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Cardioprotective and Antihypertensive Potential of *Morus alba* L. in Isoproterenol-induced Myocardial Infarction and Renal Artery Ligation-induced Hypertension

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Abstract

The present study was aimed to evaluate the cardioprotective and antihypertensive effect of ethyl acetate soluble fraction of *Morus alba* L. in rats.Rats were orally treated with the ethyl acetate soluble fraction of methanol extract of *Morus alba* leaves (EASF 25, 50, and 100 mg/kg) for three weeks. Isoproterenol (85 mg/kg, s.c.) was administered at an interval of 24 h for two days for induction of myocardial infarction. The parameters like ST segment, heart rate, mean arterial blood pressure, pressure-rate index, vascular reactivity, heart weight, cardiac markers, and antioxidant enzymes were measured. Hypertension was induced by left renal artery ligation method. The effect of EASF was evaluated on blood pressure and cumulative concentration response curve (CCRC) of $CaCl_2$ on phenylephrine pre-contracted isolated rat aortas. Oral administration of EASF significantly reduced ST segment, heart rate, arterial pressure, pressure-rate index, heart weight, LDH, CK-MB, and SGOT, whereas the levels of antioxidant enzymes were increased significantly. EASF reduced the pressor response to catecholamines and also showed protection from hypertrophy and degenerative changes on myocardial muscles. The ligation of renal artery significantly increased the blood pressure and CCRC of $CaCl_2$ shifted to the left. EASF significantly reversed these changes. The observations propose that *Morus alba* may possess beneficial role in the treatment of myocardial infarction and hypertension through the regulation of antioxidant defensive mechanisms and blockade of calcium channels, respectively.

Key words: Isoproterenol, oxidative stress, left renal artery ligation

1. Introduction

Myocardial infarction causes due to either zero flow or little flow to the particular area of heart that cannot sustain cardiac muscle function. Formation of thrombus in atherosclerosis leads to infarction [1]. During infarction, there is a rise in oxidative stress. Oxidative stress produces reactive oxygen species which contribute to the ischemic damage of the heart causing myocyte destruction [2]. Due to thrombus formation, sudden occlusion of a major branch of a coronary artery shifts aerobic or mitochondrial metabolism to anaerobic glycolysis within seconds. A compensatory increase in anaerobic glycolysis for ATP production leads to the accumulation of hydrogen ions and lactate, resulting in intracellular acidosis and inhibition of residual energy metabolism. The lack of ATP production interferes with ATPase activity in Na^+/K^+ pump, resulting in pump failure and leading to accumulation of Na^+ ions. An early increase in cytosolic Ca^{2+} also develops due to multifactorial changes in transport systems in the sarcolemma and sarcoplasmic reticulum. Ca^{2+}-induced

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activation of proteases causes alterations in contractile proteins, decreased sensitivity to Ca^{2+}, and sustained impairment of contractility despite the elevated cytosolic Ca^{2+}[3].

Hypertension (170/100 mmHg) is a medical emergency and requires urgent treatment to prevent a stroke i.e. brain damage. Increase in either cardiac output or total peripheral resistance results in increased blood pressure. Overactivity of renin–angiotensin–aldosterone system and endothelial dysfunction leading to decreased secretion of NO, generation of reactive oxygen species, hypercholesterolaemia are some causes of hypertension [4]. The molecular mechanisms and potential treatment of acute and chronic hypertension and cardioprotection is thus of paramount importance.

Morus alba L. (Moraceae), also known as white mulberry, is a popular herb in the traditional system of Indian medicine. According to Ayurveda, the plant has astringent, anthelmintic, anxiolytic, antitussive, antiasthematic, and antihypertensive actions [5]. In ancient system of medicine, it is reported that *M. alba* has brain and cardiotonic effects [6]. Experimental reports have shown that mulberry roots have cathartic, analgesic, diuretic, antitussive, sedative, and anticonvulsant actions. *M. alba* L. has antidopaminergic and antiaggresive activity [7]. The leaves are diaphoretic, emollient, and antibacterial. The plant also possesses hypoglycemic [8], hypolipidemic, hepatoprotective [9], and anxiolytic [10] activities.

The pharmacological activities of *M. alba* are due to the presence of constituents like flavonoids, such as isoflavones, flavanone, flavonols, morusin, cyclomorusin, and neocyclomorusin; novel prenylflavonoids like isoquercetin, quercetin, astragalin, and scopoline; glycosides, namely roseoside III and benzyl D-glucopyranoside which demonstrated a free radical scavenging activity [11]. The antioxidant potency of some phenolic compounds from *M. alba* has been reported in different experimental models [8, 12].

In spite of the reported cardiotonic and antioxidant properties of *M. alba*, there is no major investigative report available pertaining to its cardioprotective and antihypertensive effect. Therefore, in this study, we have investigated the cardioprotective and antihypertensive potential of EASF in isoproterenol-induced myocardial infarction and renal artery ligation-induced hypertension.

2. Materials and Methods

2.1 Plant Material

The leaves of *M. alba* were collected in the month of November (2009) from a local area in Nashik, India, and authenticated by P. S. N. Rao (Director, Botanical survey of India, Pune). A voucher specimen of the plant has been deposited at Botanical survey of India, Pune (Voucher Specimen No. NVMA2). The leaves were shade-dried and reduced to a coarse powder. The powdered plant material was defatted using petroleum ether (60–80 °C) by Soxhlet extractor and then extracted by methanol for 72 h. The extract was filtered and concentrated under reduced pressure. The yield of methanol extract of *M. alba* leaves was found to be 12.10% w/w. The methanol extract was exhaustively extracted with ethyl acetate to obtain ethyl acetate soluble (EASF 13.4% w/w) and ethyl acetate insoluble fractions (EAIF 4.6% w/w). Suspensions of EASF was prepared in distilled water using Tween 80 (0.2% v/v) as a suspending agent. The extract was administered in doses of 25, 50, and 100 mg/kg for three weeks per orally (p.o.), and ISO was administered on twentieth and twenty-first day. Control group was given only vehicle (0.2% v/v, Tween 80) in volume equivalent to that of the plant extract.

