‘Protective effect of *lagenaria siceraria* against doxorubicin induced cardiotoxicity in wistar rats’

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**Abstract**
In the present study it was aimed to evaluate the combined effect of simvastatin (SIM) and hydroalcoholic seeds extract of *Lagenaria siceraria* (H.L.S.S.E.) in doxorubicin (DOX) induced cardiotoxicity in Wister rats. DOX 10 mg/kg i.p. single dose was given to cause cardiac damage and the levels of cardiac biomarker enzyme viz LDH, CK, CK-MB and total protein & antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and lipid peroxidation (LPO) were observed and in the end of experiment histopathology was carried out for heart. SIM (15 mg/kg) and (H.L.S.S.E. 200 & 400mg/kg) were given in combination with DOX 10 mg/kg i.p. single dose to rats at the 30th day of experiment and as a pretreatment for 29 days (H.L.S.S.E. 200 & 400mg/kg) were given orally. Pretreatment with SIM and (H.L.S.S.E) significantly (P<0.001) reduced the elevated cardiac toxicity biomarker enzyme level and raised the level of antioxidants viz CAT, GSH, SOD and decrease LPO in the extract treated groups as compared to the disease control group. In the histopathological studies H.L.S.S.E and SIM showed protection against myocardial toxicity induced by doxorubicin. The result obtained from the present study indicates that the pretreatment of SIM and H.L.S.S.E may significantly reduce the (DOX) induced cardiotoxicity.

**Key words:**

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**INTRODUCTION**
The anthracycline antibiotic doxorubicin (DOX) is used widely in the chemotherapy of breast cancer, liver carcinoma. DOX is effective in curative and adjuvant chemotherapy and in the palliation of symptoms of malignancy in middle-to-late stage
disease. However, cardiotoxicity is the chief toxic effect that limits the clinical use of DOX. The mechanisms of DOX-induced cardiotoxicity have been investigated. DOX can lead to increased lipid peroxidation and oxidative damage to cardiac muscle. Free radical formation and cardiac DNA and membrane injuries are major aspects of DOX induced cardiotoxicity. The previous study on the *Lagenaria siceraria* hydro alcoholic of seed extract per-treatment enhance the antioxidant deference in myocardial damage and exhibits cardio protective property. Statins like simvastatin a widely use group of hypocholesteremic related morbidity and mortality in patients with or without coronary artery disease and with or without high cholesterol levels. The primary mechanism of action as cardio protective effects is likely through its effectiveness inhibiting lipid peroxidation preservation of antioxidant enzyme as well as seavenging of free radicals. Along with therapeutic effect stains also know to posses dose dependant side effect like liver damage rhabdomyolysis and kidney failure. It could be beneficial to use herbal supplements concurrently with conventional medicine to exhibit augmented protective efficacy than a single drug. The aim of present study is to explore whether concomitant pretreatment with simvastatin and *Lagenaria siceraria* extract better effect than simvastatin and *Lagenaria siceraria* alone.

**Materials and methods**

**Collection and authentication of *Lagenaria siceraria* seeds**

The *Lagenaria siceraria* fresh fruits/seeds was collected from the local market of Varanasi, Uttar Pradesh India. Identified and authenticated by Dr. N. K. Dubey Department of Botany, Banaras Hindu University. Where the herbarium of the specimen is deposited (voucher specimen no 2012/Jan/1).

**Reagents and chemicals**

Doxorubicin hydrochloride for injection was purchased from Celon laboratories LTD.(Gajularamram). and simvastatin was purchased from Akums Drugs & Pharmaceuticals. All solvents/chemicals used were of analytical grade and chemicals required for sensitive biochemical assays such, EDTA, H₂O, Phosphate Buffer pH 8.0, 0.02 M (potassium dihydrogen phosphate sodium Hydroxide) 5,5-dithiobis2-nitrobenzoic acid and thiobarbituric acid were obtained from Sigma Chemical Co. USA.

**Preparation of crude extract**

The shade dried seeds of *Lagenaria siceraria* were reduced to fine powder (# 40 size mesh) and around 200 g of powder was subjected to successive hot continuous extraction (soxhlet) with hydro alcohol (1:1) After the effective extraction, the solvents were distilled off the extract was then concentrated on water bath and the extract obtained with each solvent will be preserved.

**Preliminary photochemical screening**

*Lagenaria siceraria* seeds extract was subjected to preliminary photochemical screening for the detection of various plant constituents.

