Aqueous extract of *Saussurea lappa* root ameliorate oxidative myocardial injury induced by isoproterenol in rats

**ABSTRACT**

*Saussurea lappa* Clarke (Compositae), is commonly known as Kushta. In Ayurvedha, it is mentioned that the aqueous extract of the root *S. lappa* was used for treatment of angina pectoris. The present study was designed to investigate the cardioprotective effect of aqueous extract of root of *S. lappa* against isoproterenol induced myocardial injury. Myocardial injury in rat was induced by the administration of isoproterenol at a dose of 85 mg/kg, i.p., The rats were pretreated with the aqueous extract of *S. lappa* (AESL) in three different doses (100, 200 and 300 mg/kg, p.o.) through the oral route. Isoproterenol alone-treated rats showed increased serum concentration of lactate dehydrogenase (LDH), creatinine kinase (CK), and aspartate transaminase (AST), increased myocardial thiobarbituric acid reactive substances (TBARS) level, and decreased myocardial glutathione (GSH) level due to myocardial damage produced by isoproterenol. This is further conformed by histopathological changes. Chronic oral administration of AESL in three different doses significantly restored the level of myocardial LDH, CK, AST, TBARS, and GSH. The extract effect was compared with the reference standard α-tocopherol which also offered similar protection in biochemical and histopathological changes. The overall beneficial effect which was observed with the dose of 200 mg/kg indicated that AESL produced significant dose-dependent activity against isoproterenol induced myocardial injury.

**Key words:** Antioxidants, lactate dehydrogenase, myocardial infarction, oxidative stress, *Saussurea lappa*

**INTRODUCTION**

Cardiovascular disease (CVD) is known as a life-threatening problem with high mortality and morbidity at the global level. According to the World Health Organization report, the maximum mortality occurs within the age limit of 40-50 years due to myocardial infarction (MI) in both western and developing countries.[1-3] Oxidative stress due to extensive generation of free radicals with concomitant depletion of endogenous antioxidants like superoxide dismutase, catalase and reduced glutathione (GSH) play an important role in MI.[3]

Natural antioxidants play a major role in reducing the oxidative stress by scavenging the excess free radicals.[4] *Saussurea lappa* is one of the antioxidant-rich medicinal plants. *S. lappa* Clarke (Compositae), commonly known as Kushta in Sanskrit, is a tall, robust, perennial herb distributed in Kashmir. The hot water extract of the roots has been traditionally used for the treatment of asthma,[5,6] inflammations, and rheumatism.[5,7] The roots are hot, bitter, sweetish, pungent, and flattering. It is used as an analgesic, digestive, aphrodisiac and diuretic. Many authors have reported that the roots of this plant possess cortisol-lowering effect, bronchodilator, antiulcer, anticancer, anti-inflammatory, antiviral, and hepatoprotective effects.[8,9] Traditionally, aqueous extract...
of the root of *S. Lappa* was used for its anti-anginal effect.[10] So, the present research has been designed to evaluate the cardioprotective property of the aqueous extract of *S. Lappa* (AESL) root to support the traditional claim.

**MATERIALS AND METHODS**

**Chemicals**
Isoproterenol hydrochloride, thiobarbituric acid, 2,4 dinitrophenyl hydrazine, and GSH were purchased from Sigma Chemical, Bangalore. Biochemical kits for lactate dehydrogenase (LDH), creatine kinase (CK), and aspartate transaminase (AST) were purchased from Transasia Bio-Medicals Limited, Solan. All other reagents and chemicals used in this study were of analytical grade with high purity.

**Animals**
Male Wistar albino rats weighing 200-300 g were selected for the study. The animals were housed in their respective cages under hygienic and standard environmental conditions (28 ± 2°C, humidity 60-70%, 12-h light and dark cycle). The animals were allowed a standard feed (Sai Durga Feeds and Foods, Bangalore) and water *ad libitum*. They were acclimatized to the environment for 1 week prior to experimental use. The study protocol was carried out after obtaining the permission from the Institutional Animal Ethical Committee (1220/a/08/CPCSEA).

**Plant Material and Preparation of Extract**
The root powder of *S. lappa* was purchased from an Indian druggist, Tirupati and authenticated by Dr. Madhava Chetty, Professor and Head, Department of Botany, S.V. University, Tirupathi. About 1 kg of the powdered material was boiled with 5 l of distilled water for 30 min and filtered to obtain the aqueous extract. The extract was concentrated under reduced pressure and lyophilized. The freeze-dried material was weighed (about 35 g), dissolved in water (at a final concentration of 50 mg/ml) and used for this study.