2.2 Experimental Animals

Male Wistar strain rats (200–250 g) were used for the study. Animals were housed in colony cages and maintained under the standard laboratory environmental conditions; temperature 25 ± 2°C, 12 h light:12 h dark cycle, and 50 ± 5% relative humidity with free access to food and water ad libitum. Animals were acclimatised to laboratory conditions before the test. Each group consisted of five (n=5) animals. All the experiments were carried out during the light period (08:00–16:00 h). The studies were carried out in accordance with the guidelines given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India). The Institutional Animal Ethical Committee of M.V.P.S College of Pharmacy, Nashik approved the protocol of the study (IAEC/2009/05).

2.3 Drugs and Chemicals

Isoproterenol (Samarth Life Sciences Pvt. Ltd., Mumbai, India), adrenaline and noradrenaline (T. Walkers Pharmaceuticals Pvt. Ltd., Pune, India), phenylephrine (Samarth Life Sciences Pvt. Ltd., Mumbai, India), diltiazem (Cipla Pharm Ltd., Mumbai, India), thiobarbituric acid (TBA) (Research Lab Fine Chem. Industries, Mumbai, India), nitrobluetetrazolium (NBT) (Himedia Laboratories, Mumbai, India), 5,5'-dithiobis (2-nitro benzoic acid) (DTNB) (Alfa Aesar, Johnson Mathey, Chennai, India). Biochemical kits for SGOT and LDH (Agappe Diagnostic, Ernakulam, India), triglyceride, cholesterol and HDL (Pathozyme Diagnostics, Kolhapur, India), CK-MB (Aspen Labs, Baddi, India) were used. All the chemicals used were of analytical grade and purchased from standard manufacturers.

2.4 Cardioprotective Effect of EASF of *M. alba* in Isoproterenol-induced Myocardial Infarction

Myocardial infarction was induced by administration of isoproterenol (ISO) 85 mg/kg, s.c. for two consecutive days on twentieth and twenty-first day. EASF was administered for three weeks. EASF was given on the day of ISO too.

Animals were divided into eight groups (n=5 for each group) for cardioprotective activity of *M. alba*. Group I: Control (0.2% v/v, Tween 80); Group II: isoproteronol (ISO – 85 mg/kg, s.c. at an interval of 24 h for two days); Group III: EASF (25 mg/kg, p.o. for three weeks); Group IV: EASF (50 mg/kg, p.o. for three weeks); Group V: EASF (100 mg/kg, p.o. for three weeks); Group VI: EASF (25 mg/kg, p.o. for three weeks + ISO on twentieth and twenty-first day); Group VII: EASF (50 mg/kg, p.o. for three weeks + ISO on twentieth and twenty-first day); Group VIII: EASF (100 mg/kg, p.o. for three weeks + ISO on twentieth and twenty-first day).

After 12 h of the last dose of ISO, the parameters like ECG, heart rate, and mean arterial blood pressure were measured. The changes to vascular reactivity to various catecholamines were recorded and pressure-rate index was determined. The serum of animals of all the groups was assessed for their cardiac marker enzyme (LDH, SGOT, and CK-MB) levels. Hearts of animals of all the groups were excised and weighed and were subjected to histopathological studies. Heart was homogenised for determination of SOD, CAT, GSH, and LPO levels.

2.4.1 Electrocardiography and heart rate

Needle electrodes were stuck on the skin and changes in Lead II were recorded on an electrocardiograph (Powerlab, AD Instruments, Australia). Heart rate and ECG recordings in animals were anaesthetised with ketamine and xylazine (75 mg/kg and 15 mg/kg, i.p. resp.) for 1 min for every 5 min. The type of alterations (ST-segment elevation or depression) was measured [13].

2.4.2 Vascular reactivity to catecholamines

After the completion of treatment schedule, rats from each group were anaesthetised with ketamine and xylazine (75 mg/kg and 15 mg/kg, i.p. resp.). Right jugular vein was cannulated with fine polyethylene catheter for the administration of the drug. Blood pressure (BP) was recorded from left common carotid artery using pressure transducer by direct method on PowerLab data acquisition system (AD Instruments, Australia). Heparinised saline (100 IU/ml) was filled in the transducer and in the fine catheter cannulated to the carotid artery to prevent clotting. After 30 min of stabilisation, mean change in BP to adrenaline (1µg/kg), noradrenaline (1µg/kg), and phenylephrine (1µg/kg) was recorded [14].

2.4.3 Pressure-rate index (PRI)

The PRI, a parameter used as an index of myocardial oxygen demand, was calculated as the product of mean arterial blood pressure (MABP) × heart rate (HR)/1000 [15].

2.4.4 Cardiac marker enzymes

At the end of treatment, the blood was collected from retro-orbital plexus (under light ether anaesthesia) using capillary tubes. Serum was separated using R-24 research centrifuge (Remi Instruments Ltd., Mumbai, India) at 4000 rpm for 5 min. The activity of lactate dehydrogenase (LDH), creatine kinase (CK-MB), and serum glutamate oxaloacetate transaminase (SGOT) were assayed in serum using commercial kits [16].

