



CARDIOPROTECTIVE EFFECT OF *LUFFA ACUTANGULA* LEAF EXTRACT AGAINST DOXORUBICIN INDUCED MYOCARDIAL DAMAGE IN WISTAR RATS

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ABSTRACT

The present study was chosen for the leaf of *Luffa acutangula* for the scientific evaluation of cardioprotective potential based on the presence of bioactive compounds which is used to prevent cardiotoxicity. Acute oral toxicity study was carried out for evaluating the safety and toxic effect of ethanolic extract of *Luffa acutangula* leaf. From this study the result showed the ethanolic extract of *Luffa acutangula* leaves extract does not have any toxic effect upto the dose of 2000mg/kg body weight. The In vivo study was conducted with 4 groups of wistar rats, 6 animals in each group. For assessing the cardioprotective activity, Doxorubicin induced cardiotoxicity model was used. The doses of plant extract was chosen for the test to be 200 and 400mg/kg body weight of the animal. The antioxidant enzyme (SOD, CAT, GPx) level was decreased in Dox induced cardiotoxic rats heart homogenate which is compared with normal control group. The pretreatment of EELA leaf to Dox administered animals showed significantly increased antioxidants level in heart homogenate. Dox induced cardiotoxic group increased lipid peroxidation parameter like MDA level and it was significantly decreased in EELA treated group. Histopathological studies in isolated heart revealed that the pretreatment of EELA leaf prevent the myocardial damage and restored the normal architecture of the heart. From this study, it is concluded that the ethanolic extract of *Luffa acutangula* was found to possess the cardioprotective activity. Further findings to explore the possible mechanism of action of isolated compound from this plant against cardiotoxicity.

KEYWORDS: Cardioprotective Effect, *Luffa Acutangula*, Leaf Extract, Doxorubicin, Wistar Rats.

INTRODUCTION

Cardiovascular disease (CVD) is currently the main factor contributing to morbidity and mortality^[1,2], representing 30% of all deaths worldwide.^[3] Coronary heart disease (CHD) is a common disease caused by increased intake of cholesterol and saturated fat, decreased intake of polyunsaturated fatty acids (PUFAs), and increased obesity.^[4] Additionally, CHDs are more common in low-birth-weight infants.^[5] Atherosclerosis is an inflammatory disease that contributes to the major incidence and mortality of CVD.^[3] In the process of atherosclerosis, lipids accumulate on the walls of blood vessels and cause inflammatory reactions, thereby stimulating the progression of atherosclerosis.^[2] However, the atherosclerotic process starts before birth, progresses through childhood in the so-called "first thousand days," and eventually leads to CHD if

unstopped. Therefore, it is important to start prevention in the earliest stages of life.^[6,7]

The lipid profile (dyslipidemia) is a significant risk factor for CVD and early atherosclerosis.^[8,9] Dyslipidemia is a significant contributor to the risk of both coronary artery disease and stroke and is defined as an elevated plasma concentration of lipids (triglycerides and total cholesterol) and their related blood-transporting lipoproteins: high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C).^[10-12] However, the associations between the relative amount of cholesterol and total cholesterol in individual lipoprotein classes and CHD are complicated.^[13]

Plants are rich source in phytochemical constituents. Active phytochemical constituents such as flavonoids, cardiac glycosides, terpenoids and saponins are known to

responsible for antioxidant activity and hence maybe considered to be potential cardioprotective effect. The preliminary review of literature indicates that plant possess natural antioxidant property and belonging to the family Cucurbitaceae. The aim of the present study is to evaluate the cardioprotective effect of *Luffa acutangula* leaves by *in vitro* and *in vivo* method.

MATERIALS AND METHODS

Plant Collection and Identification

The fresh leaf of *Luffa acutangula* was collected from Chittoor District, AP, India and authenticated by Prof. Madhav Shetty, Dept. of botany, Taxonomist, SV University, Tirupati. A voucher was kept in the Department of Pharmacognosy for reference.