**Thin layer chromatography (TLC)**

The dried and finely powdered fruit of LS (1 g) was extracted with 10 with hydro alcohol (1:1) for 15min on the water bath. The mixture was filtered and the filtrate was evaporated to 1–1.5 ml was used for TLC investigations by use of precoated silica gel GF254 plates (Merck, Germany).The solvent system used was ethyl acetate/formic acid/water, 8:1:1 (V/V). Visualization of the flavonoids was achieved by spraying the sheets with natural products-polyethylene glycol reagent (NP/PEG) (Fluka Chemie, Switzerland).Typical intense fluorescence in UV light at 365 nm was produced immediately on
spraying flavonoids appeared as orange-yellow bands.

**Experimental animal**

Male Albino rats of Wistar strain weighing between 175 and 180 gm were used in the study. The animals were housed under standard environmental conditions (temperature 22±2 °C; humidity 45±4%) with a 12 h light/dark cycle at the Animal house of NIMS institute of pharmacy NIMS university, Shobha Nagar, Jaipur. The animals had free access to water ad libitum. All animal experiments were approved (1902/ac/09/CPCSEA) by the Animal Ethical Committee of the Institute, and all procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals.

**Dose selection**

- Simvastatin (SIM) animal dose 15 mg/kg respectively and administered
- orally for 30 day.
- hydro alcohol Lagenaria siceraria seeds extract (H.L.S.S.E.) at two different does 200mg/kg and 400mg/kg dissolved in distilled water and administered orally for 30 day.
- At the end of the treatment period, animal of all the group excluding group I. Were administered with doxorubicin (DOX) 10 mg/kg I.P.

**Experimental protocol**

Group I- ( normal control ) – normal saline (5ml/ kg b.w.)
Group II- ( disease control ) - normal saline + doxorubicin.
Group III- 200 mg/kg b.w.- of Lagenaria siceraria + doxorubicin.
Group IV- 400 mg/kg b.w.- of Lagenaria siceraria + doxorubicin.
Group V – 15 mg/kg b.w.- of Simvastatin + doxorubicin.

**Biochemical analysis**

**Serum parameters**

After 72 hrs of the doxorubicin injection animal were anaesthetized with ether. The blood was collected from by puncturing retro-orbital sinus. Serum was separated by centrifugation. Serum creatin kinase isoenzyme ( CK-MB ) 340 nm, creatin kinase ( CK ) lactate dehydrogenises ( LDH ) Total protein measured kinetically at 340 nm according to standard method by using commercially available diagnostic kits from) Reactivos GPL Barcelona,Espana).

**Tissue parameters**

**Preparation of heart tissue for estimation of oxidative stress marker**

The heart of each animals was removed after completion of experiment, weighed and the various heart were dissected out and homogenized with 10 times (w/v) ice cold phosphate buffer saline (50mM pH 7.8) in teflon glass homogenizer. The homogenate was centrifuged at 1000 rpm 4 °C for 3 min and the supernatant divided into two portions, one of which was used for measurement of lipid peroxidation (LPO). The remaining supernatant was again centrifuged at 12,000 rpm at 4 °C for 15 min and used for the measurement of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). was measured by the method of Lowry et al.

**Estimation of catalase (CAT)**

Catalase measurement was carried out by the ability of CAT to oxidize hydrogen peroxide (H2O2). 2.25 ml of potassium phosphate buffer (65 mM, pH7.8) and 100 _1 of the brain homogenate were incubated at 25 °C for 30 min. A 650 _1 H2O2 (7.5mM) was added to the brain homogenate to initiate the reaction. The change in absorption was measured at 240 nm for 2–3 min and the results were expressed as CAT mol/min mg of heart.

**Lipid peroxidation assay**
This assay was used to determine thiobarbituric acid-reactive substances as described by Slater and Sawyer (1971). In 2.0 mL of the tissue homogenate (supernatant) was added 2.0 mL of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 min, followed by centrifugation at 2500 rpm for another 15 min at 4°C. Two milliliter of clear supernatant solution was mixed with 2.0 mL of freshly prepared 0.67% w/v TBA. The resulting solution was heated in a boiling water bath for 10 min. It was then immediately cooled in an ice bath for 5 min. The absorbance of colour developed was measured by UV/VIS double beam spectrophotometer (systronic Japan) at 532 nm using 1, 1, 3, 3-tetraethoxypropane as a standard.

