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CARDIOPROTECTIVE EFFECT OF *MORUS ALBA* L. LEAVES IN ISOPRENALINE INDUCED RATS

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ABSTRACT

The study was designed to evaluate the cardioprotective effect of methanolic extract of *Morus alba* L. leaves against isoprenaline- induced myocardial infarction and was investigated by an *in vivo* method in rats. Male Wistar albino rats were divided into four groups (n=6). Group I rats served as normal control. Group II rats served as isoprenaline induced toxic control (110 mg/kg body weight) which was injected intraperitoneally (i.p.) for two consecutive days (14^{th} and 15^{th} days). Group III rats were given *Morus alba* intragastric intubation (500 mg/kg body weight) for 15 days. Group IV rats were also given *Morus alba* as in Group III and additionally isoprenaline was given for two consecutive days (14^{th} and 15^{th} days).The results described the cardioprotective effect that was observed in Group IV which showed a significant ($P< 0.05$) decreased levels of TBARS and enhanced the activities of both enzymatic and non-enzymatic antioxidants (SOD, CAT, GPx and GSH) in myocardial infarcted rats when compared to Groups II and III. In serum, the biomarkers (LDH, CK) activities were significantly ($P< 0.05$) increased in Group II compared to pretreated Group IV. Histopathological studies were also co-relating with the above biochemical parameters. These findings concluded the cardioprotective effect of *Morus alba* on lipid peroxidation and antioxidant defense system during isoprenaline -induced myocardial infarction in rats.

INTRODUCTION: Natural drugs are gaining greater acceptance from the public and the medical profession due to greater advances in understanding the mechanism of action by which herbs can positively influence health and quality [1]. Cardiovascular diseases (CVDs) such as hypertension and myocardial infarction (MI) are the most important cause of mortality in developing countries due to changing lifestyles [2].

MI is the acute condition of myocardial necrosis that occurs as a result of imbalance between coronary blood supply and myocardial demands,[3] it increases the generation of reactive oxygen species in ischemic tissue, bringing about oxidative damage of membrane lipids, proteins, carbohydrates and DNA and brings changes in the mechanical, electrical, structural and biochemical properties of the heart [4]. Although modern drugs are effective in preventing the cardiovascular disorders, their use is often limited because of their side effects and adverse reactions [5].

Isoprenaline, a synthetic catecholamine has toxic effect on the myocardium. Amongst the various mechanisms proposed to explain isoprenaline-induced cardiac damage, generation of highly cytotoxic free radicals through auto-oxidation of catecholamines has been implicated as one of the important causative factor.

The primary disturbance of isoprenaline induced myocardial infarction has been reported to enhance adenylate cyclase activity resulting in increased cAMP formation, which in turn would have lead to the higher lipid accumulation in the myocardium [6]. Increased lipolysis and peroxidation of endogenous lipids also play a major role in the cytotoxic action of isoprenaline [7]. The rat model of ISO induced myocardial necrosis serves as well accepted standardized model to evaluate several cardiac dysfunctions and to study the efficacy of various natural and synthetic cardioprotective agents [8].

The mulberry tree, a plant of the family Moraceae and the genus *Morus* , has been widely cultivated to feed silkworms. The leaves and the roots of *M. alba* have also been used in traditional medicine as a cathartic, analgesic, diuretic, antitussive, sedative, hypotensive, antiphlogistic and for the treatment of edema [9]. The decoction of the leaves is used as a gargle for relief of inflammation of the throat.

The plant contains flavonoids, coumarine, and stilbene, which have hepatoprotective and free radical scavenging activity [10]. The other uses of *M. alba* are as a hypoglycemic [11] and neuroprotective agent [12]. A piperidine alkaloid and some glycoproteins were isolated from the bark and leaves, which had antidiabetic effects [13]. Phytochemical reports on *M. alba* L. indicates that the plant contains flavonoids, tannins, triterpenes, anthocyanins, anthroquinones, phytosterols, sito-sterols, benzofuran derivatives, morusimic acid, oleanolic acid, alkoloids, steroids, saponins, and phenolic compounds [14]. A survey of the literature on *M. alba* revealed only a few pharmacological reports on the plant.

