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CARDIOPROTECTIVE AND ANTIOXIDANT POTENTIAL OF 50% HYDROETHANOLIC CRUDE EXTRACT OF *THEOBROMA COCOA* IN ISOPROTERENOL – INDUCED MYOCARDIAL RATS

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ABSTRACT

Theobroma Cocoa is an important export product of many developing countries for production of cocoa powder and chocolate. Therefore, the present study aimed to evaluate the cardioprotective effect of *Theobroma Cocoa* on isoproterenol induced myocardial in rat. The experimental albino rats were divided into four groups. The 50% hydroethanolic extract of *Theobroma Cocoa* was orally administered to group 3 and 4 rats for 28 days. Isoproterenol (20mg/100g) subcutaneously injected twice at an interval of 24 hr to group 3 rats on 29 and 30th day. At the end, rats were scarified and collected serum and heart samples for biochemical and histopathological studies. Significant ($P<0.05$) results were observed on protein, urea ,uric acid, and creatinine, serum lipid profile, cardiac marker enzymes and antioxidants in group 3 rats nearer to normal. The Histopathological observation showed the cardioprotective action of the study plant.

KEYWORDS: *Theobroma Cocoa*, Isoproterenol, Marker enzymes, Myocardial Infarction, Cardioprotective, Lipid profile.

1. INTRODUCTION

Cardiovascular diseases (CVD_s) remain the principle cause of death in both developed and developing countries, accounting roughly 20% of all deaths worldwide annually. Among various CVD_s, myocardial infarction (MI) or heart attack is the leading cause of morbidity and mortality and major cause of death by the year 2020 worldwide.[1] According to WHO 17.3 million peoples died from CVDs in 2008, over 80% of CVD death take place in low and middle income countries (WHO 2011). As estimated that by 2030 more than 23 million peoples in India's will die annually from CVDs.[2] A heart attack can occur it this blood clot completely blocks oxygen-rich blood from flowing to the heart. This is the most common cause of heart attacks.[3]

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The pathogenic mechanism of myocardial ischemic damage is still not completely understood, but the role of oxygen derived from the free radicals in myocardial ischemia is established. Oxidative stress resulting from increased production of free radicals is associate with decreased levels of antioxidant in the myocardium and plays a major role in cardiovascular diseases.[4] Administration of isoproterenol is known to produce electrocardiographic and enzymatic changes suggestive of myocardial ischemia in experimental animals.[5]

Medicinal plants are plants containing inherent active ingredients used to cure disease or relieve pain.[6] Herbal treatments have been used in the patients of cardiovascular diseases, with congestive heart failure, atherosclerosis , venous insufficiency cerebral insufficiency, and arrhythmia.[7] Due to side effect of herbal products, synthetic Products are gaining popularity in the word's market. Hence certain measures have been adopted for global promotion of India herbal products.[8]

Theobroma cocoa is an important export product of many developing countries for production of *cocoa* powder and chocolate. *Theobroma cocoa* is a small but economically important tree. It is an evergreen, 4-8m tall, of the *Sterculiaceae* family, native to the tropical region of the American .[9] Medicinal uses of *Theobroma Cocoa* seeds heart and kidney tonic; nervous system stimulant; diuretic. It is also used as base for suppositories and passaries.[10]

The following works have been carried out *Theobroma Cocoa* seed extract. Hypoglycemic properties of Malaysian *Cocoa (Theobroma Cocoa L.*) polyphenols – Rich Extract [11], Polyphenols in *Cocoa* and *Cocoa* products [12], The Productive effect of *Cocoa* (*Theobroma Cocoa L.*) in colon cancer. [13], *Cocoa* Bean *(Theobroma Cocoa L.*) dying kinetics [14], Genetic diversity and relationship of *Cocoa* (*Theobroma Cocoa L.*) in Southern Mexico [15] and Bioactive compounds and

antioxidant activity of *Cocoa* hulls (*Theobroma Cocoa L*.) from different origins.[16]

The literature does not report data showing the Cardioprotective effect of 50% hydroethanolic crude extract of *(Theobroma Cocoa L*.) in isoproterenol induced myocardial infarction.