**Estimation of glutathione (GSH)**
The assay of GSH was determined by method described by Moron et al. (1979). One milliliter of tissue homogenate (supernatant) and 1.0 mL of 20% TCA were mixed and centrifuged at 2500 rpm for 15 min at 4°C. In 0.25 mL of supernatant, 2 mL of DTNB (0.6 M) reagent was added. The final volume was made up to 3 mL with phosphate buffer (pH 8.0). The colour developed was read at 412 nm against reagent blank. Different concentrations (10-50 µg) of standard glutathione were processed as mentioned above for constructing standard curve. The amount of reduced glutathione was expressed as µg of GSH/mg of protein.

**Estimation of superoxide dismutase (SOD)**
The SOD activity was determined by the method of Misra and Fridovich (1972). 0.5 mL of heart homogenate + 0.5 mL of cold distilled water + 0.25 mL of ice-cold ethanol and 0.15 mL of ice-cold chloroform were mixed well using cyclo mixer for 5 min and centrifuged at 2500 rpm for 15 min at 4°C. To 0.5 mL of supernatant, 1.5 mL of carbonate buffer (pH 10.2) and 0.5 mL of 0.4 M ethylenediaminetetraacetic acid (EDTA) solutions were added. The reaction was initiated by the addition of 0.4 mL of epinephrine bitartrate (3 mM) and the change in optic density/minute was measured at 480 nm against reaction blank. SOD activity was expressed as units/mg of protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme was taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD. [TABLE-3]

**Histopathological studies**
Hearts were quickly removed, preserved in 10% formalin, processed and embedded in paraffin. Four micrometer thick paraffin sections were cut on glass slides and stained with hematoxylin and eosin (H and E) reagents and observed by light microscope to evaluate myocardium injury.

**Statistical analysis**
Result were expresses as mean ±SEM. The statistical significance of any difference in each parameter among the group was evaluated by one-way–ANOVA using dun nets multiple comparison test as post hoc test.

**RESULT**

**Preliminary phytochemical screening**
The phytochemical screening of the fresh seeds of *Lagenaria siceraria* showed the presence of the following: flavonoids, alkaloids, saponins, and glycosides. The results however showed that anthraquinone, tannins, and cyanogenetic glycosides were absent. [TABLE-1]

**Thin layer chromatography (TLC)**
TLC of seeds of *Lagenaria siceraria* extract on precoated silica gelGF254 plates using ethyl acetate/formic acid/water, 8:1:1 (V/V) showed under
UV (365 nm) three fluorescent zones at Rf value 0.86 flavonoids appeared as orange-yellow bands.

**Serum parameters**

Acute administration on DOX (10mg /kg ip single dose ) induced cardiotoxicity and showed significant increase in the levels of serum cardio marker enzyme viz LDH, CK-MB,CK Total Protein compare to the normal rats (p<0.001). The increase concentration of serum enzyme is a well accepted quantative index of myocardial damage cause by DOX treatment. Pretreatment with SIM and H.L.S.S.E. alone significantly (P<0.001) reduced the elevated serum enzyme levels. When compared to DOX treated rats. But concomitant pretreatment with SIM and H.L.S.S.E. normalized the activities of these enzyme in DOX induced Cardiotoxicity rats. [TABLE-2]

**Tissue parameters:**

Cardioprotective activity of H.L.S.S.E. was supported by increased myocardial antioxidant enzyme activity and decreased extent of lipid peroxidation. Lipid peroxidation is known to cause cellular damage and is primarily responsible for reactive oxygen species induced organ damage. Decreased levels of GSH, SOD, and CAT were observed in heart tissue in doxorubicin treated animal. Pretreatment with SIM and H.L.S.S.E. efficiently counteracted the doxorubicin induced cardiac tissue damage by significant increase in GSH, SOD and CAT level. The observed increase in LPO activity in doxorubicin treated animal supports above hypothesis that this increase is possibly required to overcome excessive oxidative stress. [TABILE-3]

**Table 1: Phytochemical Screening of Lagenaria siceraria**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>TEST</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>For flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>For glycoside</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>For anthraquinone</td>
<td>–</td>
</tr>
<tr>
<td>4.</td>
<td>For alkaloid</td>
<td>–</td>
</tr>
<tr>
<td>5.</td>
<td>For saponins</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>For tannins</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 2: Effect of various treatments on serum biochemical marker enzyme**

