Ameliorative effect of *Luffa acutangula* Roxb. on doxorubicin induced cardiac and nephrotoxicity in mice

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The present study reports protective effect of hydro-alcoholic extract of *Luffa acutangula* (HAELA) on doxorubicin (DXR) induced cardio and nephro toxicity in mice by studying various serum biomarkers, antioxidants in target organs and histoarchitecture alterations. Pretreatment with HAELA reversed significantly the elevated serum biomarkers, alanine amino transferase, lactate dehydrogenase and creatinine phosphokinase in heart and kidney in DXR treated mice. In addition, HAELA treatment inhibited elevated malondialdehyde formation and restored the depleted glutathione, catalase, superoxide dismutase in heart and kidney tissue. The altered histoarchitecture of heart and kidney tissue due to DXR treatment were also improved with HAELA. The protective activity observed with HAELA on DXR induced cardio and nephrotoxicity in mice was found to be related to its antioxidant property which finally results in membrane stabilization.

**Keyword:** Antioxidants, Biomarkers, Cardioprotective, Doxorubicin, *Luffa acutangula*, Nephroprotective activity

**Introduction**

Doxorubicin (DXR), a derivative of anthracycline, is a clinically established efficacious drug in the treatment of variety of cancers viz. leukemia, soft tissue, breast, small cell of the lung and esophagus cancer¹-³. Albeit, DXR elicits wide spectrum of anticancer activity, the presence of quinone moiety in the anthracycline ring structure has substantial clinical importance because of its involvement in both reductive and oxidative biotransformation, and finally generates highly reactive chemical species responsible for cardio, hepato and nephrotoxicity on acute and chronic treatment⁴-⁶. Such toxic effects of DXR are related to free radicals formation, lipid peroxidation, mitochondrial damage, decreased activity of Na⁺-K⁺-ATPase, vasoactive amine release, impairment of myocardial adrenergic signaling/regulation pathways, elevated serum cholesterol, triglyceride and low density lipoproteins⁷-¹¹,¹². DXR induced renal injury in rats manifest in increased glomerular capillary permeability and tubular atrophy, and found to be directly related to its oxidative stress¹³,¹⁴.

Many herbs/natural products having antioxidant property have showed cardioprotective activity in experimentally induced cardiac necrosis in different animal models¹⁴,¹⁵-¹⁷. The herb, *Luffa acutangula* Roxb. (Cucurbitaceae) is bitter and exhibit various pharmacological activities like CNS depressant, immunomodulatory, antitumor, anti-HIV, anti-inflammatory, hepatoprotective, larvicidal and in vitro antioxidant¹⁸-²². The major phytocomponents of *L. acutangula* are β-carotene, flavonoids, luffin, amarin, 2-deoxycucurbitacin B, acutosides A-G, acutosides H-I, oleanolic acid saponins, luffangulin, novel ribosome inactivating peptide²³-⁳⁰. Experimental studies in animals have demonstrated that antioxidant from natural products viz. lycopene and alpha-tocopherol showed protection against DXR-induced toxicity and also improved its therapeutic efficacy¹⁶,¹⁷ in cancer patients.

Considering such pharmacological activity profile and the presence of phytoconstituents and membrane stabilization property of *Luffa acutangula* (HAELA), it was thought worthwhile to study the protective effect of HAELA on DXR induced cardio-nephrotoxicity in mice.
Materials and Methods

Collection and authentication of plant—The fresh fruits of *L. acutangula* were collected from Hindustan Antibiotic Colony, Pimpri, Pune, India in the month of December. The plant was authenticated and deposited at Agharkar Research Institute, Pune, India (Voucher No. Auth 08-005).

Reagents—Thiobarbituric acid (Spectrochem Pvt. Ltd., Mumbai, India), 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB) and epinephrine (Sigma Chemical Co., St Louis, MO, USA), doxorubicin hydrochloride injection, 2 mg/mL (Khandelwal Laboratory Pvt Ltd Mumbai, India) were purchased. All other chemicals used for the assay of antioxidant enzymes (glutathione, catalase and superoxide dismutase) and chemicals used for the assay of antioxidant enzymes (glutathione, catalase and superoxide dismutase) and serum biomarkers (alanine amino transferase, lactate dehydrogenase and creatine phosphokinase) were of analytical grade and procured from local suppliers.

Extraction process—The shade dried fruits of *L. acutangula* were pulverized. The powdered plant material (850 g), was cold macerated (70%; v/v) in aqueous ethanol for 72 h, repeated for 4 times at 30 °C to ensure the complete extraction. The residue was removed by filtration and filtrate evaporated to dryness under reduced pressure at 42±2 °C using rotary evaporator. Various phytochemical constituents and physicochemical properties of HAELA were studied.

