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Correlation of Antioxidant Principles with Cardioprotective Activity of *Madhuca longifolia* (Koenig) Leaves on Isoproterenol Induced Myocardial Infarction.

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ABSTRACT

The Current pharmacological investigation was under taken to evaluate the cardioprotective effect of *Madhuca longifolia* (Koenig) against experimentally induced myocardial infarction in rats by using isoproterenol. The inducing agent significantly ($p < 0.05$) decreased the levels of superoxide dismutase, catalase, glutathione and increased leakage of cardiac injury markers; creatine kinase, lactate dehydrogenase associated to increased lipid per oxidation and histopathological perturbations. However, pre-treatmented with *Madhuca longifolia* (Koenig) restored hemodynamic parameters prevented the depletion of endogenous antioxidants and myocyte marker enzymes as well as inhibited lipid per oxidation. Results showed that *Madhuca longifolia* protected heart against cardiotoxic effects of isoproterenol by antioxidant activity with maintaining structural integrity of heart.

Keywords: Antioxidants, Isoproterenol, *Madhuca longifolia*, Myocardial infarction.

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INTRODUCTION

Myocardial infarction is the condition caused by an imbalance between the myocardial oxygen supply and demand causing myocardial hypoxia, persisting ischaemia result in to infraction causing death of myocardial tissue[1]The excessive generation of free radicals, reactive oxygen species (ROS) contains an odd number of electrons making them chemically reactive and often leading to a chain reaction which contributes to cell death[2]. Isoproterenol (ISO), a catecholamine from the synthetic origin and β-adrenergic agonist that causes severe stress in myocardial tissue and necrosis of the heart muscles[3]. The higher dose of ISO causes the cardiotoxic effects by the mechanisms like functional hypoxia and ischemia, coronary insufficiency, over load of intracellular ca^{2+}, changes in electrolyte contents and oxidative stress. Biochemical alterations in ISO induced cardiomyopathy represent the changes in cardiac marker enzymes, lipid profile, non enzymatic antioxidant levels and electrolytes [4]. Myocardial ischemia and reperfusion has been reported to be correlated with excess generation of reactive oxygen species (ROS). These ROS reported and responsible in depletion of endogenous antioxidant cluster, which resulted in increased lipid per oxidation and consequently, depressed contractile function.

Antioxidants are the active substances that delay the process of oxidation by inhibiting the polymerization chain which is initiated by free radicals and other concurrent oxidative mechanisms. A growing body of literature points to the importance of natural antioxidants from many plants, which may be used to reduce oxidative damage at cellular level. This may protect the human body against chronic diseases which includes cancer and neurodegenerative diseases, inflammation and cardiovascular diseases[5].

Madhuca longifolia (Koenig) of family sapotaceae, parts of which have various medicinal uses wound healing, bone fracture, skin disease, headache. piles, ulcer, bronchits, acute and chornic tonsillitis, pharyngitis, diabetic, stomachache, It is also using as antiburns, Laxatives, emotics, anti-earthworms, anti-snake poisoning, astringent, hepatoprotective[6]. In some research articles have proved that thee phytocontituents like phenols, flavonoids and triterpenoids have shown better organ/organs protective activity[7,8]. The study plant rich in flavonoids, phenolics, tannins, stilbenes, lignans and lignin are common in leaves, flowers, woody parts like stems and barks[9]. Therefore the present study was planned to correlate the antioxidant property with cardio protective activity of *Madhuca longifolia* (Koenig) leaves extract by ISO induced cardiotoxicity.

MATERIALS AND METHODS

Plant materials and extraction

Madhuca longifolia (Koenig) leaves were collected from our college herbal garden. The plant was identified and authenticated by Prof. K. Prabhu, Department of Pharmacognosy, S.C.S. College of Pharmacy, Harapanahalli. The dried powder of the leaves were defatted with pet ether and then extracted with 95% ethanol using soxhlet apparatus. The extracts was concentrated under reduced pressure using rota flash evaporator and stored in airtight container in refrigerator below 10ºC. 95% ethanolic extract which was used after subjecting it to preliminary qualitative phytochemical studies.