2.4.5 Heart weight

The animal heart was excised immediately after the completion of vascular reactivity and was weighed and heart weight/100 g of body weight in each group was noted.

2.4.6 Histopathological examination

The hearts were excised and immediately fixed in 10% buffered formalin. The ventricular mass was sectioned from the apex to the base of the heart, which was embedded in paraffin after being dehydrated in alcohol. Five-micrometer thick serial histological sections were obtained from the paraffin blocks by using microtome and stained with hematoxylin and eosin. The sections were examined under light microscope (Olympus, Japan) and photomicrographs were taken.

2.4.7 Sampling techniques – dissection and homogenisation

After histopathology of heart, they were rinsed thoroughly with ice-chilled 0.9% NaCl and weighed. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The post-nuclear fraction for catalase assay was obtained by centrifugation of the homogenate at 1000 g for 20 min at 4 °C; for other enzyme assays, centrifugation was at 12,000 g for 60 min at 4 °C. A Shimadzu-160A spectrophotometer was used for subsequent assays [17].

2.4.8 Catalase activity (CAT)

Catalase activity was assessed by the method of Luck (1971), where the breakdown of H_2O_2 was measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2 phosphate buffer (0.0125 M H_2O_2) and 0.05 ml of supernatant of heart homogenate (10%), and the change in the absorbance was measured at 240 nm. The enzyme activity was calculated using the millimolar extension coefficient of H_2O_2 (0.07). The results were expressed as micromole of H_2O_2 decomposed per minute per milligram of protein [18].

2.4.9 Superoxide dismutase activity (SOD)

Superoxide dismutase activity was assayed according to the method of Kono (1978), wherein the reduction of nitrobluetetrazolium chloride (NBT) was inhibited by the superoxide dismutase which was measured at 560 nm spectrophotometrically. Briefly, the reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and post-nuclear fraction of heart homogenate. The results were expressed as units per milligrams of protein, with one unit of enzyme defined as the amount of SOD required to inhibit the rate of reaction by 50% [19].

2.4.10 Lipid peroxidation assay (LPO)

The quantitative measurement of lipid peroxidation in heart was done by the method of Wills (1966). The amount of malondialdehyde (MDA) formed was measured by reaction with thiobarbituric acid at 532 nm. The results were expressed as nanomole of MDA per milligram of protein using the molar extension coefficient of chromophore (1.56×10^5 M^{-1} cm^{-1}) [20].

2.4.11 Estimation of reduced glutathione (GSH)

Reduced glutathione (GSH) in the heart was estimated according to the method of Ellman (1959). A 0.1 ml of sample of homogenate was precipitated with 0.75 ml of 4% sulphosalicylic acid. The assay mixture contained 0.5 ml of supernatant and 4.5 ml of DTNB in 0.1 M phosphate buffer, with pH 8.0. The yellow colour developed was read immediately at 412 nm. The results were expressed as nanomole of GSH per milligram of protein [21].

2.4.12 Protein estimation

The protein content was measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard and expressed as µg protein/mg of tissue [22].

2.5 Evaluation of Antihypertensive Activity of EASF of *M. alba*

The left renal artery was ligated with right artery untouched. This leads to sustained increase in blood pressure.

For antihypertensive studies, animals were divided into ten groups (n=5 for each group).

Group I: Control (0.2% v/v, Tween 80); Group II: LRA (left renal artery ligation was done); Group III: Diltiazem (30 mg/kg, p.o. for six weeks); Group IV: EASF (25 mg/kg, p.o. for six weeks), Group V: EASF (50 mg/kg, p.o.

for six weeks), Group VI: EASF (100 mg/kg, p.o. for six weeks), Group VII: EASF (25 mg/kg, p.o. for six weeks + LRA), Group VIII: EASF (50 mg/kg, p.o. for six weeks + LRA), Group IX: EASF (100 mg/kg, p.o. for six weeks + LRA), Group X: Diltiazem (30 mg/kg, p.o. for six weeks + LRA).

The hypertension was induced in experimental animals by ligation of left renal artery. Rats were anaesthetised by ketamine and xylazine (75 mg/kg and 15 mg/kg, i.p. resp.) A 3 cm retroperitoneal flank incision was done. The left kidney was exposed and the renal artery was carefully separated free of the renal vein. The renal artery was then ligated by 4–0 sterile surgical silk. The incision was closed by carefully suturing of the muscle layer with 4–0 silk using a non-cutting needle. The animals were allowed to recover [23].

Systolic blood pressure was measured after every two week by the tail cuff technique in the conscious rat on PowerLab data acquisition system. After completion of treatment schedule (six weeks after LRA), rats from each group were anaesthetized with ketamine and xylazine. Jugular vein was cannulated with fine polyethylene catheter for administration of the drug. Tracheotomy was performed and blood pressure was recorded from left common carotid artery using pressure transducer by direct method on PowerLab data acquisition system. Heparinised saline (100 IU/ml) was filled in the transducer and the fine polyethylene catheter cannulated to the carotid artery to prevent clotting.