No major investigative reports were found pertaining to its cardioprotective activity; therefore, the study was taken up to determine the cardioprotective potential of *M. alba* by using different animal models.

MATERIALS AND METHODS:

Chemicals: Isoprenaline hydrochloride 1-(3,4-dihydroxyphenyl)- 2- iso- propylaminoethanol, was obtained from the Sigma Chemical Company, St. Louis, MO, USA. All the other chemicals and reagents used were of analytical grade.

Formulation and administration of *Morus alba*: The *Morus alba* leaves were cut into pieces and air dried under shade. The powdered plant material was extracted with methanol using a Soxhlet apparatus at a concentration of 20g powder in 300 ml methanol. The extract was filtered and evaporated to dryness under reduced pressure on a rotary evaporator. The extract was dissolved in distilled water just before oral administration at a dose of 0.5 ml corresponding to 500 mg / kg body weight.

Induction of Myocardial Infarction: The myocardial infarction was induced by intraperitoneal (i.p.) injection of isoprenaline hydrochloride (110 mg/kg body weight), dissolved in physiological saline, for two consecutive 14^{th} and 15^{th} days [2].

Experimental Animals: The male Albino Wistar rats weighing 110-150 g were selected for the study. They were housed in plastic cages with filter tops under controlled conditions of 12h light and 12h dark cycles, 50% humidity at 28^{o}C. Standard pellet diet was fed to rats throughout the experimental period and water was given *ad libitum*. The study was conducted after obtaining a clearance from the institutional animal ethical committee (IAEC).

Treatment Schedule: The rats were randomly assigned in-to four groups of six animals each. The rats in Group I served as the untreated control. Group II rats served as the isoprenaline (110 mg/kg body weight) intraperitoneally twice at an interval of 24 h on the 14^{th} and 15^{th} days. The rats in Group III served as *Morus alba* via intragastric intubation at a daily dose of (500 mg/kg body weight) respectively for a period of 15 days. Group IV rats served *Morus alba* as in Group III and at the last of the experimental period on 14^{th} and 15^{th} days rats received isoprenaline (110 mg/kg body weight) injections intraperitoneally twice at an intravel of 24 h. The experiment was terminated after 15 days and all the animals were killed overnight fast.

Biochemical Estimation: Lipid peroxidation was estimated by measuring the level of thiobarbituric acid reactive substances (TBARS) in tissues [15]. The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde, a secondary product of lipid peroxidation, was measured at 535nm. The values are expressed as nmoles of MDA formed/ g tissue. LDH

was estimated via the method [16] where the lactate is acted upon by lactate dehydrogenase to form pyruvate in the presence of NAD. The pyruvate forms pyruvate phenyl hydrazone with 2,4 dinitrophenyl hydrazine. The color developed was measured at 440 nm. The values are expressed as µmoles of pyruvate liberated/min/mg protein.

Creatine kinase (CK), also known as creatine phosphokinase (CPK) or phospho-creatine kinase**.** CK was estimated [17] by the conversion of creatine and consumes adenosine triphosphate (ATP) to create phosphocreatine (PCr) and adenosine diphosphate (ADP). This CK enzyme reaction is reversible, such that also ATP can be generated from PCr and ADP whose absorbance is measured at 340 nm. The values are expressed as µmoles of phosphorus liberated/min/mg protein

Superoxide dismutase was assayed using the method [18] that involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound which subsequently reacts with naphthylamine to produce a red azo compound whose absorbance is measured at 543 nm. One unit of the enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition. The activity of catalase was determined [19] where dichromate in acetic acid was reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2), with the formation of perchromic acid as an unstable CAT intermediate.

The chromic acetate formed was measured at 610 nm. Catalase was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals via the addition of a dichromate acetic acid mixture, and heating the reaction mixture and measuring chromic acetate colorimetrically and determined the remaining H_2O_2. The values are expressed as μmoles of H_2O_2 decomposed/min/mg protein. Glutathione peroxidase (GPx) activity was assayed via the method of Rotruck *et al.,* 1973 [20] with a modification: a known amount of enzyme preparation was incubated with H_2O_2 in the presence of GSH for a specified time period. The amount of H_2O_2 utilized was determined via the method of Ellman, 1959 [21].

