckon Diagnostics Pvt. Ltd, India). (Lum and Gambino, 1974)[13]. Caspase-3 activity was estimated in heart tissue by ELISA method using commercially available kit ((Bio Vision, Inc., USA) (Jaeschke et al., 1998) [14]. and TNF-α levels was estimated in heart tissue by ELISA method using commercially available kit (eBioscience, Inc., USA) (Lehmann et al., 2008).[15] Similarly, quantitative measurement of IL-6 was performed in serum samples by ELISA method using commercial kit (Ray Biotech, Inc., USA). (Helle et al., 1991). [16] and total protein estimation in both serum and heart tissue as per the method followed by (Lowry et al., 1951)[17]

2.2.4. Histopathological examination of heart sections

Formalin fixed heart sections were embedded in paraffin wax, serially sectioned (3–5 lm), and stained with Hematoxylin and Eosin, for assessment of histopathological changes.

2.2.5. Statistical analysis

Statistical analysis was carried out using Graph pad prism 3.0 (Graph pad software San Diego, CA). All results are expressed as mean ± S.E.M. Groups of data were compared with the analysis of variance (ANOVA) followed by Dunnett's *t*-test to identify significance among groups. Values were considered statistically significant when $p < 0.05$.

3. RESULTS

In vitro antioxidant activity

The reducing potential of the *S. album* extract and ascorbic acid was very potent and the power of the extract was increased with quantity of sample [Figure 1A]. It was determined by using a modified iron (III) to iron (II) reduction assay. As shown in [Figure 1B], *S. album* extract also demonstrated H_2O_2 decomposition activity in a concentration dependent manner with an IC_{50} of 17.27µg/ml, while IC_{50} value for ascorbic acid was 6.05µg/ml.

The superoxide anion radical scavenging activity of *S. album* assayed by the PMS-NADH system is shown in [Figure 1C]. The half inhibition concentration (IC_{50}) of *S. album* extract

was 39.24 µg/ml while IC $_{50}$ value for ascorbic acid was 14.90 µg/ml. These results suggested that *S. album* extract has an effective superoxide radical scavenging effects.

Table 1. Effect of aqueous extract of *S. album* on biochemical parameters in serum and cardiac tissue

Biochemical estimation in serum					
	Control	**DOX**	**DFX**	**SA30**	**SA60**
IL-6 PG/ML	82.25±0.957**	429.25±8.261#	146.5± 4.041**	377.5±10.908**	332.5± 9.146**
CKMB IU/L	624.56± 4.64**	2133.29± 86.08#	698.96± 40.92**	1681.24±31.66**	1605.28± 43.18**
LDH IU/L	845.09± 57.5**	1914.19± 62.49#	964.69± 27.34**	1666.9± 52.61**	1574.06± 32.07**
TNF α PG/MG	0.0109±0.0003**	0.0619± 0.0009#	0.0252±0.0007**	0.051± 0.0008**	0.0490± 0.0005**
Biochemical estimation in cardiac tissue					
MDA NMOL/MG	2.491± 0.12**	10.625± 0.09#	2.701± 0.10**	7.684± 0.10**	7.499± 0.07**
CASPASE-3 %	100.06±2.32**	257.86± 11.98#	105.22± 3.33**	217.75± 12.23**	204.19± 11.70**

Each value is represented as mean ±SD. No of animals (n) =6. #P<0.01 when toxic control compared with control, **P<0.01 Vs toxic control. One way ANOVA followed by Dunnett's test