Therefore the aim of this study to investigate the Cardioprotective effect of (*Theobroma Cocoa L*.) 50% hydroethanolic crude extract on isoproterenol induced mycordial infarction rats Male albino *(wister)* rats model.

2. MATERIALS AND METHOD

2.1 Plant collection and authentication

Theobroma cocoa plant was collected in Cadbery India Pvt. Ltd in Pollachi. Before the crude was used, they were collected and carefully identified (BSI/SRC/5/23/2014-15/Tech./689) by Dr. Palanisamy Botanical Survey of India Southern Regional Centre, Coimbatore.

2.2 Preparation of plant material

About 1 kgs of *Theobroma cocoa* crude were dried over a polythene cover in shade drying method and pulverized using a mixture grinder. The coarse powder of the crude was used for the preparation of the extract.

2.3 Extraction

Fermented and dried *Theobroma Cocoa* beans purchased from Pollachi. The beans were roasted using an air-oven for 20 min at 140ºC. After cooling to room temperature, the beans were de shelled using a beaker.[17] The broken nibs were ground, the defatted with petroleum – ether (b.p 40-60ºC using a Soxhlet apparatus sample was air-dried to remove solvent residues. The extract was prepared by extracting the defatted powder with 80% (v/v) ethanol for 2 hr. The ethanol residue was removed from the extract using a rotary vaccum evaporator under reduced pressure for 20 min at 70ºC and the sample was then crystilized. Finally dark brown colored crystals of approximately 15g were obtained. It was stored in a air tightened desiccators and when needed, the residual extracts were dissolved in distilled water and used for myocardial study.

2.4 Experimental animals

Male *albino rats* of *wistar* strain weighing about 130-150g procured from the Animal House Facility, PSG Institute of Medical Science and Research, Coimbatore , Tamilndau, India.

The rats were housed in well ventilated 12+1 hour day night schedule in large spacious hygienic cages during of the experimental periods, fed pellets supplied by M/s. Hindustan Lever Limited, Bangalore, India. The place where the experimental were conducted was kept very hygienic by cleaning with antiseptic solution because the myocardial animals are easily susceptible to infections.

The experimental study was approved by the Institutional animal Ethics committee of PSG Institute of Medical Science and Research the (Proposal No: 233/2014/IAEC).

2.5 Induction of myocardial infarction

Isoproterenol hydrochloride was used to induce myocardial infarction in rats. Animals were injected subcutaneously with freshly prepared in sterile normal saline at a dose of 20mg/100 g body weighted.

2.6 Experimental design

The experimental rats were divided into 4 groups of 6 animals in each group. The animals were fasted over night before the experimental schedule begins but allowed free access of tap water.

GROUP I

The rats received only standard rat pellet for 28 days. The animals serve as healthy controls.

GROUP II

The rats were administered (20mg/100g of rat) administered subcutaneously twice at an interval of 24 hour dissolved in normal saline.

GROUP III

The rats were pretreated with *Theobroma Cocoa* extract (300mg/kg body weight) for period of 30 days and isoproterenol (20mg/100g) subcutaneously twice at an interval of 24 hour at the end of treatment period on the 29th and 30th days.

GROUP IV

The rats were pretreated with *Theobroma Cocoa* extract (300mg/kg body weight) for periods of 28 days.