<table>
<thead>
<tr>
<th>GROUP</th>
<th>L D H (IU/L)</th>
<th>CK-MB (IU/L)</th>
<th>CK (U/L)</th>
<th>Total protein (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Normal control</td>
<td>110.4 ± 2.97</td>
<td>85.49 ± 1.75</td>
<td>114.81 ± 15.10</td>
<td>6.0 ± 0.40</td>
</tr>
<tr>
<td>II Disease control</td>
<td>228 ± 6.57</td>
<td>258.6 ± 4.19</td>
<td>400.64 ± 121.67</td>
<td>8.2 ± 0.34</td>
</tr>
<tr>
<td>III L.S. 200 mg/kg +DOX</td>
<td>127.3 ± 3.87</td>
<td>112.2 ± 2.52</td>
<td>189.68 ± 51.58</td>
<td>7.9 ± 0.62</td>
</tr>
<tr>
<td>IV L.S.400 mg/kg +DOX</td>
<td>128.5 ± 3.87</td>
<td>97.27 ± 3.42</td>
<td>173.58 ± 39.44</td>
<td>7.3 ±0.52*</td>
</tr>
<tr>
<td>V(SIM 15mg/kg + DOX)</td>
<td>116.07 ± 3.95</td>
<td>94.39 ± 2.66</td>
<td>165.48 ± 29.33</td>
<td>6.9 ± 0.43*</td>
</tr>
</tbody>
</table>

Data expressed as Mean ± S.E.M n= 6 statistical significance. Compared with disease control 'P < 0.05 , **P < 0.001 , *** P < 0.001.

**Table 3: Effect of Lipid peroxidation, Glutathione , Catalase and superoxide dismutase in doxorubicin induced cardiotoxicity in rats.**

<table>
<thead>
<tr>
<th>GROUP</th>
<th>LPO(nm/mg Protein)</th>
<th>GSH(nm/g tissue w)</th>
<th>CAT(unit/mg of protein)</th>
<th>SOD(unit/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Normal control</td>
<td>2.68 ± 0.17</td>
<td>2.857 ± 0.0605</td>
<td>60.567±12.15 #</td>
<td>36.710±0.8061 †</td>
</tr>
<tr>
<td>II Disease control</td>
<td>4.67 ± 0.18</td>
<td>1.102±0.1736</td>
<td>40.632±2.027</td>
<td>23.807 ± 1.32</td>
</tr>
<tr>
<td>III L.S.200 mg/kg+DOX</td>
<td>3.67 ± 0.19 *</td>
<td>1.95±0.1033 *</td>
<td>57.28±1.053 *</td>
<td>31.610±0.8051</td>
</tr>
<tr>
<td>IV L.S.400 mg/kg+DOX</td>
<td>2.49 ± 0.26 **</td>
<td>1.59±0.1105 **</td>
<td>54.29±1.083 **</td>
<td>28.31±1.065**</td>
</tr>
<tr>
<td>V(SIM 15mg/kg + DOX)</td>
<td>2.23 ± 0.25 ***</td>
<td>1.97±0.1044 **</td>
<td>36.27±1.063 **</td>
<td>29.41±1.093**</td>
</tr>
</tbody>
</table>

Data expressed as mean ± S. E. M.; n= 6, statistical significance; ** P<0.001 vs Disease control. ** P< 0.01 vs Disease control ; * P < 0.05 vs Disease control. ane-way – ANOVA using dun nets multiple comparison test as post hoc test.

**Effect on body weight and body weight gain** It is depicted from [ TABLE-4] that the body weight body weight gain is gradually decreased in disease control group as compare to the normal control group. In treatment group ( L.S.S.E.200 mg/kg + 10 mg DOX ) it was significantly increased ( P < 0.01 ) in the body weight gain as compare to disease control group. While in ( L.S.S.E. 400 mg/kg + 10 mg DOX ) one unit of activity was taken as the enzyme reaction which 50% inhibition of adrenaline reaction in on min. # μ mole of H₂O₂ consumed / min.

Data expressed as mean ± S. E. M.; n= 6, statistical significance; *** P<0.001 vs Disease control. ** P< 0.01 vs Disease control ; * P < 0.05 vs Disease control. ane-way – ANOVA using dun nets multiple comparison test as post hoc test.
significantly increased (P < 0.001) in the body weight gain and In pre-treatment group (SIM 15mg/kg + 10 mg DOX) significantly increased in the body weight as compare to disease control group.