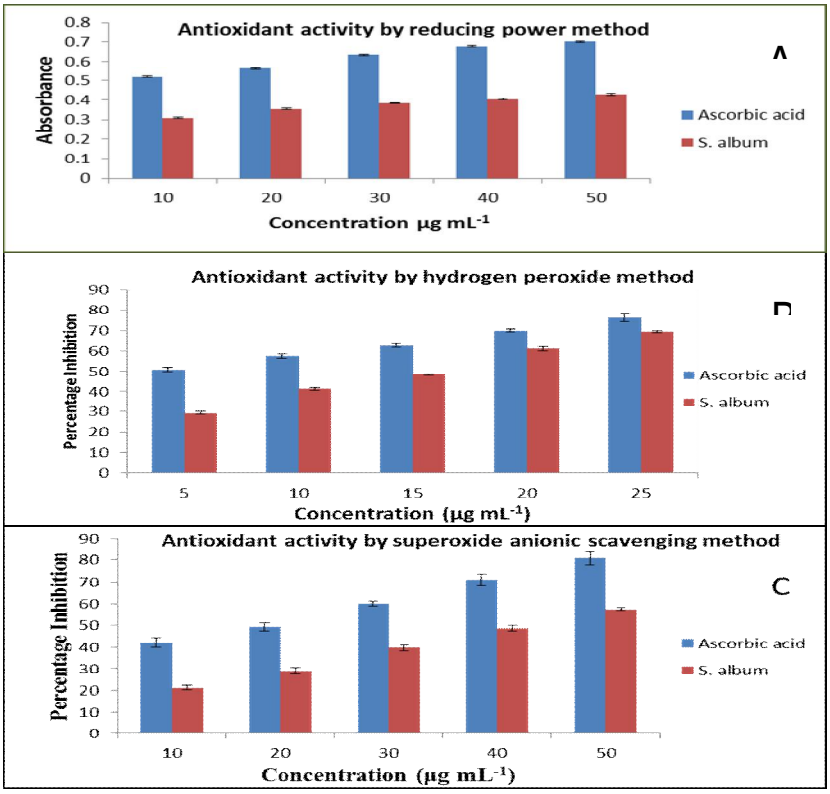

Fig. 1. *In vitro* antioxidant activity by (A) Reducing power (B) hydrogen peroxide (C) superoxide scavenging method

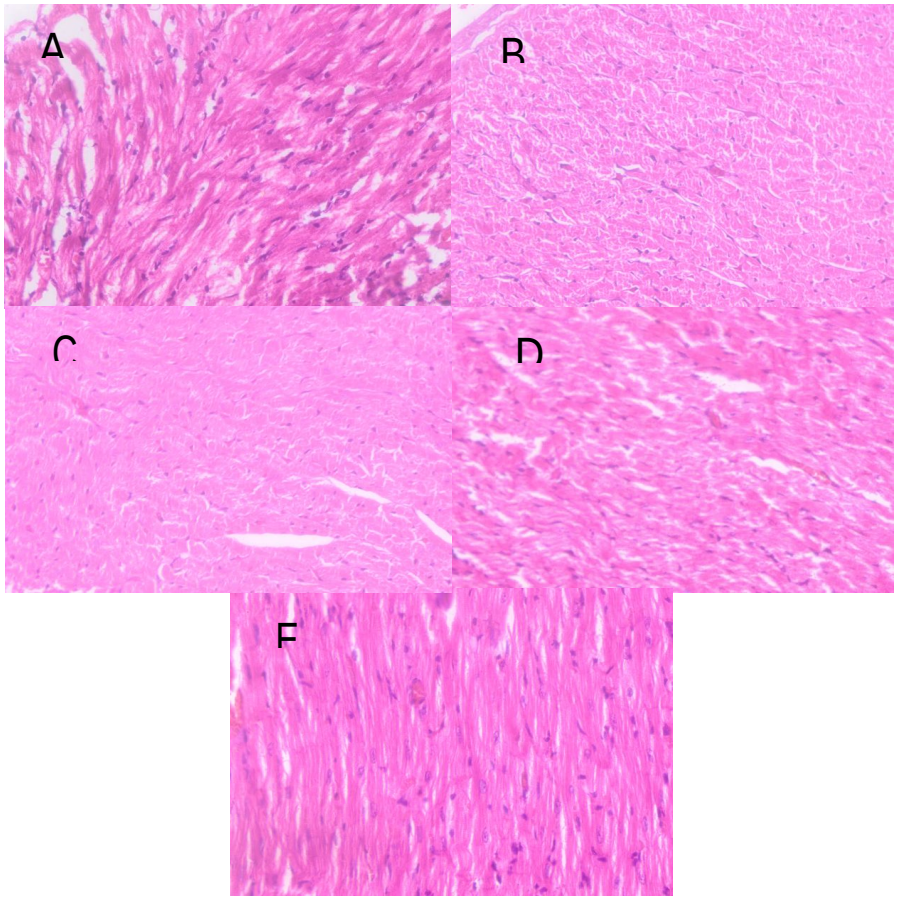

Fig. 2. Photomicrographs of heart tissue (H&E 40×).

A) Photomicrograph of pathogenic control group showing cardiotoxic myocardium with perinuclear vacoulization (H&E 40×).

B) Photomicrograph of vehicle control group showing myocardium with normal architecture (H&E 40×).