Preparation of Plant extract

The freshly collected leaf of this plant was shade dried for one week. The dried material was powdered and passed through 10-mesh sieve. The powder was then extracted with petroleum ether (500ml), ethyl acetate (500ml) and ethanol (500ml) separately for 24 hours in a Soxhlet apparatus.

ACUTE TOXICITY STUDY

Acute toxicity study was designed as per the OECD

Table 1: Experimental Design.

Group	Group name (n=6)	Treatment schedule
I	Normal Control	Normal saline or water
II	Disease Control	Doxorubicin (2.5mg/kg i.p) alternate days for 3weeks
III	EELA leaves (200mg/kg)	Doxorubicin (i.p) alternate days for 3weeks + Ethanolic extract of <i>Luffa acutangula</i> leaves (200mg/kg) orally (day 1 to day 28)
IV	EELA leaves (400mg/kg)	Doxorubicin (i.p) alternate days for 3weeks +Ethanolic extract of <i>Luffa acutangula</i> leaves (400mg/kg) orally (day 1 to day 28)

After the experimental regimen, the animal was anesthetized by using isoflurane anesthesia and blood was collected through cardiac puncture method for hematological and biochemical analysis. Then animals were sacrificed and heart was excising out for histopathological analysis.

HISTOPATHOLOGICAL STUDIES

The heart samples were excised from the experimental animals of each group and washed with ice-cold normal saline. A small portion of the heart sample from each group was quickly fixed in 10% buffered neutral formalin for 48 hours and then with bovine solution for 6 hours. Then the heart tissues were processed for paraffin embedding by standard histopathological technique i.e. dehydration through graded DOX propyl alcohol, cleaning through xylene and impregnated in paraffin wax for two hours, paraffin sections were taken at 5mm thickness- using microtome and processed in alcohol xylene series. The heart sections was stained by alumhaematoxylin and resin and the sections were examined microscopically and photographed for the evaluation of histopathological changes.

guidelines (423).

Principle and Purpose

The main purpose of acute toxicity study is to evaluate the degree of toxicity in a quantitative and qualitative manner with the purpose of comparing it with other drug substance (eg. Other drug candidates for the same indication).

Experimental Animals

Three healthy adult wistar albino rats were weighing between 150 - 250g was selected for the study. For all the three animals' food, but not water was withheld overnight prior to dosing.

IN VIVO CARDIOPROTECTIVE STUDY

The cardioprotective activity of *Luffa acutangula* leaf extract was evaluated in wistar albino rats.

Induction of cardiac toxicity: Cardiac toxicity was induced by intraperitoneal injection of Doxorubicin was dissolved in normal saline attain cumulative dose 25 mg/kg body weight.

STATISTICAL ANALYSIS

The results were expressed as Mean \pm SEM. One-way Analysis of variance (ANOVA) followed by Dunnett multiple comparison test was used to correlate the difference between the variables. $P < 0.05$, $P < 0.01$ and $P < 0.001$ is considered as statistically significant.

RESULTS AND DISCUSSION

Percentage Yield

The fresh leaves of *Luffa acutangula* was collected in the month of August, 2018. The leaves dried in shade and coarse powdered. Then the powder was extracted using various solvents like petroleum ether, ethyl acetate and ethanol by using Soxhlet apparatus. The percentage yield of various extracts are calculated and shown in **Table 2**.

Table 2: Percentage yield of Plant extract in various solvents.

S.No	Type of Extract	Yield value (%w/w)
1	Petroleum ether	4.83
2	Ethyl acetate	4.31
3	Ethanol	7.58

It results shows that the percentage yield of ethanol extract (7.58%) was higher than the petroleum ether extract (4.83%) and ethyl acetate extract (4.31%).

IN-VITRO STUDIES

Superoxide Dismutase Assay

Superoxide dismutase (SOD) is a primary antioxidant enzyme and it was directly involved in the elimination of

ROS. SOD is an enzyme that alternately catalyzes the dismutation of superoxide radical into their ordinary molecular oxygen or hydrogen peroxide and provides cellular defence against reactive oxygen species. The antioxidant effect of petroleum ether, ethyl acetate and ethanol extracts of *Luffa acutangula* leaves was evaluated and the results are tabulated in **Table 3**.