**Preliminary Phytochemical Analysis**
AESL was analyzed for the various classes of phytoconstituents such as flavonoids, phenolic acids, anthocyanins, quinones, alkaloids, tannins, and saponins using standard phytochemical methods.[11]

**Estimation of Phenolics**
Phenolic content of AESL was determined by the method of Malick and Singh, 1980.[12] Briefly, an aliquot of the sample was pipetted out in a test tube and the volume was made up to 3 ml with distilled water. Folin-Ciocalteau reagent (0.5 ml) was added to the tube and incubated for 3 min. at room temperature. Sodium carbonate (20%; 2 ml) solution was added, mixed thoroughly, and the tube was incubated for 1 min in boiling water bath. The absorbance was measured at 650 nm against a reagent blank. Standard curve using different concentrations of standard phenolic-catechol was prepared. From the standard curve, concentration of phenol in the test sample was determined and expressed as mg of catechol equivalent.

**Estimation of Flavonoids**
The flavonoid content of AESL was determined by the method of Helmja *et al.*[13] Briefly, an aliquot of the sample was pipetted out in a test tube and the volume was made up to 0.5 ml with distilled water. Sodium nitrite (5%; 0.03 ml) was added to the tube and incubated for 5 min. at room temperature. Aluminum chloride solution (10%; 0.06 ml) was added and incubated for 5 min. at room temperature. Sodium Hydroxide solution (1 M; 0.2 ml) was added and the total volume was made up to 1 ml with distilled water. Absorbance was measured at 510 nm against a reagent blank. Standard curve using different concentrations of rutin was prepared. From the standard curve, the concentration of flavonoids in the test sample was determined and expressed as mg of rutin equivalent.

**In-vitro Antioxidant Activity of Aqueous Extract of *Saussurea Lappa***

**Determination of reducing power**
The reducing power of the AESL was determined by the method described by Oyaizu (1986).[14] Different concentrations of the AESL and ascorbic acid were prepared and mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), and 2.5 ml of 1% K3Fe(CN)6. This mixture was incubated at 50°C for 20 min, 2.5 ml of 10% TCA was added to the blend and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was assorted with distilled water (2.5 ml) and FeCl3 (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated the reducing power.

**2,2-diphenyl-1-picrylhydrazyl assay**
The hydrogen atom or electron-donating group of the resultant compounds and some untainted compounds was measured from the bleaching of the purple-colored methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical, DPPH as a reagent.[15] One ml of different concentrations of the AESL and ascorbic acid in ethanol were added to 4 ml of 0.004% methanol solution of DPPH. After a 30-min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percent (I%) was calculated in the following way: 1% = (A blank – A sample/A blank) × 100 where, A blank is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations of extract.

**Determination of peroxide (H2O2) radical-scavenging activity**
The peroxide (H2O2) radical-scavenging activity of AESL
was determined by the method described by Ruch et al.,[16]
1 ml of sample solution (AESL and ascorbic acid in various
concentrations prepared in phosphate-buffered saline [PBS]) was incubated with 0.6 ml of 4 mM H$_2$O$_2$ solution
(prepared in PBS) for 10 min. The absorbance of the solution
was read at 230 nm against a blank solution containing
the extract without H$_2$O$_2$. The concentration of H$_2$O$_2$ was
spectrophotometrically stubborn from absorption at 230
nm using the molar absorptivity of 81/M/cm. The H$_2$O$_2$
radical-scavenging activity was calculated as $1\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$, where $A_{\text{blank}}$ is the absorbance of control and $A_{\text{sample}}$ is the absorbance of test.

**Acute Oral Toxicity Study**
The acute oral toxicity study was performed according to
up and down procedure. AESL up to a dose of 2000 mg/kg
did not produce any signs of toxicity and mortality.

**Induction of Myocardial Infarction**
The MI was induced in experimental rats by intraperitoneal
injection of isoproterenol hydrochloride 85 mg/kg
body weight, dissolved in physiological saline, for 2
consecutive days.[17]

**Experimental Protocol**
The rats were randomly divided into six groups with six
rats in each group. Group I, normal animals received saline
10 ml/kg bw with standard feed and water to allowed
*ad libitum* throughout the experimental period. Group II, the
rats were orally fed normal saline once daily for 28 days and
in addition, received isoproterenol (85 mg/kg body weight)
on the 29 and 30 day at an interval of 24 h. Group III-V, rats
were pretreated with AESL (100, 200 and 300 mg/kg body
weight respectively) for a period of 28 days and in addition,
received isoproterenol (85 mg/kg body weight) on the 29 and
30 day at an interval of 24 h. Group VI, rats were pretreated
with $\alpha$-tocopherol (60 mg/kg body weight, orally) for a period
of 28 days and in addition, received isoproterenol (85 mg/kg
body weight) on the 29 and 30 day at an interval of 24 h.[17]

