Protective Effect of *Solanum Torvum* on Doxorubicin-Induced Cardiotoxicity in Rats

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Summary

Oxidative stress is the main factor in Doxorubicin (DOX) induced cardiotoxicity. Wistar rats received either DOX (67.75 mg/kg, i.v, 2 days before sacrifice) or *Solanum torvum* extract (100 mg/kg and 300 mg/kg, p.o.) prior to DOX or *S. torvum* (100 mg/kg and 300 mg/kg, p.o.) extract alone for 4 weeks. Cardiotoxicity was assessed by recording changes in ECG, heart rate and measuring the levels of cardiac marker enzymes- lactic acid dehydrogenase (LDH) and creatine phosphokinase (CK-MB). The antioxidant defence enzymes superoxide dismutase (SOD) and catalase (CAT) for heart tissue and histopathological changes were also measured at the end of the treatment schedule. Treatment with *S. torvum* (100 mg/kg and 300 mg/kg) significantly (p<0.05) reversed the changes in ECG; decreased the levels of CK-MB and LDH; and increased the antioxidant defence enzyme levels of SOD and CAT. *S. torvum* treated animals showed a lesser degree of cellular infiltration in histopathological studies. The results suggest that *S. torvum* has the potential in preventing the cardiotoxicity induced by Doxorubicin.

Key words: *S. torvum*, marker enzymes, antioxidant defence enzyme, doxorubicin

Running title: *Solanum torvum* on Doxorubicin cardiotoxicity

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Introduction

Doxorubicin is an anthracycline glycoside antibiotic that possesses a potent and broad spectrum antitumour activity against a variety of human solid tumours and haematological malignancies (1, 2). However its use in chemotherapy has been limited largely due to its diverse toxicities, including cardiac, renal, hematological and testicular toxicity (3, 4). Cardiotoxicity induced by DOX is usually mediated through lipid peroxidation and inhibition of long fatty acid oxidation in cardiac tissues (5-8). Thus, oxidative stress, lipid peroxidation, and mitochondrial dysfunction have been associated with DOX induced cardiomyopathy (7).

*Solanum torvum* Sw. (Solanaceae), commonly known as Turkey berry is an erect spiny shrub of about 4 m tall, evergreen and widely branched. It is native and found cultivated in Africa and West Indies (9). The fruits and leaves are widely used in Cameroonian folk medicine. The plant is cultivated in the tropics for its sharp tasting immature fruits. It is used in the treatment of stomach pain and skin infections (10). It possesses antimicrobial (11, 12) antiviral (13), immuno-secretory (14), antiulcer (15), antioxidant (16), analgesic and anti-inflammatory (17), cardiovascular and anti-platelet aggregation activities (18). *Solanum torvum* contains a number of potentially pharmacologically active chemicals like isoflavonoid sulfate and steroidal glycosides (13, 19), chlorogenone and neochlorogenone (20) triacontane derivatives (21, 22), 22-β-o-spirostanol oligoglycosides (23), 26-O-β-glucosidase (24). The free radical scavenging effects of *Solanum torvum* on DPPH (2, 2-diphenyl-1-picrylhydrazyl) was measured *in vitro*. The antioxidant properties of flavonoids and their ability to chelate free iron could be effective in reducing cardiotoxicity of DOX (25). In view of this, since DOX induced cardiotoxicity is linked to oxidative stress, we have investigated the possible protective effect of *Solanum torvum*, against DOX-induced cardiotoxicity in rats.

Materials and Methods

**Extract preparation**

Dried fruits of *Solanum torvum* Sw. (Solanaceae) were purchased locally and authenticated by Dr. S. C. Pal, NDMPV Samaj’s College of Pharmacy, Nashik, India. The voucher specimen has been deposited at Agharkar Research Institute, Pune, India. Mature fruits were collected, sundried and grounded. The powder obtained (1kg) was defatted using pet ether (60-80°C). The marc was macerated in ethanol for 3-4 days at room temperature. The filtrate was air dried and concentrated under reduced pressure to obtain 120 g, corresponding to a yield of 12.0 % w/w. The total flavonoid content of ethanolic extract of *S. torvum* was found to be 85.26±0.02 µg rutin equivalent/ mg of extract (26). The total phenolic content of ethanolic extract of *S. torvum* was found to be 99.52± 0.42 µg gallic acid equivalent/ mg of extract (27). Appropriate concentrations of the extracts were made in distilled water. The phytoconstituents present in the crude extract were flavonoids, alkaloids, tannins and saponins (28).