2.7 Preparation of serum and tissue homogenate

After The experimental regimen, the animals were scarified by cervical dislocation under mild chloroform anaesthesia. Blood was collected on decapitation. The heart were excised immediately and thoroughly washed with ice cold physiological saline. The collected blood was centrifuged at 2500 rpm for 10 min and collects the serum. The serum was used for various biochemical experiments.1 g of heart was taken and Homogenized with 0.1M cold buffer (pH-7.4) in a potter Homogenizer fitted with Teflon plunger at 600 revolution per for 3min. the homogenate was used for various biochemical assays. The protein Lowry's *et al.*,1951) Urea[19], Uric acid (Caraway) and Creatinine [19]were estimated by colorimetric method. Lipid profile in serum Triglycerides (Philip and Mayne, 1994), Cholesterol [20], HDL [20]LDL (Beacon) and Phospholipids were assayed in serum using standard kits supplied from Aquest diagnostic services pvt.Ltd, Coimbatore. Cardiac markers enzymes such as AST, ALT, LDH, ACP, and ALP.[23]Enzymatic antioxidants such as SOD [24]Catalase[25] Lipid peroxidation [25]

2.8Histopathological studies

Heart was washed in saline and a small portion of it was quickly fixed in 10% formalin. Then the tissue were proceed by standard histopathological technique (i.e.) dehydration through graded isopropyl alcohol, cleaning through xylene and impregnated in paraffin wax for 2 hours. Then wax blocks were made, sections were used for cutting microtone and stained by haematoxylin eosin method and photographed.

2.9 Statistical analysis

The results of cardio protective and antioxidant activities are expressed as mean ± SD from six animals in each group. The results were statistically analysed using one way ANOVA followed by Tukey-Kramer post test for version 3.00 of graph Pad software, Inc. (San Dego CA), was used for statistical analysis.

3. RESULT AND DISCUSSION

The level of protein in serum and heart tissues of all experimental groups was represented in Table1. The isoproterenol induced rats Group II of serum and tissue homogenate ($p<0.001$) shows decreased levels, when compared with control rats Group I. This is due to the active necrosis in isoproterenol induced myocardial infracted rats through free radical mediated tissue damage ie. Oxygen and hydrogen peroxide radical, which in turn could bind with albumin and thus destroy it[26] In this context several authors had reported using of different medicinal plant crude extract. After treatment 300mg/kg body weight of crude extract these alteration brought back into normal. This may be due to the presence of several bioactive constituents group IV does not alter the protein value in serum and tissue so no toxicity.

The levels of serum creatinine, urea, and uric acid of control and treated animals are given in Table2. Rats induced with isoproterenol showed significant ($p<0.001$) increase in serum levels of urea, uric acid, and creatinine when compared with group I. After pretreatment with 50% hydroethanolic crude seed extract of *Theobroma cocoa* for a period of 28 days, significantly ($p<0.01$) decreased. It proved that the active constituents present in the plant may be responsible for normal profile of myocardium[27] Group IV compared with control rats (Group I).

The result presented in Table3 shows the effect of control and experimental animals on lipid profile such as triglycerides, cholesterol, HDL, LDL, and phospholipids levels in serum. Increased serum levels of triglycerides, cholesterol, phospholipids, LDL significant ($p<0.001$) decrease in HDL in rats administered with isoproterenol when compared to group I. After period of 28 days showed significant ($p<0.001$) near normal level of the lipid profile in serum when compared with normal group. No significant change in parameters was observed between group IV and I. High levels of circulating cholesterol along with triglycerides and their accumulation in the heart tissue is usually accompanied by cardiovascular damage.[28] Which could be due to the increased activity of hepatic lipoprotein lipase [29]also indicate the hypolipidermic effect of the extract. There was no significant changes were observed between group IV and group I.

Table 4,5 represent the effect of Theobroma cocoa seed extract on the activities of cardiac marker enzymes such as AST, ALT, LDH, ACP and ALP in serum and heart of the normal and experimental animal. Isoproterenol induced rats showed a significant ($p<0.001$) increase in the activities of AST, ALT, LDH, ACP and ALP in serum and a significant ($p<0.001$) decrease in the activities of these enzymes in heart, when compared to group I rats. A period of 28 days shows significant by ($p<0.001$) minimized the alternations in the activities of these enzymes in isoproterenol rats when compared to isoproterenol alone induced rats. The activities of cardiac marker enzymes which leads to subsequent increase in the activities of these enzymes in the serum. This may be due to the damage in the heart tissue, rendering the leakage of enzymes in to the serum.[30] The above findings clearly pointed out the *Theobroma Cocoa* could be highly cardioprotective against the myocardial infarction and also these results may be due to the strong antioxidant potential of secondary metabolities present in the crude seed extract .There was no significant changes were observed between group IV and group I.