Table 3: Estimation SOD activity of *Luffa acutangula* leaf extract.

S.No	Concentration (µg/ml)	SOD (U/mg Protein)			
		Standard (Ascorbic Acid)	Pet. Ether Extract	Ethyl acetate Extract	Ethanol Extract
1	200	52.89	7.84	30.95	49.71
2	400	67.83	17.23	41.46	60.08
3	600	78.31	23.39	49.86	73.24
4	800	85.9	29.41	58.82	83.89
5	1000	95.78	31.93	66.11	89.92
6	IC ₅₀ value	166.59	1528.08	613.33	209.03

Values are expressed as triplet determination

The IC₅₀ values of plant extract was compared with the IC₅₀ value of standard (Ascorbic acid). The ethanolic extract of *Luffa acutangula* leaf showed significant IC₅₀ value (209.03 U/mg Protein) than petroleum ether (1528.08 U/mg Protein) and ethyl acetate (613.33 U/mg Protein) extract. The IC₅₀ value of ethanolic extract of *Luffa acutangula* leaves has produced significant IC₅₀ value near to standard (AA) 166.59 U/mg Protein.

DPPH ASSAY

DPPH radical scavenging activity is widely used method

for assessing antioxidant effect of the plant extracts. DPPH is a well-known stable radical and scavenger for other radicals. The rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. A strong absorption band centered at 520nm. DPPH radical has a deep violet colour in solution and it becomes colourless or pale yellow when it neutralised. The DPPH radical scavenging activity of petroleum ether, ethyl acetate and ethanol extract of *Luffa acutangula* leaves was given in following Table 4.

Table 4: DPPH Free Radical Scavenging Assay of *Luffa Acutangula* Leaf Extract.

S.No	Concentration (µg/ml)	DPPH (U/mg Protein)			
		Standard (Quercetin)	Pet Ether Extract	Ethyl acetate Extract	Ethanol Extract
1	200	61.53	5.66	13.76	29.96
2	400	70.85	8.91	24.29	44.53
3	600	78.54	14.17	39.67	60.32
4	800	85.02	18.62	55.06	73.68
5	1000	91.49	24.29	60.32	84.21
6	IC ₅₀ value	142.14	2117.48	784.28	476.09

Values are expressed as triplet determination

From the results the IC₅₀ values of various extracts of *Luffa acutangula* leaf and standard are depicted. The IC₅₀ values of plant extract was compared with the IC₅₀ value of standard (Ascorbic acid). The ethanolic extract of *Luffa acutangula* leaf showed significant IC₅₀ value (476.09 U/mg Protein) than petroleum ether (2117.48 U/mg Protein) and ethyl acetate (784.28 U/mg Protein) extract. The IC₅₀ value of ethanolic extract of *Luffa acutangula* leaves has produced significant IC₅₀ value near to standard (Quercetin) 96.59 U/mg Protein.

ethyl acetate extract of *Luffa acutangula* leaves.

IN-VITRO CARDIOPROTECTIVE STUDY USING H9c2 (RAT CARDIOMYOCYTE) CELL LINE

H9c2 (rat cardiomyocyte) cell line derived from rat heart ventricle of embryo and it was widely used as an *in-vitro* cellular cardiac model because they have most of the molecular and functional features of adult cardiomyocytes. Cardiac hypertrophy is a major risk factor for heart failure. H9c2 cell line has the advantage of being animal-free alternative and it can accurately mimic the hypertrophic responses.^[90,91] So this finding are helps to choose H9c2 cell line as a model for the study of *In-vitro* cardioprotective effect of *Luffa acutangula* leaf extracts.