2.5.1 In vitro studies

At the end of the experiment, rats were killed by air emboli method. Thoracic aorta was rapidly dissected out and immersed in chilled Kreb's solution, composed of (mM): NaCl, 118.0; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; and glucose, 11.0, and bubbled with 95% O_2 + 5% CO_2 (pH 7.4). After the perivascular tissue was carefully removed, aortic rings approximately 4 mm in lengths were cut. Care was taken to avoid abrading the intimal surface in order to maintain the integrity of the endothelial layer. Aortic rings were suspended in organ chambers containing 10 ml Kreb's solution at 37 °C and aerated. The aortic segments were allowed to equilibrate for 1 h at a resting tension of 2 g. During the equilibration period, the solution was replaced every 15 min. Changes in tension were recorded by isometric transducers connected to a PowerLab data acquisition system. After equilibration period, aortic rings were washed 4–5 times with Ca^{2+}-free Kreb's solution before pre-contracted with phenylephrine (10^{-6} M) and then Ca^{2+} was added cumulatively to obtain a concentration–response curve (10^{-5} to 1×10^{-3} M). Ca^{2+}-free Kreb's solution composed of (mM): NaCl, 118.0; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; and glucose, 11.0; and 5×10^{-5}M EDTA [24].

Later on, aortic rings were incubated with EASF (6 mg/ml) for 30 min and again aortic rings were pre-contracted with phenylephrine and then Ca^{2+} was added cumulatively to obtain a concentration–response curve.

In second set of experiment, the effect of EASF was observed on endothelium-intact and endothelium-denuded aortic rings. The endothelium was removed by gently rubbing the intimal space with a cotton swab, and the same experiment was performed with endothelium-denuded aortic rings.

2.6 Statistical Analysis

Results are expressed as mean±SEM, and the statistical analysis of data was done using one-way analysis of variance (ANOVA) followed by Dunnett's test. Probability level less than 0.05 was considered statistically significant.

3. Results

3.1 Measurement of ST Segment

The control animals showed normal ECG. The animals treated with ISO showed significant ($p<0.001$) elevation of ST segment as compared to control group. Pretreatment with EASF showed no change in ST segment after three weeks. Administration of EASF (100 mg/kg) for three weeks followed by ISO treatment on twentieth and twenty-first day showed significant ($p<0.001$) depression in ST segment compared to ISO group. EASF (25 and 50 mg/kg) treatment reduced the ST changes ($p<0.05$, $p<0.01$ resp.) (Fig. 1).

3.2 Measurement of Heart Rate

The control group showed normal heart rate. Heart rate was significantly ($p<0.001$) increased after treatment

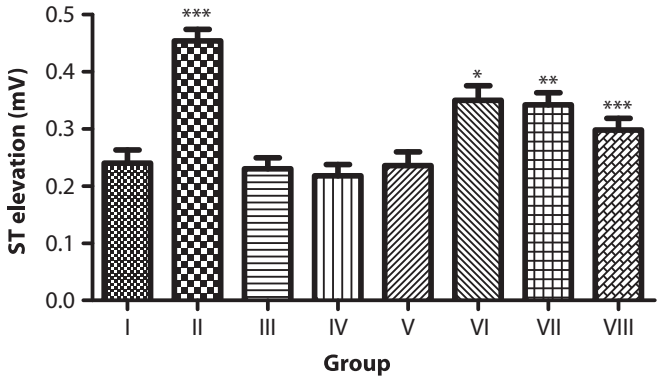

Fig. 1. Effect of EASF of *M. alba* on ST-segment elevation in ISO-induced myocardial infarction

Each column represents mean±SEM (n = 5).

Groups II, III, IV, and V compared to Group I. Groups VI, VII, and VIII compared to Group II.

*p< 0.05; **p< 0.01; ***p < 0.001; (one-way ANOVA followed by Dunnett's test).

Table 1: Effects of EASF of *M. alba* on heart rate and heart weights in ISO-induced myocardial infarction

Groups	Treatment (Beats/min)	Heart rate	Heart weights (g/100 g of body weight)
I	Control	319.0 ± 3.7	0.37 ± 0.0
II	ISO	445.8 ± 3.4***	0.62 ± 0.0***
III	EASF-25	320.3 ± 2.9	0.37 ± 0.0
IV	EASF-50	302.9 ± 5.2	0.38 ± 0.0
V	EASF-100	305.5 ± 5.1	0.40 ± 0.0
VI	EASF-25+ISO	407.0 ± 3.5***	0.53 ± 0.0***
VII	EASF-50+ISO	366.4 ± 2.6***	0.50 ± 0.0***
VIII	EASF-100+ISO	357.6 ± 2.8***	0.48 ± 0.0***

Values are expressed as mean±SEM (n=5).

Groups II, III, IV, and V compared to Group I. Groups VI, VII, and VIII compared to Group II.

*p<0.05, **p<0.01, *** p<0.001; (one-way ANOVA followed by Dunnett's test).

with ISO compared to control group. The administration of EASF (25, 50, and 100 mg/kg) for a period of three weeks followed by ISO significantly attenuated ISO-induced elevation of heart rate (Table 1).

3.3 Measurement of Mean arterial Pressure (MAP) by Invasive (direct) Method

The mean arterial pressure was significantly (p<0.001) increased in ISO-treated group compared to control

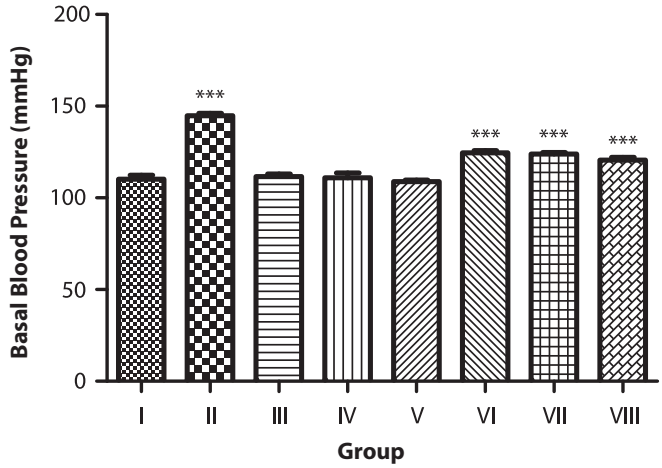

Fig. 2. Effect of EASF of *M. alba* on mean arterial pressure ISO-induced myocardial infarction

Each column represents mean ± SEM (n = 5).