At the end of the treatment period, blood samples were
collected by the retro-orbital plexus puncture method
under light ether anesthesia and serum was separated by
centrifugation and used for the biochemical estimations
(LDH, CK and AST) using the respective kits. The heart was
excised immediately and immersed in physiological saline.
It was suspended in 10% (w/v) ice-cold saline; then fixed in 10% buffered formalin; 10% stored buffered formalin were embedded in paraffin; 5-µm thick sections were cut and stained with hematoxylin and
eosin. These sections were then examined under a light
microscope for histological changes.

**Statistical Analysis**
Values are expressed as mean ± SD and analyzed using
Graph Pad Prism Version 5.1 using ANOVA followed by
Tukey’s multiple comparison Test. $P < 0.05$ was considered
significant.

**RESULTS**

**Phytochemical Investigation**
Preliminary phytochemical investigation of AESL revealed
the presence of flavonoids, terpenoids, alkaloids, and
phytosterols as important active constituents. Phenolic and
flavonoid content of AESL were determined and the results
indicated that AESL contains 3.5 mg/g of catechol-equivalent
phenolics and 16.0 mg/g of rutin-equivalent flavonoids.

**In-vitro Antioxidant Activity**
In the present study, increasing the concentration of AESL
and ascorbic acid shows increased absorbance, indicating
the reducing power. The reducing power of AESL was very
potent but lower than that of ascorbic acid and the reducing
power of the AESL was increased with the quantity of
sample [Figure 1]. AESL and ascorbic acid show DPPH-inhibition activity in different concentrations. And in higher
concentration (500 µg), AESL shows higher % inhibition
activity (100%) and when compared with ascorbic acid
(100%), it was equal. The IC$_{50}$ value of AESL and ascorbic
acid is found to be 70, 120 µg ml respectively [Figure 2].
AESL and ascorbic acid show H$_2$O$_2$-inhibition activity in
different concentration. While increasing concentration,
% inhibition activity also increased. When compared with
ascorbic acid, the % inhibition activity is low in AESL. The
IC$_{50}$ value of AESL and ascorbic acid is found to be 150, 120
µg ml respectively [Figure 3].

![Figure 1: Reducing power of aqueous extract of *Saussurea lappa*](image-url)
In acute toxicity study, it was found that the animals were safe up to a maximum dose of 2,000 mg/kg b.w. There were no changes in the normal behavioral pattern and no signs and symptoms of toxicity and mortality were observed. The results obtained in the different groups subjected to ISO-induced ischemic injury are presented in Tables 1-3.

**Myocardial and Serum Lactate Dehydrogenase**

Myocardial LDH in Group II was significantly ($P < 0.001$) lower than that in Group I. In Group III-VI the level of LDH was restored to normal when compared with Group II. There was a significant ($P < 0.001$) increase in the level of serum LDH in Group II when compared with Group I and the level significantly decreased in the Groups III ($P < 0.001$), IV ($P < 0.001$), V ($P < 0.001$), and VI ($P < 0.001$).

**Myocardial and Serum Creatinine Kinase**

Myocardial CK in Group II was significantly ($P < 0.001$) lower than that in the control group, i.e., Group I. In Groups III-VI, the level of CK was restored to normal when compared with Group II. There was significant ($P < 0.001$) increase in the level of serum CK in Group II when compared with control Group I and the level significantly decreased in the Groups III ($P < 0.001$), IV ($P < 0.001$), V ($P < 0.001$), and VI ($P < 0.001$).

**Myocardial and Serum Aspartate Transaminase**

There were no significant changes in the level of myocardial

### Table 1: Serum level of lactate dehydrogenase, creatinine kinase and aspartate transaminase

| Treatment group                      | LDH U/l | Creatinine kinase U/l | AST U/l |
|--------------------------------------|---------|-----------------------|---------|
| Control (Saline 10 ml/kg)             | 29.8±5.8| 58.4±20.2             | 27.7±1.3|
| Isoproterenol (85 mg/kg)              | 139.7±10.1*** | 264.7±23.7*** | 95.4±23.2*** |
| AESL (100 mg/kg)                     | 46.6±35.3*** | 86.3±73.6*** | 50.8±13.4*** |
| AESL (200 mg/kg)                     | 34.7±5.3*** | 64.1±20.6*** | 65.4±7.7**  |
| AESL (300 mg/kg)                     | 37.7±7.7*** | 142.3±23.5*** | 51.8±5.9**  |
| α-tocopherol (60 mg/kg)              | 55.8±33.4*** | 72.3±18.5*** | 40.6±5.8*** |