Preparation of HAELA suspension for animal experiments—A uniform suspension of HAELA was prepared in (1%, w/v) carboxymethyl cellulose (CMC) and used for cardio-nephroprotective activity evaluation mice.

Animals—Swiss albino mice (20-25 g) of either sex were obtained from National Toxicology Center, Pune, India. The animals were housed and maintained in clean polypropylene cages under standard conditions: 50 ± 5 %, RH at 25 ± 2 °C and (12:12 h L:D), fed with standard diet (Amrut Laboratory Animal Feed, Sangli, Maharashtra, India) and water ad libitum. Experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee and conform to the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in Research. Animal house registration number with Govt of India is 198/99/CPCSEA.

Acute oral toxicity studies—Acute toxicity study of HAELA was performed on mice following OECD guidelines. Different doses of HAELA were administered up to 2000 mg/kg orally (po) to mice and they observed for a period of 72 h for behavioural changes, toxic reactions and mortality if any.

Experimental design—The mice were allowed to acclimatize for 7 days in animal house conditions prior to experimentation. Mice were fasted overnight and randomly divided into following 5 groups (10 mice/group). The treatment schedule and experimental design for DXR cardio and nephro toxicity was performed by the method of Singal and Iliskovic32 with minor modification.

Group I - mice received CMC (1 %, w/v; 10 mL/kg, bw, po) daily from 1st to 10th day.

Group II - mice received DXR (2 mg/kg, bw, ip) on 1st, 3rd, 5th and 7th days and CMC (1 %, w/v; 10 mL/kg, bw, po) daily from 1st to 10th day.

Group III, IV and V - mice received DXR (2 mg/kg, bw, ip) on 1st, 3rd, 5th and 7th and HAELA (100, 200 and 400 mg/kg, bw, po), respectively daily from 1st to 10th day.

Serum biomarkers—Mice were anaesthetized with diethyl ether 24 h after the administration of the last dose (on day 11) and blood was collected by cardiac puncture, allowed clotting at room temperature. Serum was separated by centrifugation at 3000 rpm at 30 °C for 15 min. The separated serum was used for the assay of serum biomarkers viz. serum aspartate aminotransaminase (AST)32 lactic dehydrogenase (LDH)33 and creatine phosphokinase (CPK)34.

Histopathological studies—After the collection of blood, heart and kidneys were surgically removed and weighed accurately. One kidney and half heart (cut vertically into 2 parts) were used for histoarchitecture studies. The slices of heart and kidney were initially washed with cold normal saline and then fixed in 10% formalin solution. After dehydration, the slices of heart and kidney were embedded in paraffin wax cut into 4 × 6 µm thick section and stained using haemotoxylin and eosin. The tissue sections were observed under 100 X microscope for changes in histoarchitecture and mycographs were taken.

Preparation of cardiac and renal homogenates—Other part of heart and kidney were washed with cold saline solution and 10% (w/v) homogenates were prepared using motor driven glass homogenizer in ice cold tris buffer (10 mM, pH 7.4). An aliquot of the homogenate was used for the assay of malondialdehyde (MDA) formation. The homogenates further centrifuged at 7000 rpm for 10 min at 4 °C and supernatant were used of assays of GSH, SOD and CAT.
**Determination of cardiac and renal MDA**—Quantitative assay of MDA was performed by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in heart and kidney tissue homogenates by the method of Ohkawa and Nobuko. The amount of MDA formed was quantified by reaction with TBARS and used as an index of lipid peroxidation. The results were expressed as nmol of MDA/g of wet tissue using molar extinction coefficient of the chromophore \((1.56 \times 10^5/M/cm)\) and 1, 1, 3, 3-tetraethoxy propane as standard.

**Determination of cardiac and renal glutathione**—Glutathione (GSH) was assayed in the supernatant of heart and kidney homogenates using DTNB method described by Ellman and the results were expressed as µmol of GSH/g of wet tissue.

**Determination of cardiac and renal catalase (CAT)**—CAT was assayed in the supernatant of heart and kidney homogenates as per Clairborne method which is a quantitative spectroscopic method, developed for the breakdown of \(H_2O_2\) into \(H_2O\) and \(O_2\) at 240 nm in a unit time for routine studies of CAT kinetics. The results are expressed in µmoles of \(H_2O_2/g\) of wet tissue/min.