From the results of SOD and DPPH assay showed that the ethanolic extract of *Luffa acutangula* leaves possess the potent antioxidant effect than petroleum ether and

CELL VIABILITY ASSAY (MTT ASSAY)

The MTT assay is most commonly used method for determining the viability of the cells. It is colorimetric assay for assessing the cell metabolic activity. Viable cell contains NAD (P) H - dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple colour. Formazan crystals are dissolved using a solubilising solution and absorbance was measured at 500-600nm. When the cells are die they lose their ability to convert MTT into formazan. If more cells are in viable they shows thick colour formazan formation. The colour formation serves as a convenient marker of the viable cells. The cell line is treated with Doxorubicin alone and Plant extract with doxorubicin and percentage of cell viability is depicted in following **Table 5**.

Table 5: Cytotoxic effect of different concentrations of Doxorubicin on H9c2 cell line.

S.No	Concentration (µg/ml)	Dilutions	Cell viability (%)
1	200	1:1	13.75
2	100	1:2	23.99
3	50	1:4	31.82
4	25	1:8	41.33
5	12.5	1:16	48.69
6	6.25	1:32	57.48
7	Cell control	-	100

Values are expressed as triplet determination

In doxorubicin treated cell line significantly decreased the cell viability in a dose dependent manner and it shows increased cell death because of its excessive ROS production activity. Doxorubicin has the several mechanism for the cardiotoxic effects, but an excessive generation of free radicals (ROS) is the major mechanism behind drug induced toxicity.^[92] Doxorubicin has Quinone moiety in their molecule which is converted enzymatically or non-enzymatically by cytochrome P₄₅₀ into its semiquinone moiety by the acquisition of electrons. This semiquinone form is then oxidised by molecular oxygen to yield free radicals which is leading to cardiotoxicity. Doxorubicin was reduced the half percentage of cell viability on H9c2 cell line at the concentration of 4.38µg/ml after 24hrs. So the Doxorubicin has cardiotoxic effect in a dose dependent manner.

Table 6: Cardio protective effect of Pet. Ether extract of *Luffa acutangula* leaves on H9C2 cell line induced cardiotoxicity using Doxorubicin.

S.No	Concentration (µg/ml)	Dilutions	Cell viability (%)
1	200	1:1	70.48
2	100	1:2	62.17
3	50	1:4	50.55
4	25	1:8	39.69
5	12.5	1:16	29.01
6	6.25	1:32	19.76
7	Cell control	-	100

Values are expressed as triplet determination

Table 7: Cardio protective effect of Ethyl acetate extract of *Luffa acutangula* leaves on H9C2 cell line induced cardiotoxicity using Doxorubicin.

S.No	Concentration (µg/ml)	Dilutions	Cell viability (%)
1	200	1:1	89.31
2	100	1:2	77.60
3	50	1:4	64.37
4	25	1:8	52.16
5	12.5	1:16	38.93
6	6.25	1:32	26.72
7	Cell control	-	100

Values are expressed as triplet determination

Table 8: Cardio protective effect of Ethanol extract of *Luffa acutangula* leaves on H9C2 cell line induced cardiotoxicity using Doxorubicin.

S.No	Concentration (µg/ml)	Dilutions	Cell viability (%)
1	200	1:1	93.38
2	100	1:2	80.15
3	50	1:4	67.93
4	25	1:8	56.99
5	12.5	1:16	47.16
6	6.25	1:32	36.39
7	Cell control	-	100

Values are expressed as triplet determination

From the result of antioxidant study and cardioprotective cell line study (cell viability assay) the ethanolic extract of *Luffa acutangula* leaf possess significant antioxidant and cardioprotective activity than petroleum ether and ethyl acetate extract. So, furtherly the ethanolic extract of *Luffa acutangula* leaf was chosen for *in-vivo* cardioprotective study.

IN VIVO CARDIOPROTECTIVE STUDY

After completion of the acute toxicity study, the animals were grouped and the cardioprotective effect of ethanolic extract of *Luffa acutangula* leaves against DOX induced cardiotoxicity was established by assessing haematological, biochemical and histopathological studies.

GENERAL OBSERVATIONS

The general observations like behavioural changes and appearance of the animals was noted throughout the experimental period. In DOX treated group have shown the characteristic changes as the animal fur became scruffy, had red exudates around the eyes and nose, soft watery faeces, abdomen enlargement and animals looked weaker and lethargic when compared with normal control. These observations are markedly less in the animals treated with ethanolic extract of *Luffa acutangula* leaves (EELA) when compared with DOX treated group.