C) Photomicrograph of desferrioxamine [50 mg/kg, i.v. +DOX] showing myocardium with no evidence of necrosis, inflammation or fibrosis (H&E 40×).

D) Photomicrograph of SA 30 [30 mg/kg SAAE single p.o. + DOX] showing that pre-treatment moderately restored the myocardium with no evidence of vacuolization, inflammation or fibrosis (H&E 40×).

E) Photomicrograph of SA 60 [60 mg/kg SAAE single p.o. +DOX] pre-treatment shows sporadic sign of vacuolization and myocardium structure appears to be restored (H&E 40×).

3.1. Effect on Lipid Peroxidation

Estimation of LPO level in the serum of animals from different groups showed that mean serum LPO level were significantly ($P<0.01$) elevated in doxorubicin treated group (i.e. group II, TOX) compared to normal control group (i.e. Group I).). Treatment with SAAE at

30mg/kg (i.e. SA30, group III) and 60 mg/kg (i.e. SA60, group IV) respectively showed significant (P<0.01) decrease in LPO level compared to TOX group (i.e. group II). Desferrioxamine (i.e. Group V, STD) at a dose of 50 mg kg^{-1} (i.v.) also showed significantly (P<0.01) reduced LPO level compared to toxicant group (Table 1).

3.2. Effect on CK-MB level

Estimation of CK-MB level in the serum of animals from different groups showed that mean serum CK-MB level were significantly (P<0.01) elevated in doxorubicin treated group (i.e. group II, TOX) compared to normal control group (i.e. Group I). Treatment with SAAE at 30mg/kg (i.e. SA30, group III) and 60 mg/kg (i.e. SA60, group IV) respectively showed significant (P<0.01) decrease in CK-MB level compared to TOX group (i.e. group II). Desferrioxamine (i.e. Group VII, STD) at a dose of 50 mg kg^{-1} i.v. also showed significantly (P<0.01) reduced CK-MB level compared to toxicant group (Table 1).

3.3. Effect on LDH level

Serum LDH level were significantly (P<0.01) elevated in TOX group (i.e. Group II) compared to CNT group (i.e. normal control, Group I). Treatment with SAAE at 30mg/kg (i.e. SA30, group III) and SA60 (i.e. 60mg/kg, group IV) reduced LDH level significantly (P<0.01) compared to doxorubicin induced group (i.e. Group II, TOX). Treatment with DFX also showed significant reduction (P<0.01) in LDH level compared to toxicant (i.e. TOX, group II) (Table 1).

3.4. Effect on IL-6 level

Estimation of mean serum IL-6 level in doxorubicin induced group (i.e TOX, Group II) showed significantly (P<0.01) elevated IL-6 level as compared to group treated with normal saline (i.e. CNT, group I). Groups treated with SAAE at doses of 30mg/kg (i.e. SA30, group III) and 60mg/kg (i.e. SA60, group IV) showed significantly (P<0.01) less serum IL-6 levels compared to TOX group (i.e. group II). Similarly, treatment with standard drug (i.e. STD, group VII) showed significant (P<0.01) reduction in IL-6 level compared to TOX (Table 1).

3.5. Effect on caspase-3 activity

Caspase-3 activity in doxorubicin treated was increased significantly (P<0.01) to more than 2.5 fold of baseline level of control (i.e. CNT, group I) group. Treatment with SAAE at 30mg/kg (i.e. SA30, group III) and at 60mg/kg (i.e. SA60, group IV) caspase activity was significantly (P<0.01) decreased in comparison to toxicant to approximately 1.4 fold and 1.6

fold of the control level (i.e. CNT, group I) respectively. The STD group also showed significant reduction (P<0.01) compared to toxicant (i.e. TOX, group II) (Table 1).

3.6. Effect on TNF-alpha level

The TOX group (i.e. group I) showed significant (P<0.01) elevation in TNF-α level compared to normal healthy rats (i.e CNT, group I). Whereas, treatment with SA at 30 mg/kg (SA30, group III) and 60 mg/kg (SA60, group IV) decreased TNF-α level significantly (P<0.01) compared to toxicant treated group (i.e. TOX, group II). The STD group also showed significantly lower (P<0.01) TNF alpha level compared to TOX group (Table 1).