**Animals**

Laboratory breed Wistar albino rats of either sex weighing between 150-200 g, maintained under standard laboratory conditions of 25 ± 1°C, and photo period (12 h dark/12 h light) were used for the experiment.

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Commercial pellet diet (Amrut laboratory rat and mice feed, Sangli, India.) and water were provided *ad libitum*. The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethical Committee.

**Chemicals**

Doxorubicin (Oncodria, Sun Pharmaceutical Ind. Ltd. Gujarat, India) was obtained from the local market. 1,1-diphenyl, 2- picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, Mumbai. All chemicals for sensitive biochemical assays were obtained from Sigma Chemicals Co. India and Hi Media Chemicals, Mumbai, India. Distilled water was used for biochemical assays. LDH and CK-MB kits were obtained from Aspen Laboratories Pvt. Ltd., Baddi, India. and Teco Diagnostics, Anaheim, USA respectively.

**Experimental protocol**

During the acclimatization period, the baseline ECG was recorded (Chart 5.0 AD Instrument, Austria). The animals were then randomly divided into the following experimental groups with 5 animals in each group.

- **Group 1**: Vehicle treated group
- **Group 2**: DOX (67.75 mg/kg, i.v, 2 days before the sacrifice)
- **Group 3**: *Solanum torvum* (100 mg/kg, p.o.) daily for four weeks
- **Group 4**: *Solanum torvum* (300 mg/kg, p.o.) daily for four weeks
- **Group 5**: *Solanum torvum* (100 mg/kg, p.o.) daily for four weeks + DOX (67.75 mg/kg, i.v, 2 days before the sacrifice)
- **Group 6**: *Solanum torvum* (300 mg/kg, p.o.) daily for four weeks + DOX (67.75 mg/kg, i.v, 2 days before the sacrifice)

At the end of the treatment schedule, the animals were anaesthetized with diethyl ether, ECG recorded, and then sacrificed by a high dose of diethyl ether. Blood was withdrawn immediately for enzyme assays and the heart was dissected out and weighed. Heart tissue was washed with ice-cold 0.9% saline and homogenized quickly with ice cold 0.1 M Tris HCl buffer (pH 7.5) using Remi homogenizer to give a 10% homogenate.

**Electrocardiography (ECG)**

ECG was recorded before and after the treatment schedule. For ECG recording (Chart 5.0, ADI Instruments) rats underwent light ether anesthesia. Needle electrodes were inserted under the skin. For each ECG tracing, QRS complex, QT interval and ST intervals were measured (29).

**Preparation of Serum and tissue homogenate**

Blood was collected and allowed to clot. Serum was separated by centrifugation of the clotted blood at 5000 rpm for 4 min and used for estimation of LDH, CK-MB. Known amount of tissue was weighed and homogenized in ice cold 0.1 M Tris HCl buffer for estimation of SOD and CAT.

**Antioxidant Parameters**

Superoxide dismutase activity (SOD)
The assay of SOD was based on the ability of SOD to inhibit spontaneous oxidation of adrenaline to adrenochrome (30, 31). 0.05 ml supernatant was added to 2.0 ml of carbonate buffer and 0.5 ml of 0.01mM EDTA solution. The reaction was initiated by addition of 0.5ml of epinephrine and the autoxidation of adrenaline (3x10^4 M) to adrenochrome at pH 10.2 was measured by following change in OD at 480 nm. The change in optical density every minute was measured at 480 nm against reagent blank. The results are expressed as units of SOD activity (mg/wet tissue). One unit of SOD activity induced approximately 50% inhibition of adrenaline.

Catalase activity (CAT)
Catalase(CAT): The reaction mixture consisted of 2 ml phosphate buffer (pH 7.0), 0.95 ml of hydrogen peroxide (0.019M) and 0.05ml of supernatant in a final volume of 3 ml. Absorbance were recorded at 240 nm every 10 sec for 1 min. One unit of CAT was defined as the amount of enzyme required to decompose 1 µmol of peroxide per min, at 25°C at pH 7.0. The results were expressed as units of CAT U/g of wet tissue (32).