Groups II, III, IV, and V compared to Group I. Groups VI, VII, and VIII compared to Group II.

*p< 0.05; **p< 0.01; *** p < 0.001; (one-way ANOVA followed by Dunnett's test).

group. Administration of EASF (25, 50, and 100 mg/kg) for a period of three weeks followed by ISO showed a significant (p<0.001) fall in mean arterial pressure compared to ISO group (Fig. 2).

3.4 Pressure-rate Index (PRI)

ISO-treated group showed significant (p<0.001) increase in PRI compared to control group. The treatment with EASF (25, 50, and 100 mg/kg) followed by ISO showed a significant (p<0.001) reduction in PRI compared to ISO-treated group (Fig. 3).

3.5 Vascular Reactivity to Various Catecholamines Like Adrenaline (Adr), Noradrenaline (NA), and Phenylephrine (PE)

Control group showed normal responses to the various catecholamine like Adr (1µg/kg), NA (1 µg/kg), and PE (1µg/kg) in vascular reactivity, whereas ISO-treated groups showed a significant (p<0.001) elevation in mean change in BP to Adr, NA, and PE. Groups undergoing EASF treatment followed by ISO showed significant decrease in mean change in BP compared to ISO-treated group (Table 2).

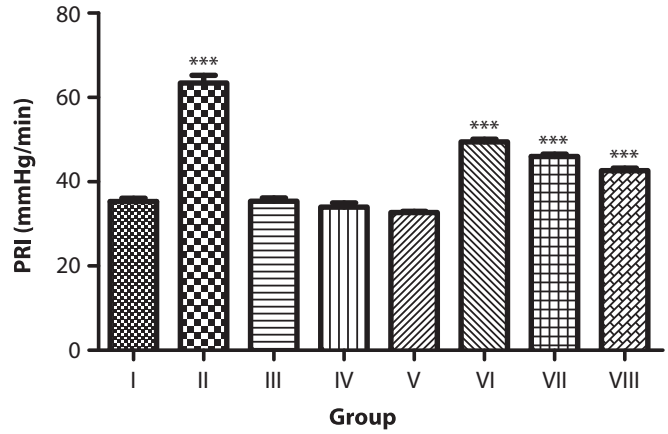

Fig. 3. Effect of EASF of *M. alba* on pressure-rate index in ISO-induced myocardial infarction
Each column represents mean ± SEM (n = 5).
Groups II, III, IV, and V compared to Group I. Groups VI, VII, and VIII compared to Group II.
*p< 0.05; **p< 0.01; ***p < 0.001; (one-way ANOVA followed by Dunnett's test).

Table 2: Effect of EASF of *M. alba* on vascular reactivity to noradrenaline, adrenaline, and phenylephrine in ISO-induced myocardial infarction

Groups	Treatment Phenylephrine	Noradrenaline (mmHg)	Adrenaline (mmHg)	(mmHg)
I	Control	38.2 ± 2.3	41.2 ± 3.8	40.3 ± 2.0
II	ISO	47.0 ± 3.1**	56.4 ± 5.4***	54.7 ± 2.1***
III	EASF-25	36.3 ± 2.2	40.3 ± 6.2	38.4 ± 0.3
IV	EASF-50	37.6 ± 0.1	39.8 ± 2.2	36.9 ± 3.4
V	EASF-100	36.8 ± 0.3	38.4 ± 3.6	37.1 ± 0.1
VI	EASF-25+ISO	44.8 ± 3.7	50.6 ± 3.2*	45.4 ± 0.2***
VII	EASF-50+ISO	41.2 ± 4.1*	44.0 ± 2.2**	42.3 ± 2.1***
VIII	EASF-100+ISO	40.4 ± 3.1**	42.4 ± 3.1**	41.9 ± 2.1***

Values are expressed as mean±SEM (n=5).
Groups II, III, IV, and V compared to Group I. Groups VI, VII, and VIII compared to Group II.
*p< 0.05; **p< 0.01; ***p < 0.001; (one-way ANOVA followed by Dunnett's test)

3.6 Heart Weight

The heart weight of experimental animals was significantly increased (p<0.001) in ISO-treated group compared to control group. Pretreatment with EASF (25, 50, and 100 mg/kg) significantly (p<0.001) decreased heart weights compared to ISO-treated group (Table 1).

3.7. Effects of EASF on ISO-induced Alterations in Rat Heart SOD, CAT, GSH, and LPO

3.7.1 Effect on SOD level

The ISO treatment significantly decreased the SOD activity in heart tissues. Pretreatment with EASF significantly (p<0.001) increased level of SOD at all doses (Table 3).

3.7.2 Effect on brain CAT and GSH level

Due to ISO treatment, the levels of CAT and GSH were significantly reduced. Pretreatment with EASF (50 and 100 mg/kg) significantly (p<0.001) increased the CAT and GSH levels (Table 3).

3.7.3 Lipid peroxidation assay (LPO)

The ISO treatment leads to significant increase in LPO level. Pretreatment with EASF (50 and 100 mg/kg) significantly (p<0.001) reduced elevated level of LPO (Table 3).