3.7. Histopathology

Samples from cardiac tissue from animals belonging to different treatment groups were examined with special reference to myocardial fibre integrity and histological evidence of DOX induced cardiac damage. CNT group (i.e. group I) showed normal myocardial structure (Fig.2B). The doxorubicin treated group (i.e. TOX, group II) showed disarray of myocardial cells with small and large vacuolar myopathy (Fig.2A). Cardiac tissue of groups treated with SA at 30 mg/kg (i.e. SA 30, group IV) showed moderate vacuolated myopathy with partially restoration of typical myocardium structure (Fig. 2D). Cardiac tissue of groups treated with SA at 60mg/kg (i.e. SA60, group IV) (Fig 2E) showed less evidence of vacuolar myopathy and fully restored typical myocardium structure and STD group (i.e. group VII) (Fig. 2C) showed a normal myocardium with no evidence of vacuolar myopathy.

4. DISCUSSION

Doxorubicin, is a synthetic anthracycline derivative, has toxic effect on the myocardium. According to proposed mechanism, generation of reactive oxygen species (ROS) lead to degenerative changes in cardiac tissue that culminate in depletion in the indigenous antioxidants results in doxorubicin-induced cardiac damage. CK-MB and LDH are normally found in the blood at low levels, DOX treated rats herein showed significant increment in serum levels of these enzymes.[18,19] However, meticulous ability of SAAE to effectively prevent these changes clearly points towards its cardio-protective ability and maintenance of myocardium integrity.

Oxidative stress induced by administration of Doxorubicin significantly reduces antioxidant capacity in heart and blood.[20] From the present study, it is clear that DOX significantly increases the cardiac lipid peroxidation as expressed by increased MDA level. Treatment

with 30 mg/kg and 60 mg/kg doses of SAAE for 30 days inhibited the DOX induced oxidative stress and significant decrease in tissue MDA levels.

Doxorubicin induces cell death of monocytes and macrophages. The acute inflammation induced by doxorubicin is associated with apoptosis of monocytes/macrophages which increases levels of inflammatory cytokines such as IL-6 and TNF-α .[21,22] TNF-α level is enhanced due to increased oxidative stress in various pathophysiological conditions.[23] these changes promotes the initiation of various other cell toxicants on endothelial cell and myocytes. Doxorubicin, in our study significantly increases the IL-6 and TNF-α level in serum. This confirmed the previous studies which reported that DOX cardiotoxicity is also mediated by inflammatory cytokines. Our results shows reduction in IL-6 levels suggest that treatment with SAAE is potentially protective against doxorubicin cardiotoxicity by decreasing apoptosis in cardiac tissues. TNF-α is one of the proinflammatory cytokines which mediate cardiac damage,[24] Similar to IL-6, TNF-alpha level in SAAE group at 30 and 60 mg kg^{-1} showed significant difference compared to TOX group. Decreased TNF-alpha levels were observed after treatment with SAAE. These observations of TNF-alpha levels indicate that the antioxidant property of SAAE protect by decreasing free radical release and the apoptosis caused due to 20 mg kg^{-1} i.p. single dose of DOX.

Increased ROS production leads to activation of caspases, the executioners of apoptotic pathway. These changes lead to myocytes damage and ultimately dysfunction of heart. Estimation of caspase-3 enzyme in our study showed significantly higher levels in doxorubicin treated groups. Previously, Pathan et al. and Abeer E. El-Mehy et al [25, 26], have confirmed that DOX induced cardiotoxicity is mediated via caspase-3 dependent apoptotic pathway.

Further the histopathological studies suggest that treatment of SAAE greatly inhibited the DOX induced changes in cardiac tissue supporting the protective action of SAAE against DOX induced cardiotoxicity.

Santalum album contains mixture of primary sesquiterpene alcohols, tannins, terpenes, resins and waxes. It has been reported to have nitrous oxide scavenging activity and DPPH antioxidant activity. [6,27] This antioxidant activity is may be due to the presence of these sesquiterpene alcohols and tannins. Results of our in vitro antioxidant activity showed that *S. album* is having antioxidant potential. Results of our study showed that treatment with SA reduce levels of inflammatory and pro-apoptotic biomarkers due to DOX. So it can be

concluded from the present study that SA can protect cardiac tissue from oxidative stress induced cell injury and lipid peroxidation. And it also interferes with DOX-induced inflammatory and apoptotic induction in cardiac tissue. Therefore, present experiment can justify the further development of *Santalum album* as a protective agent against DOX-induced cardiotoxicity.

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