Cardiac marker enzymes
Blood samples were collected from each animal and the serum obtained by centrifugation was used for determination of LDH (LDH kit-Aspen Laboratories Pvt. Ltd.,Baddi; India.) and CK-MB (Teco Diagnostics, Anaheim, USA).

Histopathological examination
The hearts were excised and immediately fixed in 10% buffered formalin. The tissue specimen was embedded in paraffin after being dehydrated in alcohol and subsequently cleared with xylene. Five-micrometer thick serial histological sections were obtained from the paraffin blocks and stained with hematoxylin and eosin. The sections were examined under light microscope and photomicrographs were taken.

Statistical analysis
All data were expressed as the mean ± SEM. For statistical analysis of the data, group means were compared by one-way analysis of variance (ANOVA) followed by Dunnett’s test, P<0.05 was considered significant.

Results

ECG, heart rate and heart weight
Animals treated with DOX (67.75 mg/kg, i.v, 2 days before sacrifice) showed a significant (p<0.05) prolongation of QT interval and ST interval as compared to vehicle treated animals. Treatment with S. torvum extract (100 mg/kg and 300 mg/kg) alone has not shown any significant change as compared to vehicle treated group. S. torvum extract (100 and 300 mg/kg) significantly (p<0.05) decreased the prolongation of QT and ST intervals in animals treated with DOX as compared to DOX treated group. There was a significant increase in heart rate and a significant decrease in heart weight in DOX treated animals which was prevented by S. torvum extract (100 and 300 mg/kg) treatment for four weeks (Table 1).
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**Table 1:** Effect of ethanolic extract of *Solanum torvum* on ECG changes, heart rate and heart weight in DOX treated animals

<table>
<thead>
<tr>
<th>Sr no</th>
<th>Treatment group (mg/kg)</th>
<th>QT interval (mV)</th>
<th>ST changes (mV)</th>
<th>Heart rate (beats/min)</th>
<th>Heart weight per 100 gm BW (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 0.06±0.001</td>
<td>0.05±0.01</td>
<td>334.2±2.32</td>
<td>0.45±0.013</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DOX (67.75) 0.09±0.006*</td>
<td>-0.06±0.01*</td>
<td>397.8±3.22*</td>
<td>0.39±0.003*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ST (100) 0.05±0.01</td>
<td>0.03±0.004</td>
<td>331.1±14.7</td>
<td>0.45±0.007</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>ST (300) 0.05±0.002</td>
<td>0.02±0.001</td>
<td>277.4±5.65</td>
<td>0.45±0.003</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>ST (100) + DOX (67.75)</td>
<td>0.08±0.002</td>
<td>0.07±0.002#</td>
<td>352.6±3.61#</td>
<td>0.40±0.006#</td>
</tr>
<tr>
<td>6</td>
<td>ST (300) + DOX (67.75)</td>
<td>0.04±0.005#</td>
<td>0.00±0.000#</td>
<td>272.6±5.21#</td>
<td>0.45±0.004#</td>
</tr>
<tr>
<td>F (5,24)</td>
<td>10.72</td>
<td>57.19</td>
<td>44.04</td>
<td>43.39</td>
<td></td>
</tr>
</tbody>
</table>

N=5. The observations are mean ± SEM. * p<0.05 as compared to control and # p<0.05 as compared to DOX (ANOVA followed by Dunnett’s test).

**Cardiac marker enzymes**

Animals treated with DOX (67.75 mg/kg, i.v, 2 days before sacrifice) significantly increased the levels of cardiac injury markers i.e. serum LDH and CK-MB levels. Animals treated with *S. torvum* extract (300 mg/kg) alone has shown a significant increase in cardiac marker enzymes as compared to control group. Treatment with *S. torvum* extract (100 and 300 mg/kg) significantly (p<0.05) decreased the levels of LDH and CK-MB in DOX treated animals as compared to DOX group (Table 2).