Chemicals

Isoproterenol was obtained from sigma chemical company. The biochemical kits were purchased from Erba Mannheim, Germany and all the other chemicals and reagents used were of analytical grade.

DPPH radical-scavenging activity[10]

The stable 2, 2-diphenly-1-picryl hydroxyl radical (DPPH) was used for determination of free radical-scavenging activity of the extract. To 1 ml of 0.135mM DPPH prepared in methanol was mixed with 1.0 ml of 95% ethanolic extract *Madhuca longifolia* leaves (EEML) ranging from 100 to 600µg/ml. the reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured at 517nm. The scavenging ability of the extract was calculated using the formula.

% Inhibition = Control OD – Test OD / Control OD X 100

Total Phenolic Content (TPC)[11]

The total phenolic content was determined by adopting the method as described in Malik E.P and singh M.B et al[11]. Aliquots of the extract was taken in a 10 ml glass tube and made up to a volume of 3 ml with distilled water. Then 0.5 ml Folin ciocalteau reagent (1:1 with water) and 2 ml 20% Na_2CO_3 were added sequentially in each tube. A blue color was developed in each tube and the intensity of the color is directly proportional to the phenolic content. The blue colouration in the tube is due to the formation of molybdenum blue as a result of complex redox reaction between phenols and phosphomolibdic acid in Folin ciocalteau reagent in alkaline medium. The test solutions were warmed for 1minute, cooled and absorbance was measured at 650 nm. The calibration curve was prepared using catechol. The phenolic content of the plant was expressed as a mg. equivalent of phenol per gm. of extract.

Total tannin content[12]

The quantitative tannin content in samples was estimated by the method of Price and Butler (Price and Butler, 1977) with some modifications. In short, 0.1 g of a dry plant sample was transferred to 100 ml flask; 50 ml water was added and boiled for 30 min. After filtration with cotton filter, the solution was further transferred to a 500 ml flask and water was added ad 500 ml mark. 0.5 ml aliquots were finally transferred to vials , 1 ml 1% $K_3Fe(CN)_6$ and 1 ml 1% $FeCl_3$ were added and water was added upto 10 ml volume. After five min time period, the solutions were measured spectrophotometrically at 720 nm. The actual tannin concentrations were calculated on the basis of the optical absorbance values obtained for the standard solutions in range 5 -30 µg /10 ml.

Animals

In present study Wister albino rats (200-250g) of either sex were used . The animals were acclimatized for one week – 10days at laboratory conditions. They were housed in standard animal cages and maintained at 27°C ± 2°C under 12 hrs dark/light cycle. They were fed with standard rat feed (Gold Mohur Lipton India Ltd.) and water ad labium was provided. Ethical clearance for this project was obtained from the institutional animal ethical committee prior to beginning of the work, the Registration no. SCSCP/626/3/2012-2013.

Experimental protocol[13,14]

Albino rats were randomly allotted in to 6 groups of 6 animals. The group I animals were treated as negative control and were fed with normal saline for 16 days. The animals of group II, III, IV, V and VI were fed with normal saline, Vit.C (200 mg/kg p.o), test extract of three doses (250, 333 and 500 mg/kg) for IV, V and IV groups, daily by p.o, respectively for 16 days. Then, animals of all the groups were given ISO (200mg/kg) by s.c for two consecutive days at 24h interval.

At the end of experimental period (after 24h of second ISO injection or 16th day of extract/vehicle treatment) all the rats were anaesthetized with urethane (1g/kg, i.p) and blood was collected from the retro-orbital plexus; the serum was separated and used for the determination of diagnostic marker enzymes like ALT, AST, LDH, CK, TC, TG, LDL and HDL. The heart was dissected out, washed immediately in ice-chilled physiological saline. The samples of heart tissue were analyzed for tissue GSH, lipid peroxidation**,** SOD and CAT. The hearts were also stored in 10% formalin for histological studies to evaluate the details of myocardial architecture in each group microscopically.