The values are expressed as μmoles of GSH utilized/min/mg protein. Reduced glutathione (GSH) content was determined via the method Moron *et al*., 1979 [22]. GSH determination is based on the development of yellow colur when 5, 5' dithio 2-nitro benzoic acid (DTNB) is added to compounds containing sulfhydryl groups. The values are expressed as mg/g tissue.

Preparation of Tissue Homogenate: Heart tissue were removed immediately and washed with ice-cold saline and homogenized in the appropriate buffer in a tissue homogenizer.

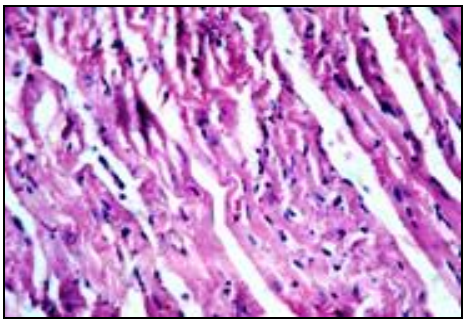
Histopathological Studies: At the end of the study, all the rats were sacrificed by cervical decapitation and the hearts were dissected out, washed in ice cold saline. Then myocardial tissue was immediately fixed in 10% buffered neutral formalin solution. After fixation, tissues were embedded in paraffin and serial sections were cut and each section was stained with hematoxylin and eosin. The slides were examined under light microscope and photographs were taken.

Statistical Analysis: The results presented here are the means ± SD of 6 rats in each group. The results were analyzed using one-way analysis of variance [ANOVA] and the group means were compared using Duncan's multiple range test [DMRT] using SPSS version 12 for Windows. The findings were considered as statistically significant if $P<0.05$ [23].

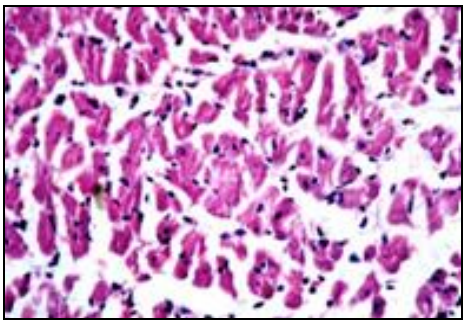
RESULTS: Normal architecture of the cardiac cells was observed with no evidence of microscopic changes in the control and *Morus alba* treated groups [**Figure 1**]. In isoprenaline-treated rats heart, histological changes such as perivascular cuffing of vasa vasorum with intimal fibrosis, disruption of medial elastic fibers with diffuse interstitial fibrosis and myocytolysis were seen. In *Morus alba* (500 mg/kg body weight) and isoprenaline (110 mg/kg body weight) treated rats there was no appreciable change in the heart.

Histopathology changes in the myocardial infarction of control and experimental rats. Effect of *Morus alba* on the histopathology of the myocardium. (a) Normal rats (group I) showing normal cardiac cells. (b) Isoprenaline (110 mg/kg bodyweight)- induced rats (group II) showing perivascular cuffing of vasa vasorum with intimal fibrosis, disruption of medial elastic fibers with

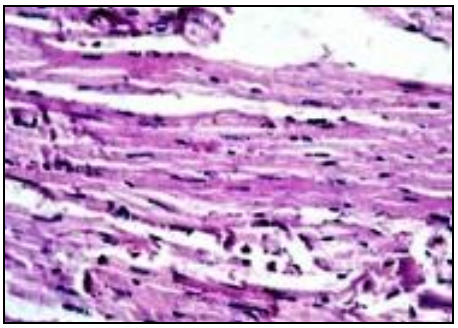
diffuse interstitial fibrosis and myocytolysis. (c) *Morus alba* (500mg/kg body weight) treated rats (group III) showing normal architecture of the cardiac cells. (d) *Morus alba* (500 mg/kg body weight) and isoprenaline (110 mg/kg body weight) induced rats showing normal architecture of the cardiac cells.