**Table 2:** Effect of ethanolic extract of *Solanum torvum* on cardiac marker enzymes-CK-MB and LDH in DOX treated animals

<table>
<thead>
<tr>
<th>Sr no</th>
<th>Treatment group (mg/kg)</th>
<th>CK-MB (IU/L)</th>
<th>LDH (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>255.2±4.13</td>
<td>83.67±3.36</td>
</tr>
<tr>
<td>2</td>
<td>DOX (67.75)</td>
<td>570.4±15.17*</td>
<td>606.5±10.11*</td>
</tr>
<tr>
<td>3</td>
<td>ST (100)</td>
<td>266.4±5.04</td>
<td>90.25±7.55</td>
</tr>
<tr>
<td>4</td>
<td>ST (300)</td>
<td>423.3±32.5*</td>
<td>251.9±97.24*</td>
</tr>
<tr>
<td>5</td>
<td>ST (100)+DOX (67.75)</td>
<td>302.6±1.96#</td>
<td>98.33±2.20#</td>
</tr>
<tr>
<td>6</td>
<td>ST (300)+DOX (67.75)</td>
<td>474.8±32.5#</td>
<td>503.6±45.01#</td>
</tr>
<tr>
<td>F (5,24)</td>
<td>51.84</td>
<td>27.2</td>
<td></td>
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</table>

N=5. The observations are mean ± SEM. * p<0.05 as compared to control and # p<0.05 as compared to DOX (ANOVA followed by Dunnett’s test).

ST = Ethanolic extract of *Solanum torvum*, DOX= Doxorubicin, BW=Body weight.
Antioxidant enzymes

Animals treated with DOX (67.75 mg/kg, i.v, 2 days before sacrifice) significantly (p<0.05) decreased the levels of SOD and CAT. Animals treated with *S. torvum* extract (100 and 300 mg/kg) alone has significantly increased the levels of CAT as compared to vehicle treated group. Treatment with *S. torvum* extract (100 and 300 mg/kg) in DOX treated animals significantly (p<0.05) increased the levels of SOD and CAT as compared to DOX group (Table 3).

**Table 3:-** Effect of ethanolic extract of *Solanum torvum* on superoxide dismutase and catalase in heart of DOX treated animals

<table>
<thead>
<tr>
<th>Sr no</th>
<th>Treatment group (mg/kg)</th>
<th>SOD (mg/wet tissue)</th>
<th>Catalase (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>83.72±3.1</td>
<td>0.3481±0.0031</td>
</tr>
<tr>
<td>2</td>
<td>DOX (67.4)</td>
<td>38.27±0.58*</td>
<td>0.01407±0.0076*</td>
</tr>
<tr>
<td>3</td>
<td>ST (100)</td>
<td>70.8±7.1</td>
<td>0.3664±0.0014*</td>
</tr>
<tr>
<td>4</td>
<td>ST (300)</td>
<td>68.35±4.86</td>
<td>0.4433±0.0054*</td>
</tr>
<tr>
<td>5</td>
<td>ST (100)+DOX (67.4)</td>
<td>129.88±7.48#</td>
<td>0.0923±0.0016#</td>
</tr>
<tr>
<td>6</td>
<td>ST (300)+DOX (67.4)</td>
<td>215.9±24.58#</td>
<td>0.3392±0.0044#</td>
</tr>
<tr>
<td>F</td>
<td>(5,24)</td>
<td>32.65</td>
<td>1467.44</td>
</tr>
</tbody>
</table>

N=5. The observations are Mean ± SEM. * is p<0.005 as compared to control and # is p<0.005 as compared to DOX (ANOVA followed by Dunnett’s Test).

ST = Alcoholic extract of *Solanum torvum*, DOX= Doxorubicin.

**Histopathology**

In histopathological examination, normal architecture was observed in control animals whereas DOX induced cardiac changes were recognized by the presence of marked peripheral cell inflammation, necrosis and congestion (Fig 1). These changes were reduced significantly in animals which received *Solanum torvum* (100 and 300 mg/kg) prior to DOX treatment.
Fig. 1 Photomicrographs of histopathological examination (10 X) of heart tissue. Section A) Control group treated with vehicle shows normal architecture, section B) group treated with DOX (67.5 mg/kg) shows significant peripheral cell inflammation, necrosis and congestion, section C) group treated with ST (100mg/kg) shows normal architecture, section D) group treated with ST (300mg/kg) shows very mild congestion that may be due to increased blood flow to organ, section E) group treated with ST (100mg/kg) and DOX (67.5 mg/kg) shows significant reduction in peripheral cell inflammation, and mild congestion, section F) group treated with ST (300mg/kg) and DOX (67.5 mg/kg) shows mild congestion, which may due to the vasodilatation.