All value expressed as mean±SD; One-way analysis of variance followed by Tukey’s multiple comparison test, **P<0.001, *P<0.01, LDH: Lactate dehydrogenase, AST: Aspartate transaminase, AESL: Aqueous extract of Saussurea lappa

### Table 2: Myocardial tissue level of lactate dehydrogenase, creatinine kinase and aspartate transaminase

| Treatment group                      | LDH U/l | Creatinine kinase U/l | AST U/l |
|--------------------------------------|---------|-----------------------|---------|
| Control (Saline 10 ml/kg)             | 37.6±10.3| 130.8±26.8            | 69.6±4.1 |
| Isoproterenol (85 mg/kg)              | 5.9±1.4*** | 43.4±25.6*** | 62.5±2.3*** |
| AESL (100 mg/kg)                     | 38.7±7.7*** | 152.3±31.4*** | 68.1±16.5** |
| AESL (200 mg/kg)                     | 35.9±6.9**  | 192.9±5.9*** | 77.2±0.77** |
| AESL (300 mg/kg)                     | 37.3±19.2*** | 196.2±43.8*** | 87.6±17.6** |
| α-tocopherol (60 mg/kg)              | 29.9±14.9*** | 110.9±9.3*** | 83.6±9.1*   |

All value expressed as mean±SD; One-way analysis of variance followed by Tukey’s multiple comparison test, **P<0.001, *P<0.01, LDH: Lactate dehydrogenase, AST: Aspartate transaminase, AESL: Aqueous extract of Saussurea lappa

### Table 3: Myocardial tissue level of thiobarbituric acid reactive substances and glutathione

| Treatment group                      | TBARS nmol/g wet wt | GSH µg/g wet wt |
|--------------------------------------|---------------------|-----------------|
| Control (Saline 10 ml/kg)             | 31.8±1.2            | 138.4±0.71      |
| Isoproterenol (85 mg/kg)              | 103.2±29.5**        | 85.2±14.3*      |
| AESL (100 mg/kg)                     | 48.6±31.6**         | 118.5±12.2**    |
| AESL (200 mg/kg)                     | 23.5±4.6**          | 198.1±4.4***    |
| AESL (300 mg/kg)                     | 66.6±4.5**          | 244.8±5.1**     |
| α-tocopherol (60 mg/kg)              | 20.1±4.9**          | 188.2±6.6***    |

All value expressed as Mean±SD; One-way analysis of variance followed by Tukey’s multiple comparison test, **P<0.001, *P<0.01, *P<0.05, NS: Non-significant, TBARS: Thiobarbituric acid reactive substances, AESL: Aqueous extract of Saussurea lappa, GSH: Glutathione

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Figure 2: 2,2-diphenyl-1-picrylhydrazyl inhibition activity of aqueous extract of *Saussurea lappa*

Figure 3: Hydrogen peroxide radical scavenging activity of aqueous extract of *Saussurea lappa*
AST in Group II and Group III-IV when compared with Group I and II respectively. In Group V and VI there was a significant ($P < 0.01$ and $P < 0.05$ respectively) increase in the level of AST than that in Group II. There was a significant ($P < 0.001$) increase in the level of serum AST in Group II when compared with control Group I and the level significantly decreased in the Groups III ($P < 0.001$), IV ($P < 0.01$), V ($P < 0.001$), and VI ($P < 0.001$).

**Myocardial Thiobarbituric Acid Reactive Substances**

Myocardial TBARS in Group II was significantly ($P < 0.01$) higher than that in control Group I. In Groups IV and VI, there was a significantly ($P < 0.01$) lower TBARS level in comparison to Group II. There were no significant changes in the level of TBARS in Group III and V when compared with Group II.

**Myocardial Glutathione**

There was significant ($P < 0.05$) decrease in the level of GSH in Group II when compared with Group I. The level of GSH was significantly increased in the Groups III and VI ($P < 0.001$) and Group V ($P < 0.01$) when compared to Group II. There were no significant changes in the level of GSH in Group III when compared with Group II.

**Histological Changes**

Light microscopy of the tissue sections of Group I showed the normal cyto architecture of the heart tissue. Group II showed the necrotic changes in myocardial tissue. The tissue sections of both the Groups III-IV showed regenerative changes in heart tissue and Group V-VI showed normal architecture of the heart tissue. The results are presented in Figure 4.