BODY WEIGHT

The body weight changes of the animals was

measured throughout the study period and given it into the **Table 10**.

Table 10: Body weight changes.

Days	Body weight (g)			
	Normal control	Disease control	EELA (200mg/kg)	EELA (400mg/kg)
0	123.5±1.43	139.66±1.47	134±1.73	136.5±1.38
7	136.5±2.15	122.33±2.12 ^{###}	130.83±2.75 ^{NS,*}	131.5±1.72 ^{NS,**}
14	138.83±1.70	118±1.41 ^{###}	131.5±2.92 ^{NS,*}	135.5±1.96 ^{NS,**}
21	140.83±4.26	117±1.52 ^{###}	133.83±2.85 ^{NS,*}	137.33±1.97 ^{NS,**}
28	142.16±3.77	112.75±1.07 ^{###}	136.66±2.84 ^{NS,*}	138.66±1.98 ^{NS,**}

HEART WEIGHT

After end of the treatment period the heart weight of the animals was measured and tabulated in **Table 11**.

Table 11: Heart weight changes.

S.NO	Group	Heart Weight (g)
1	Normal control	0.37±0.0049
2	Disease control	0.49±0.0210 ^{###}
3	EELA (200mg/kg)	0.42±0.0170 ^{NS,**}
4	EELA (400mg/kg)	0.37±0.0066 ^{NS,***}

HEMATOLOGICAL PARAMETER ANALYSIS

Blood is an important index for physiological and pathological index for humans and animals and the parameters usually measured are haemoglobin (Hb), RBC and WBC. Alterations in blood parameters may be due to the changes in cellular integrity and membrane permeability of cells or even exposure to toxic chemicals. DOX intoxication might be lead to anaemia as a result of either altered activity of hematopoietic tissue, impaired erythropoiesis and defective iron metabolism.

HAEMOGLOBIN

Haemoglobin (Hb) is the oxygen carrying protein which is present in RBC. Hb concentration may affect the cardiovascular system through oxygen supply and viscosity.^[94] An increased or decreased levels of Hb are independently associated with increased risk factor of CVDs. The Hb concentration of the animals were given in following **Table 12**.

Table 12: Concentration of Hemoglobin.

S.NO	GROUP	HEMOGLOBIN (g/dl)
1	Normal control	13.5±0.18
2	Disease control	10.3±0.34 ^{###}
3	EELA (200mg/kg)	12.8±0.24 ^{NS,***}
4	EELA (400mg/kg)	13.6±0.26 ^{NS,***}

TOTAL RBC

Red Blood Cells the are constituents of intravascular clots and carry oxygen. RBCs may play a prothrombotic role in blood coagulation by increasing blood viscosity

and forcing platelets towards the vessel wall. Incorporation of RBCs into fibrin clot affects clot structure and mechanical properties. Even small structural differences in RBCs may have large influence on pathophysiology. RBCs may actively participate in thrombin generation. The reduction in RBC level may increasing the heart rate and cause chest pain. The Total RBC level of animals were given in following **Table 13**.

Table 13: Concentration of RBC.

S.NO	GROUP	TOTAL RBC (ml/cmm)
1	Normal control	7.16±0.033
2	Disease control	6.06±0.039 ^{###}
3	EELA (200mg/kg)	7.001±0.071 ^{NS,***}
4	EELA (400mg/kg)	7.05±0.102 ^{NS,***}

OTAL WBC

The WBC and WBC subtypes are important for body immune system. The increased and decreased level of WBC may cause inflammatory responses, auto immune disorder and other diseases. In cancer, the chemotherapy drugs are destroying the bone marrow and reduce WBC count and cause several infections and disorders. The total WBC concentrations of animals are given in following **Table 14**.

Table 14: Concentration of WBC.