Table 4: Initial and final body weight and body weight gain

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INITIAL BODYWEIGHT (g)</th>
<th>FINAL BODYWEIGHT (g)</th>
<th>WEIGHT DIFFERENCE (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I normal control</td>
<td>175.0 ± 3.209</td>
<td>164.4 ± 1.394</td>
<td>10.40 ± 2.638</td>
</tr>
<tr>
<td>II disease control</td>
<td>173.0 ± 3.010</td>
<td>155.0 ± 3.234</td>
<td>-18.00 ± 2.168*</td>
</tr>
<tr>
<td>III (L.S. 200 mg/kg + DOX)</td>
<td>175.0 ± 3.479</td>
<td>171.4 ± 1.879</td>
<td>-3.600 ± 1.600*</td>
</tr>
<tr>
<td>IV (L.S. 400 mg/kg + DOX)</td>
<td>174.0 ± 3.654</td>
<td>167.6 ± 0.622</td>
<td>-6.400 ± 3.032**</td>
</tr>
<tr>
<td>V (SIM 15 mg/kg + DOX)</td>
<td>180.0 ± 2.457</td>
<td>175.5 ± 1.155</td>
<td>-4.500 ± 1.302***</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM; n=6 in each group, ns= not significant, when compared Norma control * P<0.05, **P<0.01 and*** P<0.001 when compared to disease control by One-way ANOVA.

DISCUSSION

The results showed that the seeds extract of the Lagenaria siceraria is rich in flavonoids, alkaloids, saponins, glycosides and alkaloids. Flavonoid containing plants have influence on arachidonic acid metabolism, thus could have anti-inflammatory, cardio protective and anti-allergic effect. In the present study the serum levels of CK, CK-MB, LDH and total protein in H. L.S.S.E. treated group decreased significantly. A single dose administration of DOX (10 mg kg⁻¹) caused a significant increase in lipid peroxidation in cardiac tissues and decrease in myocardial GSH, CAT and SOD in disease control group indicating an increase in oxidative stress. Administration of H.L.S.S.E. improved the biochemical marker levels indicating decrease in oxidative stress as evident by increased level of GSH,CAT and SOD with decreased lipid peroxidation.H.L.S.S.E. contain flavonoids. The studies on H.L.S.S.E. also provide a clear evidence that the pretreatment enhances the antioxidant defense in myocardial damage and exhibit cardio protective property and it also has been provided that statins (SIM) provides protection from DOX induced cardiac damage. The mechanism of simvastatin involves inhibition of lipid peroxidation.
peroxidation, tissue fibrosis preservation of antioxidant enzyme and scavenging of free radicals.\textsuperscript{21,22,23} H.L.S.E. dose dependent protection action against DOX concomitant treatment could be a beneficial therapy that is increase dose of H.L.S.E 400 mg/kg with dose of SIM than a combination of lower amount of H.L.S.E. 200 mg/ kg in order to prevent the dose depend side effect of SIM like hepatomagaly and stetosis.\textsuperscript{24,25} The results of this study based on biochemical and histopathological studies revealed myocardial atrophy, nuclear pyknosis, cytoplasmic vacuoles and cytoplasmic eosinophilia in the DOX treated rat hearts.\textsuperscript{26} Although the protection is seen with all the two doses, i.e, 200 mg/kg and 400 mg/kg body weight, the best protective effect is indicated by 400 mg/kg dose. In treatment group that H.L.S.E. (200mg/kg) + DOX significantly increase (P<0.05) in the body weight gain in compare to disease control group while in H.L.S.E.( 400mg/kg) + DOX) group (p<0.001).

Conclusion

In the present experiment we found that the concomitant pretreatment SIM and L.S.S.E. restored the level of cardiac marker enzyme and body weight pattern in DOX induced cardio toxicity rats. The therapy of L.S.S.E. Have some the cardio protective effect. In this present study we demonstrated that treatment of L.S.S.E. significantly ameliorated DOX induced cardio toxicity in rats as compare to disease control and L.S.S.E. alone. These effects might be due to the presence of polyphenolic components in the seed of H. L.S.S.E. Further extensive study on L.S.S.E. required to confirm its Cardioprotective activity

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\textbf{References}