Table 6 illustrates the effect of Theobroma Cocoa on activities of antioxidant enzymes SOD and Catalase in serum and heart tissue homogenate of control and experimental rats. SOD and Catalase were lowered significantly ($p<0.001$) owing to the myocardial infarction in isoproterenol induced rats when compared with control group. After oral pretreatment for a period of 28 days successfully ($p<0.001$) prevented the decrease in the activity of these enzymes. The decrease in SOD and Catalase may be due to the involvement of $O^{\cdot}{}_2^{-}$ free radical in heart cell damage mediated by Isoproterenol.[31] This effect was probably owing to removal of excess free radicals generated by isoproterenol.[32] Which demonstrated on important role of *Theobroma Cocoa* in regulating antioxidant capacity. Non significant changes were observed between Group IV and Group I on serum and heart tissue homogenate.

The activities of LPO levels in serum and heart tissue homogenate of control and experimental rats were shown in Table 7. The group II showed a significant ($p<0.001$) increase levels of lipid peroxides both in serum and heart tissue homogenate when compared with group I. The oral administration of pretreatment for a period of 28 days showed a significant ($p<0.001$) decreased level of LPO both in serum and tissue as compared with myocardial infracted rats. group I. Lipid peroxidation is well established mechanism of cellular injury and has been used as an indicator of oxidative stress that leads to pathogenesis of MI.[33] Pretreatment of *Theobroma*

Cocoa shows decreased levels of LPO both serum and tissue as compared with myocardial infracted rats. This may be due to the free radical scavenging activity potential of *Theobroma Cocoa* seed. There was no significant change observed between group IV and group I.

The histopathological studies of control and experimental rat heart were shown in Figure (1). The heart tissue homogenate was observed under light microscope and assessed using H&E stain. In group I Hematoxylin and Esoin staining of heart tissue shows normal feature heart muscle and normal myocardium. In group II H&E section of heart tissue shows thrombus formation, contraction band necrosis minimal mononuclear inflammatory cell infiltrate in focal areas. In group III H&E section of heart tissue shows very minimal mononuclear inflammatory cell infiltrate in a focal area (at one place). In group IV the H&E sections of heart muscle tissue showing very minimal mononuclear inflammatory cell infiltrate in a focal area i.e. normal morphology of cardiac muscle when compared to group I. Thus *Theobroma Cocoa* has some protective effect on the myocardium against isoproterenol and possess a significant medicinal value in the prophylactic treatment of MI. Similar study was carried out the different medicinal plants.[34,35]

Table: 1 Effects of *Theobroma Cocoa* on serum and heart tissue protein levels in control and experimental rats

Protein levels		
Groups	**Serum (g dL^{-1})**	**Heart (mg g^{-1} tissue)**
I	6.9 ± 0.75	5.3 ± 0.71
II	3.5 ± 0.36a***	2.5 ± 0.25a***
III	5.7 ± 40.8b***	3.7 ± 0.24b***
IV	8.7 ± 7.0c^{ns}	4.9 ± 0.9sc ^{ns}

Values are given by means ± S.D. for groups of six animals in each group Group comparsion a)Group II vs I, b)Group III vs II c) Group IV vs I Statistical significance* p<0.05, ** p<0.01, ns Non significant

Table: 2 Effects of *Theobroma Cocoa* on urea, uric acid, and creatinine levels in control and experimental rats