Table 3: Effects of EASF of *M. alba* on the levels of CAT, SOD, LPO, and GSH in ISO-induced myocardial infarction

Groups	Treatment	CAT µmol of H_2O_2 decomposed/min/mg protein	SOD U/mg protein	LPO nmol of MDA/mg protein	GSH µmol/mg protein
I	Control	5.26 ± 1.1	16.8 ± 1.3	24.7 ± 3.1	5.40 ± 1.7
II	ISO	2.47± 0.8***	5.7 ± 1.9***	58.4 ± 0.5***	1.72± 1.8***
III	EASF-25	6.18 ± 5.3	14.5 ± 2.4	25.8 ± 2.1	5.52 ± 3.6
IV	EASF-50	6.34 ± 1.7	15.0 ± 0.9	23.4 ± 1.9	4.82 ± 1.3
V	EASF-100	6.86 ± 2.0	14.8 ± 3.4	32.8 ± 1.3	5.79 ± 3.4
VI	EASF-25+ISO	3.26± 4.7	9.4 ± 5.2***	44.2± 2.8***	2.64 ± 4.1***
VII	EASF-50+ISO	4.38 ± 1.2***	11.5 ± 3.1***	32.8 ± 2.5***	3.66 ± 3.7***
VIII	EASF-100+ISO	5.06 ± 1.4***	14.9 ± 1.4***	30.2 ± 3.1***	4.53 ± 0.9***

Values are expressed as mean±SEM (n=5).

Groups II, III, IV, and V compared to Group I. Groups VI, VII, and VIII compared to Group II.

*p< 0.05; **p< 0.01; ***p < 0.001; (one-way ANOVA followed by Dunnett's test).

Table 4: Effects of EASF of *M. alba* on the levels on cardiac marker enzymes in ISO-induced myocardial infarction

Groups	Treatment	LDH (IU/L)	CK-MB (IU/L)	SGOT (IU/L)
I	Control	338.3 ± 2.1	290.2 ± 0.2	58.1 ± 2.7
II	ISO	629.4 ± 2.7***	569.4 ± 0.1***	88.2 ± 1.2***
III	EASF-25	362.7 ± 3.3	278.3 ± 4.3	56.4 ± 0.8
IV	EASF-50	272.4 ±0.1	242.4 ± 3.7	54.7 ± 0.3
V	EASF-100	374.3 ± 0.5	226.6 ± 2.5	51.2 ± 2.9
VI	EASF-25+ISO	468.1 ± 0.3**	462.7 ± 3.1*	72.2 ± 3.2***
VII	EASF-50+ISO	494.2 ± 0.9*	418.3 ± 1.7**	64.5 ± 4.7***
VIII	EASF-100+ISO	412.7 ± 1.1**	403.4 ± 2.3**	67.3 ± 5.2***

Values are expressed as mean±SEM (n=5).

Groups II, III, IV, and V compared to Group I. Groups VI, VII, and VIII compared to Group II.

*p< 0.05; **p< 0.01; ***p < 0.001; (one-way ANOVA followed by Dunnett's test).

3.8 Effects of EASF on ISO-induced Alterations in Cardiac Marker Enzymes Lactate Dehydrogenase (LDH), Creatine Kinase–MB (CK-MB), and SGOT

Exposure to ISO significantly increased the LDH, CK-MB, and SGOT levels. Pretreatment with EASF of *M. alba* (25, 50, and 100 mg/kg) significantly (p<0.01) decreased levels of cardiac damage marker enzymes compared to ISO-treated group (Table 4).

3.9 Histopathological examination (10X) of Rat Heart from Various Groups

A section of heart (Haematoxyline and Eosine stained) from control group showed normal architecture. A section of the heart of ISO-treated group revealed focal myonecrosis and lymphocytic infiltration (myocarditis). A section of heart of *M. alba per se* showed normal architecture of myocardial fibres. A section of the heart of ISO group pretreated with *M. alba* revealed less intensity and distribution of myonecrosis and lymphocytic infiltrations (myocarditis) indicating protection from ISO-induced changes in the hearts as compared to ISO group (Fig. 4).

3.10 Evaluation of Anti-hypertensive Effect of EASF

3.10.1 Measurement of blood pressure by non-invasive (indirect) and invasive (direct) method

The control group showed normal blood pressure. LRA-ligated group showed significant (p<0.001) rise in blood pressure. Administration of EASF for a period