Discussion

The dose of DOX induced in this study corresponds to the dose that is currently being used in the clinical practice (33). The overall incidence of cardiotoxicity is about 3% at a total dose of 400 mg/m2 in human subjects (34). In rats the corresponding dose was found to be 67.75 mg/kg using the dose calculator results. Pilot studies in our laboratory indicated that the dose of DOX used in this study was effective to induce cardiotoxicity in rat.
The mechanism of DOX induced cardiomyopathy is not completely understood, but several hypotheses have been postulated which include inhibition of nucleic acid (35), protein synthesis (36), release of vasoactive amines (37), alterations in sarcolemmal Ca++ transport (38), alterations in membrane bound enzymes (39), abnormalities in mitochondria and lysosomal alterations (40) and an imbalance of myocardial electrolytes (41). Data derived from several laboratories suggest that Ca++ transport abnormalities occur in cardiac (42, 43) tissues exposed to DOX and that the myocardium eventually becomes overloaded with calcium (44, 45). This conclusion is based on total tissue calcium levels in hearts from treated animals and the observation of calcium phosphate accumulations in necrotic cells. The generation of free radical by DOX in the form of DOX semiquinone also has been suggested to play a major role in its cardiotoxic effects (46) by increasing oxygen free radical activity (47) and inducing the peroxidation of unsaturated lipids within the membranes (48). Semiquinones are unstable under aerobic conditions thereby generating superoxide anion radicals.

The ECG changes induced by DOX consisted of prolongation of QT interval, ST interval and widening of QRS complex. These ECG changes have been partly explained by Jensen (49). Whereas the QRS-intervals are directly related to cell depolarization, the QT interval is an expression of the late repolarization phase; the anthracyclines specifically prolong the later phase by disturbing the ion flux across the cellular membrane. It has been found that the above parameters are the most reliable ECG parameters for the assessment of DOX-induced cardiotoxicity (29). S. torvum extract (100 and 300 mg/kg) significantly decreased the prolongation of QT and ST intervals and heart rate in DOX treated groups. The heart weight was significantly decreased in DOX treated group which was prevented by S. torvum extract (100 and 300 mg/kg) treatment.

Administration of DOX to rats increased cardiotoxicity manifested by elevation in the levels of cardiac injury markers i.e. serum LDH and CK-MB levels. Our results are in good agreement with those previously reported (50, 51). However treatment with S. torvum extracts (100 and 300 mg/kg) in DOX treated group has resulted in reversal of cardiac enzyme activities (Table 2). The increase in cardiac marker levels in serum suggests an increased leakage of these enzymes from mitochondria as a result of toxicity induced by treatment with DOX.

The heart is particularly susceptible to free radical injury, because it contains less free radical detoxifying substances than do metabolic organs like liver or kidney (52, 53). Moreover DOX is known to have a higher affinity for cardiolipin, a major phospholipid component of the mitochondrial membrane in heart cells resulting in selective accumulation of doxorubicin inside cardiac cells (54).

The cardioprotective mechanism also appears to be through modulation of various antioxidant parameters thereby improving the overall antioxidant defence of the myocardial tissue. Free radical scavenging enzymes such as catalase, superoxide dismutase are the first line cellular defense enzymes against oxidative injury, decomposing O2 and H2O2 before their interaction to form the more reactive hydroxyl radical (OH·). The equilibrium between these enzymes is an important process for the effective removal of oxygen stress in intracellular organelles (55). In our study, a decrease in concentration of cardiac SOD and CAT levels in DOX treated group was observed. S. torvum extract (100 and 300 mg/kg) treatment significantly reversed the changes in antioxidant levels induced by DOX treatment. The fall in SOD levels may be due to the involvement of superoxide...
free radical in myocardial cell damage. A decrease in the activity of SOD can result in the
decreased removal of superoxide ion, which can be harmful to the myocardium (56).
There is a general agreement that flavonoids act as scavengers of reactive oxygen species
(57). Flavonoids have been found to protect heart from DOX induced cardiotoxicity
when co-administered with DOX in mice (58), which suggests that these compounds are
potential cardioprotectors against DOX, induced chronic cardiotoxicity. The antioxidant
properties of Solanum torvum could be attributed to the presence of flavonoid
phytoconstituent in it.
Cardiac histopathological features induced by DOX treatment was observed as significant
peripheral cell inflammation, necrosis and congestion. Pretreatment with Solanum torvum
extract reversed these abnormal changes. Thus, in conclusion the above data suggests that
Solanum torvum which is rich in flavonoids has the potential in preventing the
cardotoxic effects induced by doxorubicin.

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