**Determination of cardiac and renal superoxide dismutase (SOD)**—The SOD activity was assayed in the supernatant of heart and kidney homogenates by the method of Sun and Zigman in which the activity of SOD was inversely proportional to the concentration of its oxidation product adrenochrome, which was measured spectrophotometrically at 320 nm. One unit of SOD activity is defined as the enzyme concentration required to inhibit the rate of auto-oxidation of adrenaline by 50% in 1 min at pH 7.0. The results were expressed as units/g wet tissue.

**Statistical analysis**—The results were expressed as (mean ± SE) and statistically analyzed by one-way ANOVA followed by Tukey-Kramer test. A value of \(P<0.05\) was considered to be statistically significant.

**Results**

**Extraction process**—Yield of HAELA was found to be 3.2% (w/w) Preliminary phytochemical analysis, physiochemical and analytical profiles of HAELA are outlined in Table 1.

**Acute oral toxicity studies**—The exact LD₅₀ value by oral route could not be determined as neither mortality nor toxic symptoms observed in mice treated with 2000 mg/kg up-to 72 h. Hence, 2000 mg/kg was considered as maximum tolerable dose as per OECD guidelines. Therefore, 100 \(1/20\), 1/10 as 200 and 1/5 400 mg/kg were selected for cardio and nephroprotective activity evaluation in mice.

**Serum biomarkers**—DXR treated mice showed significant \((P<0.001)\) elevation in serum biomarkers as compared to vehicle treated normal mice (Table 2). Mice treated with different doses of HAELA (200 and 400 mg/kg, b.w. po) with DXR significantly \((P<0.01)\) prevented the elevation in serum biomarkers due to DXR (Table 2).

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**Table 1**—Phytochemical constituents and physiochemical property of HAELA

<table>
<thead>
<tr>
<th>Physical appearance</th>
<th>Brown amorphous powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>Freely soluble in methanol, ethanol and water</td>
</tr>
<tr>
<td>UV Etoh λ Max nm [E¹% 1cm]</td>
<td>273</td>
</tr>
<tr>
<td>Rₜ values (TLC)*of HAELA</td>
<td>0.76, 0.52, 0.77</td>
</tr>
<tr>
<td>Phytochemicals</td>
<td>Flavonoids, Saponins Steroids Tannins, and amino acids</td>
</tr>
</tbody>
</table>

*solvent system: chloroform: methanol (8:2); n-hexane: ethyl acetate (4:1); ethyl acetate chloroform: methanol (2:6:3)

**Table 2**—Effect of HAELA on serum biomarkers in DXR induced cardiotoxicity

<table>
<thead>
<tr>
<th>Treatment and dose (mg/kg,po)</th>
<th>AST</th>
<th>LDH</th>
<th>CPK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control (10 mL/kg)</td>
<td>38.2±1.3</td>
<td>43.3±2.2</td>
<td>5.1±0.4</td>
</tr>
<tr>
<td>DXR control</td>
<td>81.4±3.8**</td>
<td>179.6±7.5##</td>
<td>19.9±1.1##</td>
</tr>
<tr>
<td>HAELA 100</td>
<td>74.6±2.9</td>
<td>165.3±6.8</td>
<td>15.2±1.0</td>
</tr>
<tr>
<td>HAELA 200</td>
<td>62.9±2.7*</td>
<td>123.1±6.2*</td>
<td>9.4±0.8*</td>
</tr>
<tr>
<td>HAELA 400</td>
<td>52.3±1.9*</td>
<td>101.2±4.8**</td>
<td>7.3±0.7*</td>
</tr>
</tbody>
</table>

\(P\) values: **< 0.001 compared with vehicle control group*< 0.01, **< 0.001 compared with DXR control
**Antioxidants in heart and renal tissues**—Heart and kidney MDA were significantly elevated (P<0.001) in mice treated with DXR. Administration of HAELA (200 and 400 mg/kg) with DXR reversed significantly (P<0.001) the elevated MDA level in heart and kidney tissues (Tables 3 and 4). A significant depletion (P<0.01) of heart and kidney GSH, SOD and CAT were observed in DXR treated mice as compared to vehicle treated normal mice. Treatment with HAELA (200 and 400 mg/kg) along with DXR significantly (P<0.001) restored the depleted GSH, SOD and CAT in heart and kidney tissue of mice due to DXR (Table 3 and 4). The restoration of depletion of GSH, SOD, and CAT were found to be dose dependent. It was observed that HAELA at higher dose 400 mg/kg able to restore the depleted antioxidants to a greater extent but failed to bring back to normal level.