Histopathological studies

Slices of heart tissue were collected in 10% formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Section of 5-6 microns in thickness were cut and stained with hematoxylin and eosin. All the sections of the tissues were examined under microscope for the analyzing the altered architecture of the heart tissue due to isoproterenol challenge and improved heart architecture due to pretreatment with test extracts.

Statistical analysis

Results were expressed as mean ± SEM, (n=6). Statistical analyses were performed with one way analysis of variance (ANOVA) followed by Tukey's Kramer comparison test by using Graph Pad Instant Software. P value less than 0.05 was considered to be statistically significant. *P<0.05, **<0.01 and ***<0.001, when compared with control and toxicant group as applicable.

RESULTS

Since GSH is considered as inbuilt antioxidant which prevents lipid per oxidation, estimation of tissue GSH, SOD, CAT and extent of lipid per oxidation were considered as parameters of screening *in-vivo* antioxidant properties. Treatment with EEML leaves of higher dose (500 mg/kg) replenished the depleted tissue levels of GSH, SOD, CAT and lowers the elevated LPO in Isoproterenol induced MI (table 4).

In present study ISO treated rats showed significant elevation in the levels of diagnostic marker enzymes and decreased HDL levels, whereas in EEML (500 mg/kg) leaves treated group showed significant reduction in ISO induced elevated diagnostic marker enzymes (table 3). This reduction in enzymes levels could be due to extract action in maintaining membrane integrity, which prevents leakage of these enzymes and hence protected against MI.

DISCUSSION

Studies have demonstrated the effectiveness of medicinal plants and phytochemicals as a phytotherapeutic agent in ischaemic heart disease as these serve as excellent candidates against oxidative stress associated conditions[15]. The experimental results witnessed that *Madhuca longifolia* (Koenig) maintained the levels of antioxidants and lipid peroxides of heart in myocardial infracted rats.

The total phenolic content of 95% EEML was 58.4mg/g, where as in aqueous extract 32.04mg/gm expressed as equivalent to catechol. Similarly tannin content was found to be 55 mg/g, 30.0mg/gm expressed as equivalent to tannic acid, in aqueous and ethanolic ext respectively as shown in table 1, fig.1 and 2. EEML have demonstrated concentration dependent increase in the DPPH radical scavenging activity. Whereas at 600µg ascorbic acid has 80.20% DPPH radical scavenging activity. However, test extracts even at 600µg showed lesser inhibition i.e. 68.00% than standard in the antioxidant model. The results are summarized in table 2. Myocardial infarction results from any interruption in the blood supply to any part of the heart, and leads to the death of the cardiac tissue (myocardial necrosis). The consequences of myocardial infarction include hyperlipidemia, per oxidation of membrane lipids, and loss of plasma membrane integrity[16].

During the last few decades, research data has prompted a passionate debate as to whether oxidation, or specifically, oxidative stress mediated by free radicals/reactive oxygen species (ROS)/reactive nitrogen species (RNS), is a primary or secondary cause of many chronic diseases[17]. Hence in this study, leaves of EEML reported to posses polyphenolic compounds. The preliminary phytochemical screening of leaves showed that, they contain flavonoids, tannins and polyphenols, saponins in ehanolic and aqueous extract. After quantitative estimations alcoholic extract yielded more flavonoidal, tannin and polyphenols compared to aqueous extract, hence EEML selected in this study.

Table 1: Total phenolic and tannin content

Particulars	Phenolic content		Tannin content	
Extracts	95% ethanolic extract	Aqueous extract	95% ethanolic extract	Aqueous extract
Standard curve	Catechol		Tannic acid	
Absorbance	650nm		720nm	
Amount of content in extract per gram	58.4 mg/g	32.04 mg/g	55.00 mg/g	30.00 mg/g
R^2 value	0.998		0.993	