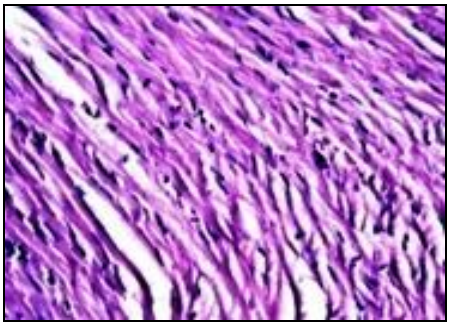
(A) GROUP I: NORMAL APPEARING CARDIAC MYOCYTES



(B) GROUP II: SEVERE NECROSIS AND CHRONIC INFLAMMATORY INFILTERATE



(C) GROUP III: NORMAL APPEARING MYOCYTES



(D) GROUP IV: NORMAL APPEARING MYOCYTES

The circulatory and tissue levels of TBARS in normal and experimental rats are shown in **table 1**. Rats treated with isoprenaline showed a significant (P<0.05) increase in these levels when compared with normal control rats. Rats pre-treated with *Morus alba* (500 mg/kg body weight) and isoprenaline-induced rats significantly (P<0.05) decrease the levels of TBARS when compared with the isoprenaline induced rats.

TABLE 1: EFFECT OF *MORUS ALBA* ON CIRCULATING AND TISSUE LIPID PEROXIDATION (TBARS) OF CONTROL AND EXPERIMENTAL RATS

Groups	control	ISO induced	Morus alba 500mg	Morus alba 500 mg + ISO Induced
LPO	0.98±0.14[b]	5.62±0.31[a]	1.01±0.09[b]	1.16±0.27[b]

Table 2 represents the activities of antioxidants in the normal, control and the isoprenaline-induced rats. The isoprenaline-induced rats showed a significant (P<0.05) decrease in the antioxidants (SOD, CAT, GPx and GSH) when compared with normal control rats. On treatment with *Morus alba* (500 mg/kg body weight) daily for a period of 15 days, a significant (P<0.05) increase in the activities of antioxidant was observed in the isoprenaline-treated rats when compared with the isoprenaline induced rats.

TABLE 2: EFFECT OF *MORUS ALBA* ON CIRCULATORY AND TISSUE ANTIOXIDANTS OF CONTROL AND EXPERIMENTAL RATS

Groups	control	ISO induced	Morus alba 500mg	Morus alba 500 mg + ISO Induced
SOD	1.59± 0.14[b]	0.88± 0.21[a]	1.58±0.2[b]	1.57±0.29[b]
CAT	7.75± 0.81[b]	4.33± 0.54[a]	7.78±0.42[b]	7.50±0.51[b]
GPx	29.40± 3.6[b]	15.28±1.56[a]	29.10±2.15[b]	29.01±2.14[b]
GSH	5.04± 0.13[b]	2.34± 0.49[a]	5.14±0.32[b]	4.93±0.23[b]

Table 3 depicted that the group II(ISO induced) rats showed a decreased biomarkers (CK and LDH) level in heart tissue than groupI. In group IV (pretreatment with *Morus* significantly (p<0.05) reversed the level of (CK and LDH) to the near normal level compared to that of group II. Compared with the control, isoprenaline caused significant increased in the activities of serum myocardial injury marker enzyme

creatine kinase and lactate dehydrogenase, pretreatment with *Morus* almost restored all the isoprenaline-induced alterations in creatine kinase and lactate dehydrogenase activity, to normal levels.

TABLE 3: EFFECT OF *MORUS ALBA LIN* ON THE ACTIVITIES OF BIOMARKER ENZYMES OF CONTROL AND EXPERIMENTAL RATS

Groups	control	ISO induced	Morus alba 500mg	Morus alba 500 mg + ISO Induced
Heart				
LDH	91.41± 4.38[b]	54.28± 3.42[a]	90.85± 5.56[b]	91.25± 3.78[b]
CK	123.25± 0.85[b]	84.4± 0.54[a]	123.73± 0.3[b]	122.90± 0.78[b]
Serum				
LDH	180.30± 6.94[b]	122.90± 0.78[b]	182.30± 4.75[b]	209.31± 8.41[b]
CK	43.72± 0.22[b]	80.69± 0.04[a]	43.49± 0.15[b]	42.92± 0.08[b]