**Histopathology**—Histopathological examination of the cardiac tissue of vehicle treated normal mice showed clear integrity of myocardial cell membrane and no inflammatory cells infiltration (Fig. 1A). The DXR treated mice showed myocytic necrosis and infiltration of lymphocytes, macrophages, inflammatory cells along with vacuolization and fibroblastic proliferation (Fig. 2B). Administration of HAELA (200 and 400 mg/kg) prevented infiltration of inflammatory cells and vacuolar changes induced by DXR treatment. The distribution of necrosis, lymphocytes and macrophage infiltration prevented with HAELA (200 and 400 mg/kg) treatment (Fig. 1D and 1E). However, group of mice treated with HAELA (100 mg/kg) showed non-significant improvement in infiltration of inflammatory cells as well as proliferation process (Fig. 1C).

Histopathological examination of the kidney of vehicle treated mice showed clear integrity of renal cell membrane (Fig. 2A). It was observed a significant development of glomerulosclerosis and tubulointerstitial lesions in DXR treated mice (Fig. 2B). Treatment of HAELA (200 and 400 mg/kg) along with DXR prevented glomerulosclerosis and tubulointerstitial lesions in mice (Fig. 2D and 2E). The mice treated with HAELA (100 mg/kg) did not show any improvement in glomerulosclerosis or tubulointerstitial lesions induced by DXR treatment.

### Table 3—Effect of HAELA on cardiac antioxidants in DXR induced cardiotoxicity

<table>
<thead>
<tr>
<th>Cardiac antioxidants</th>
<th>Vehicle Control</th>
<th>DXR control</th>
<th>HAELA-100</th>
<th>HAELA-200</th>
<th>HAELA-400</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA formation (nM of MDA/g of wet tissue)</td>
<td>3.5±1.41</td>
<td>22.9±2.16#</td>
<td>19.40±2.4</td>
<td>12.9±1.38**</td>
<td>10.5±0.55***</td>
</tr>
<tr>
<td>GSH (µmol/g of wet tissue)</td>
<td>6.6±0.76</td>
<td>2.4±0.63#</td>
<td>3.43±0.36</td>
<td>5.6±0.21**</td>
<td>8.2±0.50***</td>
</tr>
<tr>
<td>CAT (µM of H₂O₂/g of wet tissue)</td>
<td>27.6±1.20</td>
<td>11.9±0.75#</td>
<td>18.58±1.57</td>
<td>27.9±5.71**</td>
<td>46.1±1.70***</td>
</tr>
<tr>
<td>SOD (Units/ g of wet tissue)</td>
<td>30.2±1.91</td>
<td>15.2±1.88#</td>
<td>15.68±1.94</td>
<td>26.22±3.04**</td>
<td>27.6±2.30***</td>
</tr>
</tbody>
</table>

P values: \#< 0.001 compared with vehicle control group, ***< 0.001, **< 0.01, compared with DXR control

### Table 4—Effect of HAELA on renal antioxidants in DXR induced nephrotoxicity in mice

<table>
<thead>
<tr>
<th>Renal antioxidants</th>
<th>Vehicle Control</th>
<th>DXR control</th>
<th>HAELA-100</th>
<th>HAELA-200</th>
<th>HAELA-400</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA formation (nM of MDA/g of wet tissue)</td>
<td>4.79±0.41</td>
<td>19.03± 1.82##</td>
<td>18.29±1.53</td>
<td>12.63±1.09**</td>
<td>10.54±0.57***</td>
</tr>
<tr>
<td>GSH (µmol/g of wet tissue)</td>
<td>3.32±0.12</td>
<td>0.95± 0.10#</td>
<td>1.54±0.25</td>
<td>1.93±0.27*</td>
<td>2.17±0.11**</td>
</tr>
<tr>
<td>CAT (µM of H₂O₂/g of wet tissue/ min.)</td>
<td>32.44±1.58</td>
<td>19.86±1.90#</td>
<td>17.41±1.81</td>
<td>24.32±0.81*</td>
<td>28.98±0.83**</td>
</tr>
<tr>
<td>SOD (Units/ g of wet tissue)</td>
<td>33.96±3.52</td>
<td>15.90± ±2.26#</td>
<td>27.88±1.84</td>
<td>30.04±4.71*</td>
<td>32.16±1.36**</td>
</tr>
</tbody>
</table>

P values: \#< 0.01 and \##< 0.001 compared with vehicle control group ***< 0.001, **< 0.01, *< 0.05 compared with DXR control
Fig. 1—Haematoxylin and eosin staining of heart tissue of (A) normal rats; (B) DXR treated rats; (C) rats treated with DXR and 100 mg/kg of HAELA; (D) rats treated with DXR and 200 mg/kg of HAELA; (E) rats treated with DXR and 400 mg/kg of HAELA [Figs A-E:100 ×].