**DISCUSSION**

Isoproterenol is a well-known cardiotoxic agent due to its ability to destroy myocardial cells. As a result of this, cytosolic enzymes such as LDH, AST, and CK were released into the blood stream and serve as the diagnostic markers of myocardial tissue damage. The amount of these cellular enzymes present in heart reflects the alterations in plasma membrane integrity and/or permeability. Changes in the level of myocardial markers LDH and CK in both serum and heart homogenate in ISO-treated rats [Tables 1 and 2] confirms the onset of myocardial necrosis. Chronic oral administration of AESL in three different doses (100, 200, and 300 mg/kg p.o) caused significant change in the level of cardiac markers (LDH, CK, and AST) in both serum and myocardium. But, there was no significant alteration in the level of myocardial AST in ISO-treated and AESL (100 and 200 mg/kg) groups and AESL in higher dose (300 mg/kg) significantly increased the level of myocardial AST when compared with baseline level (Group I).

Administration of ISO generates the free radicals in the myocardium through oxidative stress and produces myocardial necrosis. The principal finding of the present study is that ISO-induced myocardial necrosis was associated with oxidative stress, as evidenced by increase in myocardial

Figure 4: Histopathological report. (a) Group I: Vehicle-received rat heart shows the normal cyto-architecture of the myocardium. (b) Group II: ISO-treated rat heart shows the necrotic changes in myocardial tissue. (c) Group III: Aqueous extract of *Saussurea lappa* 1 (100 mg/kg)-treated rat heart shows regenerative changes in myocardial tissue. (d) Group IV: Aqueous extract of *Saussurea lappa* 2 (200 mg/kg)-treated rat heart shows regenerative changes in myocardial tissue. (e) Group V: SL (300 mg/kg)-treated rat heart shows normal cyto-architecture of myocardium. (f) Group VI: α-tocopherol (60 mg/kg)-treated rat heart shows normal cyto-architecture of myocardium. N=Nucleus; ID=Intercalated disks; NC= Necrotic changes; MC= Myocardial cells; DC= Degenerative changes; RC= Regenerative changes
TBARS in Group II [Table 3]. Similar observations were made earlier by other studies.\[^{[33-27]}\] Chronic oral administration of AESL prevents the oxidative stress and the structural changes associated with ISO-induced myocardial necrosis. The mechanism of such protection by the chronic oral administration of AESL may be due to myocardial adaptation, oxidative stress is mediated through reduction in the TBARS level.\[^{[28]}\] In the present study, we observed that AESL at 200 mg/kg only significantly reduced the oxidative stress and lower (100 mg) or higher (300 mg) doses do not offer significant protection against oxidative stress [Table 3].

Antioxidants play a vital role in eliminating the reactive oxygen species. GSH is one of the major antioxidant enzymes to scavenge the free radicals during tissue damage.\[^{[24]}\] GSH scavenges singlet oxygen, superoxide, and peroxide radicals to form oxidized GSH and other disulfides. Also, antioxidant compounds have been shown to increase GSH reductase activity, that maintains GSH in a reduced state.\[^{[23,25]}\] Decrease in the level of GSH in ISO-treated animals [Table 3] indicated that the depletion of GSH resulted in enhanced lipid peroxidation, and excessive lipid peroxidation caused increased GSH consumption.\[^{[23]}\] AESL-treated groups showed that the significant increase in the level of GSH may be due to its enhanced synthesis. But, this effect is not observed with the lower dose (100 mg) of AESL [Table 3]. Cardio protective effects of AESL was compared with α-tocopherol as the standard natural antioxidant also offered significant protection against ISO-induced depletion of marker enzymes and oxidative stress. This action may be probably due to suppression of membrane damage and reduction in membrane fluidity. Light microscopy examination of rat heart-section treated with AESL and α-tocopherol restored the myocardial damage with no evidence of focal damage produced by isoproterenol, which showed the cytoprotective action of AESL [Figure 4].

Administration of antioxidant-rich natural drugs decreases the mortality from CVD and also promises a therapeutic approach to combat oxidative stress associated with cardiac diseases.\[^{[1]}\] AESL also offered significant inhibition activity in in-vitro antioxidant model which confirmed the protective role via the antioxidant mechanism. As per phytochemical investigation, the AESL contain flavonoids and phenolic compounds in high concentrations, which might be a responsible active principle for the cardio protective action.

**CONCLUSION**

In summary, it has been concluded from the biochemical and histopathological evidence that the AESL produced significant dose-dependent cardioprotection in isoproterenol-induced MI. This is the first report to support the ayurvedic recommendation and further study is ongoing to isolate and elucidate the mechanism of action of the active principle.

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