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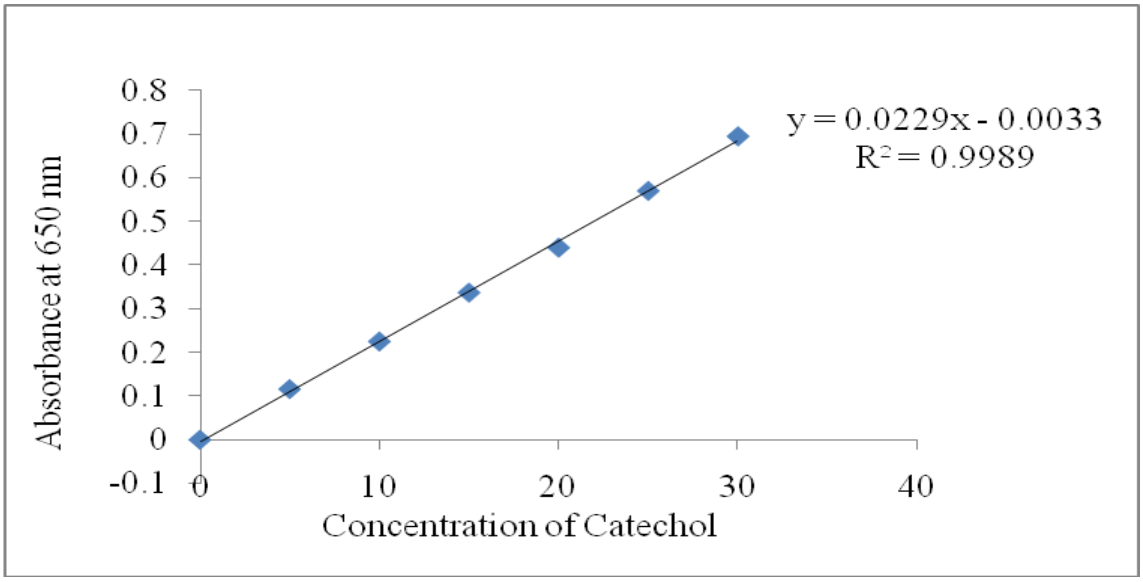