Discussion

DXR is an important and therapeutically effective anticancer drug with wide spectrum of activity\(^5\); however, its clinical application is hampered by cardio and nephrotoxicity on chronic treatment\(^{39,40}\). The DXR, in the form of DXR semiquinone has been attributed to play a major role in nephrotoxic effect, because semiquinones are known to generate superoxide anions by interacting with molecular oxygen leading to oxidative stress and ultimately cause cellular injury\(^{13,41-43}\). The impairment of oxidant-antioxidant systems, initiates peroxidation of membrane bound polyunsaturated fatty acids and protein oxidation, leads to alterations in permeability of myocytes and nephrons, intracellular calcium overload causing finally irreversible damage to the tissues\(^{14}\). Furthermore, increased free radicals formation augment infiltration of neutrophils, activate glomerular mesangial cells, which ultimetly manifest in renal tissue damage\(^{44,45}\).

In present experiments, kidney and heart MDA content was increased and considered to be an index of excessive formation of free radicals largely arising from oxidative products of DXR, causing injury to heart and kidney. The oxidative products of DXR stimulate NADPH dependent microsomal lipid peroxidation by generating reactive species. The documented scientific reports suggest that DXR induces heart and kidney damage by disturbing antioxidants defense mechanism and also augmenting membrane lipid peroxidation, free radicals formation, mitochondrial damage, and iron dependent oxidative damage to biological macromolecules\(^{46-50}\).
Reduced glutathione (GSH), a non enzymatic antioxidant abundantly present in the body and distributed intracellularly known to play a critical role of coenzyme and participate in the removal of free radicals, peroxides, maintains membrane thiols, and also a substrate for glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) and thiol transferase. SOD, CAT and GPx constitute a mutually supportive enzyme system of the first line cellular defense against oxidative injury by decomposing \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) prior to their interaction to form the more harmful hydroxyl, alkoxyl radicals. Further, GSH also plays a major role in the removal of hydroperoxides, free radicals, and amino acid transport across membranes. Depleted glutathione in the target organs (heart and kidney) by DXR was significantly restored by HAELA treatment. It may be understood that elevated level of GSH could be attributed either to (a) its enhanced synthesis, (b) and/or improved GR, GPx and GST by HAELA treatment. Furthermore, enzymatic antioxidant, CAT destroys hydrogen peroxide \( \text{H}_2\text{O}_2 \) by dismutation and also exhibit peroxidative activity. SOD metallo-enzymes are available in Mn-superoxide dismutase (Mn'SOD), Cu/Zn- superoxide dismutase (Cu/ZnSOD) and Fe- superoxide dismutase (FeSOD) which are known to catalyze the dismutation of superoxide anion into oxygen and hydrogen.
peroxide\textsuperscript{56-59}. Further, maintenance of equilibrium between these enzymes is a critical factor for the effective removal of oxidative stress especially in intracellular organelles\textsuperscript{17}. Thus, the protective mechanism(s) of HAELA appears to be through modulation of various antioxidants, which in turn helps to improve the overall antioxidant defense system in renal and cardiac tissues.

The phytocomponents, flavonoids, saponins and tannins present in HAELA could allow to augment scavenging/removing/neutralizing superoxide anions, reactive hydroxyl and other free radicals by improving the antioxidant system.

Heart and kidney contain abundant biomarker enzymes released into the extra cellular fluid during myocardial or renal injury either due to oxidative stress or metabolic changes\textsuperscript{58,59}. In the present study too, serum biomarkers, (AST, LDH and CPK) were elevated following DXR administration. Elevation of serum biomarkers confirm the onset of cardiac and kidney necrosis due to DXR. HAELA treatment prevented the elevated AST, LDH and CPK significantly, thereby indicating cardio-nephroprotective effect, presumably by membrane stabilization process. Both clinically and experimentally, it has been demonstrated that combined treatment by using alpha-tocopherol and DXR, improved the therapeutic index of DXR\textsuperscript{15}. Thus, using phytocomponents of potent antioxidant with DXR, the therapeutic index of DXR can also be improved, and simultaneously the toxic effects on vital organs (heart and kidney) can be ameliorated or prevented.

Administration of HAELA reduced toxicity of DXR moderately and improved the histoarchitecture of both heart and kidney. Prevention of oxidative stress by (a) scavenging/neutralizing free radicals; (b) augmenting enzymatic and non-enzymatic antioxidants, and finally; (c) stabilization of membrane (myocytes as well as nephrons) ultimately leading to cardio-nephroprotection with HAELA treatment.

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