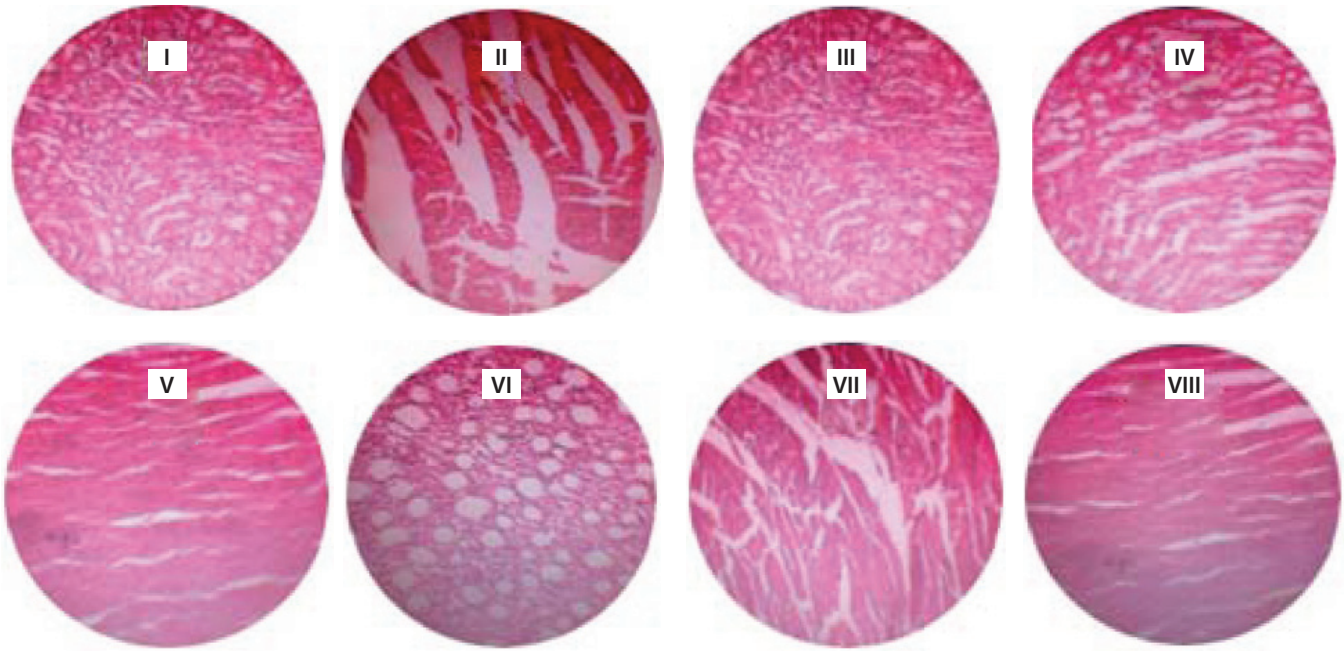

Fig. 4. Histopathological changes of rat hearts

I: Vehicle treated group, histological structure of rat heart showing normal myocardial fibers; II: ISO treated group revealed focal myonecrosis and lymphocytic infiltration (myocarditis); III, IV, V: *M. alba per se* showed normal architecture of myocardial fibers; VI, VII, VIII: ISO + *M.alba* treated groups revealed less intensity and distribution of myonecrosis and lymphocytic infiltrations (myocarditis) indicating protection from ISO-induced changes in the hearts as compared to ISO group.

of six weeks (25, 50, and 100 mg/kg) caused significant ($p<0.001$) fall in the blood pressure (Figs. 5 and 6).

3.10.2 In vitro study

The control group showed normal response to $CaCl_2$ on isolated rat aorta pre-contracted with phenylephrine, whereas in LRA ligation group, there was a shift in the cumulative concentration response curve (CCRC) of $CaCl_2$ to the left. The incubation of aortic rings of EASF (6 mg/ml) for 30 min before PE significantly ($p<0.001$) shifted the CCRC of $CaCl_2$ to the right with suppression of maxima as compared to CCRC of LRA group.

In the second set of experiment, $CaCl_2$ on isolated rat aortic rings pre-contracted with PE produced dose-dependent contractions in both endothelium-intact and endothelium-denuded rat aortas. The effect of EASF on endothelium-intact and endothelium-denuded rat was similar. The incubation of aortic rings of EASF (6 mg/ml) for 30 min before $CaCl_2$ also significantly inhibited contractions due to $CaCl_2$ in both endothelium-intact and endothelium-denuded aortic rings (Fig. 7a, b, and c).

4. Discussion

Isoproterenol, a potent synthetic catecholamine, induces subendocardial myocardial ischemia, hypoxia, necrosis, and finally fibroblastic hyperplasia with decreased myocardial compliance which closely resembles local myocardial infarction-like pathological changes seen in human myocardial infarction [25]. The cardiotoxicity occurs primarily *via* adrenoceptor activation. The excess catecholamines affect calcium transport mechanism primarily *via* oxidation reactions involving free radical mediated damage result in the development of intracellular Ca^{2+} overload due to depressed sarcolemmal Ca^{2+} transport and cause ventricular dysfunction [26]. The therapeutic intervention with antioxidant activity may be useful in preventing these deleterious changes. ISO-induced myocardial infarction is widely used as a model of evaluating cardioprotective drugs [1].

The loss of cell membrane of injured myocardium in myocardial infarction is characterised by rapid ST elevation in ECG, increased ventricular excitability, conduction disturbances, and tachycardia [13]. The