Serum (mg/dL)			
Groups	**Urea**	**Uric acid**	**Creatinine**
I	26.46 ± 0.87	3.17± 0.42	0.30 ± 0.04
II	43.83±1.27a***	5.72 ± 0.45a***	0.84 ± 0.16a***
III	27.11±1.12b***	3.77± 0.32b***	0.47± 0.07b***
IV	25.21 ± 0.72c^{ns}	2.94 ± 0.43c^{ns}	0.28 ± 0.04c^{ns}

Values are given by means ± S.D. for groups of six animals in each group Group comparsion a)Group II vs I, b)Group III vs II c) Group IV vs I Statistical significance* p<0.05, ** p<0.01, ns Non significant

Table: 3 Effects of *Theobroma Cocoa* of Lipid profile levels in serum and heart tissue of Control and Experimental rats

SERUM LIPID PROFILE (mg/dl)					
Group	**Triglycerides**	**Cholesterol**	**HDL**	**LDL**	**VLDL**
I	92.3 ± 1.03	94 ± 1.41	34.8 ± 3.18	26.0±2.09	76.8 ± 1.9
II	125 ± 3.2a***	124±2.09a***	21.5±1.87a***	47.0±1.7a***	104.1±2.8a***
III	115.6±3.01b***	107.5 ±1.87b***	27.3±1.63b**	36.1 ±2.3b**	96.1 ± 2.1b***
IV	96.5 ± 1.87c^{ns}	87.3 ± 1.63c^{ns}	32.8 ± 1.47c^{ns}	30.8 ± 3.4c^{ns}	71.6 ± 2.16c^{ns}

Values are given by means ± S.D. for groups of six animals in each group Group comparsion a)Group II vs I, b)Group III vs II c) Group IV vs I Statistical significance* p<0.05, ** p<0.01, ns Non significant

Table: 4 Effects of *Theobroma Cocoa* of AST, ALT, LDH, ACP and ALP levels in Serum of Control and Experimental rat.

Serum (IU/L)					
Groups	**AST**	**ALT**	**LDH**	**ACP**	**ALP**
I	43.2± 1.05	13.4 ± 1.16	133.5 ± 1.66	23.5 ± 1.83	16.1 ± 1.11
II	80.5 ± 2.33a***	24.6± 1.48a***	212.7±2.60a***	85.9 ± 2.47***	65.4±1.17a***
III	51.9 ± 4.32b***	17.3 ± 1.66b***	153 ±1.67b***	54.2±1.49b***	47.7±1.41b***
IV	41.3 ± 2.91c^{ns}	12.9 ± 0.83c^{ns}	128.5 ± 0.68c^{ns}	27.5 ± 1.53c^{ns}	22.8 ± 2.40c^{ns}

Values are given by means ± S.D. for groups of six animals in each group Group comparsion a)Group II vs I, b)Group III vs II c) Group IV vs I Statistical significance* p<0.05, ** p<0.01, ns Non significant .

Table: 5 Effects of *Theobroma Cocoa* of AST, ALT, LDH, ACP and ALP levels in heart tissue of Control and Experimental rats

Heart tissue Homogenate (µ mole of pyruvate liberated /min/ mg of protein)					
Groups	**AST**	**ALT**	**LDH**	**ACP**	**ALP**
I	53.6 ± 1.24	20.1 ± 0.71	125.5 ± 3.03	85.9 ± 2.4	52.0 ± 2.5
II	45.0 ± 1.56a***	24.1 ± 1.28a***	54.7 ± 0.98a***	27.5 ±1.49a***	27.2±0.89a***
III	49.5 ±1.61b***	17.6 ± 0.87b**	92.5 ±2.05b***	92.5 ±2.05b***	35.4 ± 1.0b**
IV	52.0 ± 1.32c^{ns}	14.2 ± 0.49c^{ns}	113.6 ± 2.55c^{ns}	54.2 ± 1.53c^{ns}	37.2 ± 1.9c^{ns}