Figure 1: Catechol calibration curve

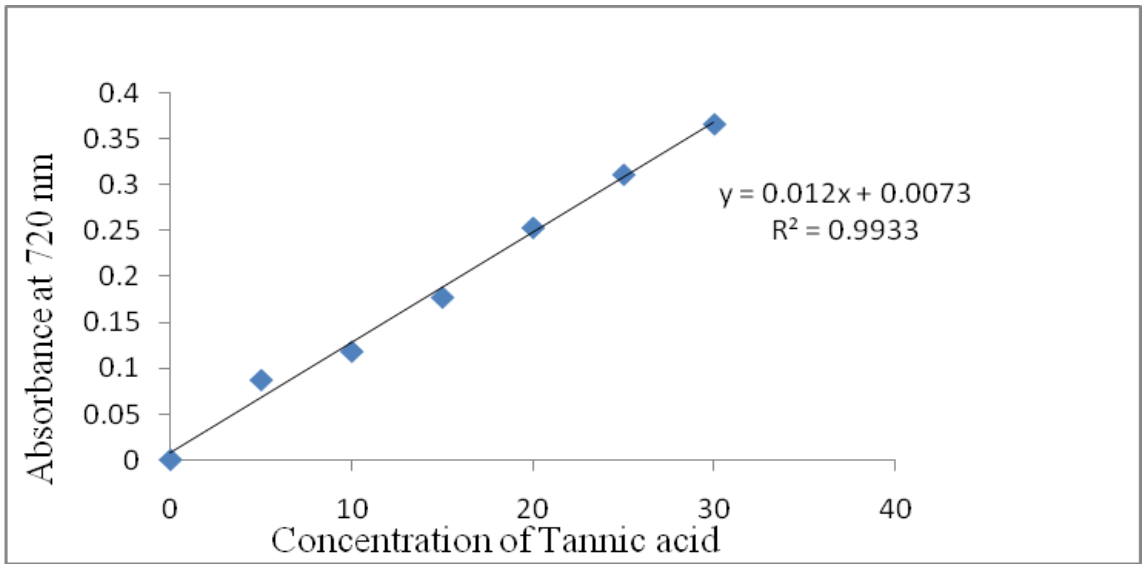

Figure 2: Tannic acid calibration curve

Table 2: DPPH antioxidant activity of EEML

Sl. No.	Conc	Standard (vitamin c)		95% ethanolic extract	
		Mean±SEM	%inhibition	Mean±SEM	%inhibition
1.	Control	0.0500±0.0000	000	0.0420±0.0001	000
2.	100µg/ml	0.0212±0.00075	60.0%	0.0442±0.0062	8.57%
3.	200µg/ml	0.0162±0.0047	66.80%	0.0335±0.0065	18.50%
4.	300µg/ml	0.0157±0.0025	68.70%	0.0320±0.0040	36.80%
5.	400µg/ml	0.0127±0.0026	74.50%	0.0260±0.0016	44.70%
6.	500µg/ml	0.0107±0.0023	78.00%	0.0210±0.0042	58.90%
7	600µg/ml	0.0102±0.0021	80.20%	0.0150±0.0013	68.00%

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Table 3: Effect of 95% EEML on Biochemical markers in Isoproterenol induced Cardiotoxicity

Groups	AST	ALT	CK	LDH	LDL	HDL	TC	TG
Normal control	38.69 ± 0.429	38.49± 0.523	168.5± 5.226	234.8± 14.99	72.22± 2.332	66.18± 5.898	182.9± 5.513	134.9± 8.113
Positive control	76.72± 1.414	58.89± 0.525	346.3± 6.884	356.0± 6.655	162.1± 3.240	36.98± 1.519	312.3± 20.74	310.5± 3.868
Std. Vit C	36.18± 0.580***	35.77± 0.824***	156.0± 1.646***	227.6± 15.87***	80.86± 2.65 ***	62.66± 5.610**	175.3± 6.306***	129.5± 9.013***
EEML 250 mg/kg	71.60 ± 2.689	57.90± 0.536	344.6± 6.399	353.9± 5.874	120.4 ± 5.460	35.08± 1.461	310.7± 21.21	309.9± 4.458
EEML 333mg/k	65.79 ± 1.457**	55.29± 1.578*	343.4± 7.808	361.1± 4.334	119.2± 5.340**	39.10± 1.639	169.8± 4.654***	266.1± 13.83**
EEML 500 mg/kg	52.05 ± 2.775***	45.60± 1.074***	151.0± 2.461***	221.5± 17.19***	110.5± 5.220**	58.15± 6.803***	157.7± 6.193***	130.2± 7.495***

Values are mean ± SEM of six rats/treatment. *p<0.05,** p<0.01,***p<0.001 as compared to positive control

Table 4: Effect of 95% EEML on GSH, LPO, SOD, and CAT on Isoproterenol induced Cardiotoxicity in Rats.

Treatment	GSH	LPO	SOD	CAT
Normal control	0.3250±0.0025	0.4062±0.0095	0.3448±0.005	0.3677±0.0032
Positive control	0.2093±0.413	0.6430±0.0054	0.1648±0.0046	0.1820±0.0043
Standard	0.3073±0.0109**	0.3748±0.0055***	0.3278±0.0049***	0.3462±0.0073***
95%EEML 250mg/kg	0.2037±0.0214	0.6145±0.0084	0.1832±0.0124	0.1923±0.0038
95%EEML 333mg/kg	0.3048±0.0127**	0.5973±0.0201*	0.2010±0.0102*	0.2275±0.0095**
95% EEML 500mg/kg	0.3248±0.0047**	0.4430±0.0151**	0.2923±0.0084***	0.3283±0.0064***

Values are the mean Mean ± SEM of six rats/treatment. Significance *p<0.05,**p<0.01 and ***p<0.001 compared to Positive control.

CONCLUSION

The results of present study propose that the test extract successfully quenched oxidative stress induced by ISO by the virtue of its phyto-antioxidants and offered cardioprotection. However, further investigations are required to elucidate its exact mechanism of action and to establish possible basis for its clinical utility.

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