S.NO	GROUP	TOTAL WBC($\times 10^3/m$)
1	Normal control	10116.67±504.25
2	Disease control	6066.67±456.55 ^{###}
3	EELA (200mg/kg)	7783.33±314.55 ^{###,*}
4	EELA (400mg/kg)	10833.33±560.16 ^{NS,***}

The results of haematological parameters showed that the Doxorubicin treated animals significantly decreased the Hb, RBC and WBC counts by changing the cellular integrity and permeability. Dox toxicity leads to the condition of anaemia, which increase the oxygen demand in the blood supply associated with leukopenia. Groups pre-treated with EELA has been reversed the Dox toxicity and significantly increased the Hb, RBC and WBC contents. Therefore, the results suggested that the

extract has a significant protective effect on the hematopoietic system.

SUMMARY AND CONCLUSION

The present study was chosen for the leaf of *Luffa acutangula* for the scientific evaluation of cardioprotective potential based on the presence of bioactive compounds which is used to prevent cardiotoxicity.

Doxorubicin is an anthracycline antibiotic which is effectively used in the treatment of different malignancies. Nowadays its use is limited because of the drug induce cardiotoxicity in dose dependent manner. Several mechanisms are involved in DOX induced cardiotoxicity, but the major mechanism is oxidative stress. Antioxidant properties of several plants are experimentally proved and widely used as most effective agents against oxidative stress.

ROS is the major causative factor for many diseases. Antioxidants are prevent the deleterious effect caused by these free radicals. *In vitro* antioxidant study and cardioprotective study was carried out in three extracts (petroleum ether, ethyl acetate and ethanol) of *Luffa acutangula* to choose effective extract for further *in vivo* studies. The antioxidant effect was evaluated by SOD assay and DPPH radical scavenging assay. The cardioprotective study was evaluated by using H9c2 (Rat Cardiomyocyte) cell line. Cell viability assay (MTT assay) was carried out. From this, the ethanolic extract of *Luffa acutangula* leaf possess potent antioxidant effect which exhibit their action at lower concentration. In cell viability assay the DOX treated cells showed decreased cell viability and increased cell death. The pretreatment with the plant extracts increased the cell viability and reduce the cell death. From this also the ethanolic extract of *Luffa acutangula* possess potent cardioprotective activity. So the ethanolic extract was chosen for further *in vivo* studies.

Acute oral toxicity study was carried out for evaluating the safety and toxic effect of ethanolic extract of *Luffa acutangula* leaf. From this study the result showed the ethanolic extract of *Luffa acutangula* leaves extract does not have any toxic effect upto the dose of 2000mg/kg body weight.

The *In vivo* study was conducted with 4 groups of wistar rats, 6 animals in each group. For assessing the cardioprotective activity, Doxorubicin induced cardiotoxicity model was used. The doses of plant extract was chosen for the test to be 200 and 400mg/kg body weight of the animal.

In doxorubicin induced cardiotoxic rats showed significant decrease in body weight and looked very weaker when compared with the normal control group. The pre-treatment of EELA leaf to Dox administered animals showed significant increase in body weight. The

heart weight of Dox treated rats showed significant increase when compared with normal control group. The pretreatment of EELA leaf to Dox administered animals showed significant decrease in heart weight.

In Dox induced cardiotoxic group significantly decreased the haematological parameter values and the pretreatment of EELA leaf to Dox administered animals shows that the haematological parameter values were increased. The elevation of cardiac marker enzyme level in serum was showed in Dox induced cardiotoxic group and it confirms the onset of myocardial injury. The pretreatment of EELA leaf to Dox administered animals significantly decreased the cardiac marker enzyme level in serum.

The antioxidant enzyme (SOD, CAT, GPx) level was decreased in Dox induced cardiotoxic rats heart homogenate which is compared with normal control group. The pretreatment of EELA leaf to Dox administered animals showed significantly increased antioxidants level in heart homogenate. Dox induced cardiotoxic group increased lipid peroxidation parameter like MDA level and it was significantly decreased in EELA treated group.

From this study, it is concluded that the ethanolic extract of *Luffa acutangula* was found to possess the cardioprotective activity. Further findings to explore the possible mechanism of action of isolated compound from this plant against cardiotoxicity.

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