Values are given by means ± S.D. for groups of six animals in each group Group comparsion a)Group II vs I, b)Group III vs II c) Group IV vs I Statistical significance* p<0.05, ** p<0.01, ns Non significant

Table: 6 Effects of *Theobroma Cocoa* of SOD and Catalase levels in serum and heart tissue of Control and Experimental rat

Serum and heart tissue 50% inhibition of nitrate/min/mg prote				
Groups	**SOD (Serum)**	**SOD (Tissue)**	**Catalase(Serum)**	**Catalase(Tissue)**
I	7.09 ± 0.85	5.4 ± 0.84	42.9 ± 3.83	76.06 ± 1.1
II	4.42 ± 0.36a***	2.9± 0.50a***	35.54 ±1.47a***	43.25 ± 0.41a***
III	9.07 ± 0.49b***	3.9 ± 0.54b**	50.8 ± 1.32b***	57.43 ± 0.63b***
IV	7.14 ± 0.42c^{ns}	5.9 ± 0.76c^{ns}	42.8 ± 1.72c^{ns}	71.86 ± 1.3c^{ns}

Values are given by means ± S.D. for groups of six animals in each group Group comparsion a)Group II vs I, b)Group III vs II c) Group IV vs I Statistical significance* p<0.05, ** p<0.01, ns Non significant

Table: 7 Effects of 50% hydroethanolic crude extract of *Theobroma Cocoa* of LPO levels in serum and heart tissue of Control and Experimental rats

LPO (nmole malondiadehyde released/mg protein)		
Groups	**Serum**	**Tissue**
I	3.39 ± 0.28	6.32 ± 1.2
II	5.43 ± 0.41a***	15.0 ± 2.3a***
III	2.14 ± 0.22b***	8.99 ± 0.43b***
IV	3.81 ± 0.31c^{ns}	7.3 ± 0.35c ^{ns}

Values are given by means ± S.D. for groups of six animals in each group Group comparsion a)Group II vs I, b)Group III vs II c) Group IV vs I Statistical significance* p<0.05, ** p<0.01, ns Non significant

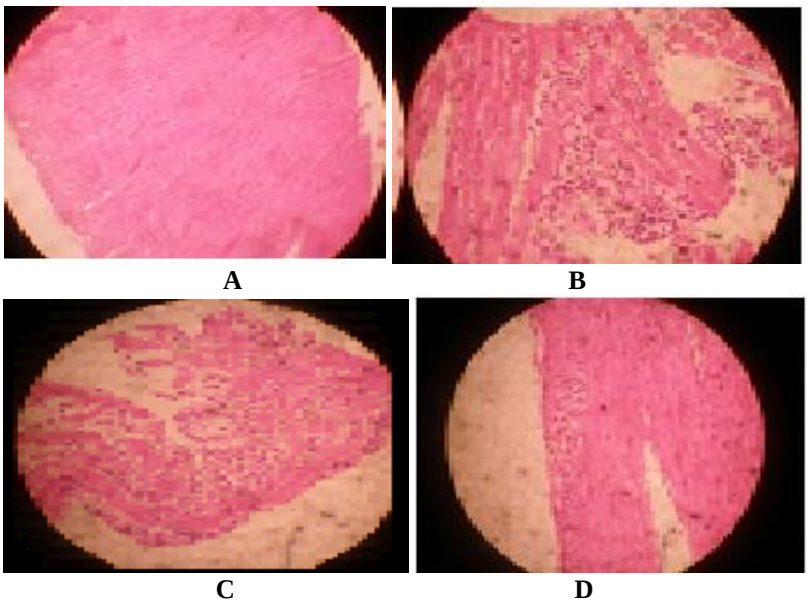

Figure: 1 Histopathological Observation of Heart

A) Shows normal heart muscle B) Shows features of mild chronic myocarditis C)Shows similar features as that of (B), However myocarditis is less intense D) Shows similar features as that of (C), in addition showing fatty change in the myocarditis area.

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