DISCUSSION: Our result showed that *Morus alba* maintained the levels of lipid peroxides and antioxidants of heart in myocardial infarcted rats. Histopathological findings also support the findings of this study. The isoprenaline induced myocardium showed perivascular cuffing of vasa vasorum with intimal fibrosis and disruption of medial elastic fibers with diffuse interstitial fibrosis. Normal rats treated with *Morus alba* (500mg/kg body weight) showed normal cardiac fibers without any pathological changes. Myocardial section of Group IV rats showed normal architecture of cardiac cells. This action produced by the *Morus alba* reflects the protection offered during myocardial infarction.

In our study we observed an increased the concentration TBARS which has been suggested to be due to an enhanced oxidative stress in experimentally induced myocardial injury. Oral treatment with *Morus alba* significantly decreased the concentration of TBARS in the isoprenaline-induced rats. *Morus alba* protected the heart against lipid peroxidative damage by removal of excess free radicals generated by isoprenaline. The presence of phenolic groups in phytochemicals of *Morus alba* probably protected the heart from myocardial damage by scavenging free radicals and thereby suppressing the peroxidation of lipids.

Thus, *Morus alba* being a phenolic compound might have inhibited lipid peroxidation in our study. SOD and CAT are considered as primary enzymes since they are involved in the direct elimination of ROS. We have observed significant decrease in the activities of SOD and CAT in the isoprenaline -induced rats (Group II): this might be due to excessive generation of free radicals by isoprenaline. Administration of *Morus alba* restored the levels of SOD and CAT to near normal.

This shows the antioxidant effect of *Morus alba* against myocardial injury caused by free radicals. Significant decrease in the level of GSH and Gpx may be due to its increased utilization during the burst of reactive oxygen species production, in protecting 'SH' group containing proteins from lipid peroxidation [24]. The decreased activities of glutathione peroxidase in the heart of isoprenaline -induced myocardial infarction might be due to decreased availability of their substrate, reduced glutathione. Inactivation of the enzyme Gpx in the heart tissue leads to the accumulation of oxidized glutathione which in turn inactivates many enzymes containing SH groups.

Administration of *Morus alba* significantly prevented the alterations in the levels of GSH, GPx and restored the levels to near normal. This effect may be due to the free radical scavenging properties of *Morus alba* [25]. The scavenging activities of the phenolic substances are attributed to the active hydrogen-donating ability of the hydroxyl substitutions [26] .The presence of the phenolic groups in the phytochemicals especially naringenin and quercetin in *Morus alba* could be responsible for ·OH radical scavenging activity. Thus, it is quite clear that changes observed in SOD, CAT, GPx and GSH could be attributed to the enhancement in antioxidant status in blood and tissues of normal rats.

Myocardium contains an abundant amount of diagnostic marker enzymes (CK, LDH) for myocardial infarction and once metabolically damaged, it releases its intracellular contents into the extracellular fluid [27]. Hence, the serum levels of these marker enzymes reflect the alterations in membrane integrity and/or permeability. Creatine kinase (CK) is a cardiac enzyme that helps in converting creatine to creatinine, a reaction that is necessary for metabolism and energy production. LDH is an enzyme that helps in converting lactic acid to pyruvic acid.

LDH-1 isozyme is normally found in the heart muscle and LDH-2 is found predominately in blood serum. Isoprenaline is well known cardio-toxic agent and due to its ability it destructs myocardial cells. As a result of this, cytosolic enzyme Lactate Dehydrogenase (LDH) was released into blood stream and serves as a diagnostic marker of myocardial tissue damage. Previously it has been reported that *Morus alba* extracts have been shown to produce beneficial effects, such as neutralizing the free radicals and enhancing the antioxidant status. Phenolic compounds of *Morus alba* act by scavenging free radicals [28] and quenching the lipid peroxidative chain. Thus, *Morus alba* being a phenolic compound might have inhibited lipid peroxidation in our study.

CONCLUSION: In conclusion, our study reveals that administration of *Morus alba* proved to be more effective in reducing the extent of myocardial damage and significantly counteracted the oxidative stress during isoprenaline -induced myocardial infarction in rats.

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