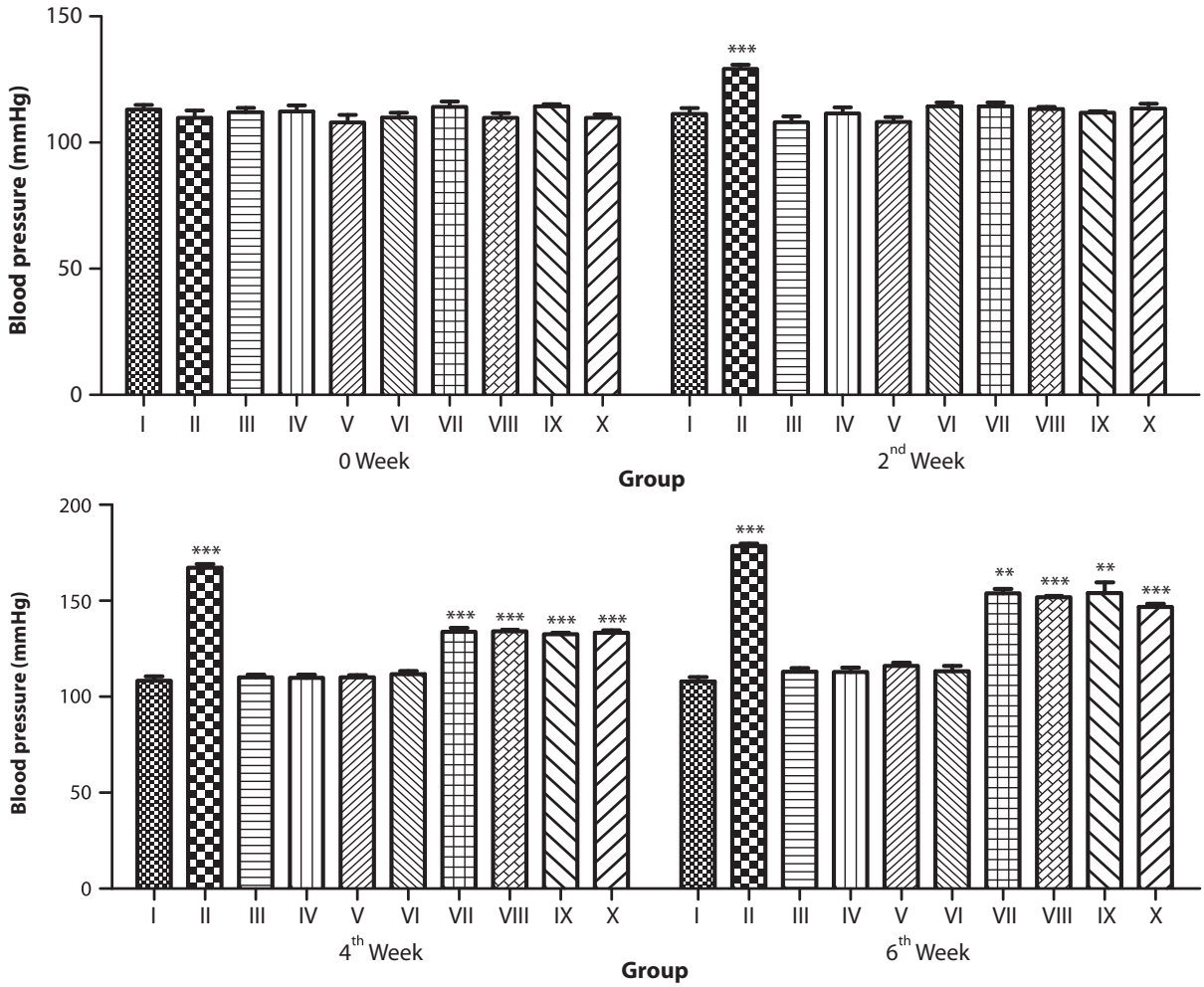

Fig. 5. Effect of EASF of *M. alba* on blood pressure (non-invasive) in LRA-induced hypertension
Each column represents mean ± SEM (n = 5).
Groups II, III, IV, V, and VI compared to Group I. Groups VII, VIII, IX, and X compared to Group II.
*p< 0.05; **p< 0.01; ***p < 0.001; (one-way ANOVA followed by Dunnett's test).

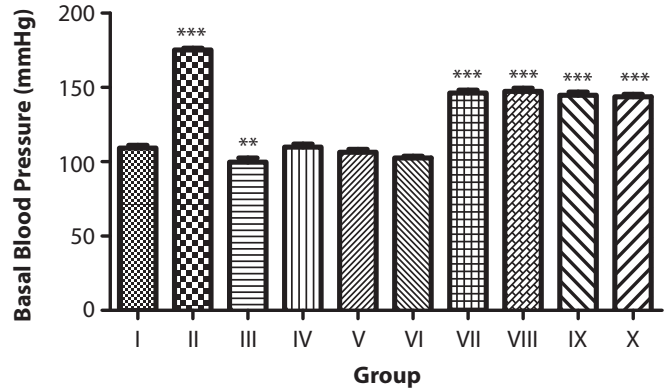

Fig. 6. Effect of EASF of *M. alba* on blood pressure (invasive) in LRA-induced hypertension
Each column represents mean ± SEM (n = 5).
Groups II, III, IV, V, and VI compared to Group I. Groups VII, VIII, IX, and X compared to Group II.
*p< 0.05; **p< 0.01; ***p < 0.001; (one-way ANOVA followed by Dunnett's test).

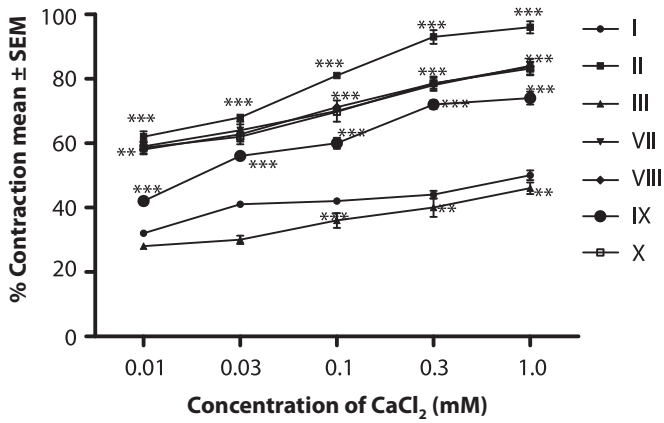

Fig. 7a. Effect of EASF of *M. alba* on cumulative concentration response curve (CCRC) of $CaCl_2$ on PE pre-contracted rat aortas in LRA-induced hypertension

Values represents mean ± SEM (n = 5).

Groups II and III compared to Group I. Groups VII, VIII, IX, and X compared to Group II.

*p< 0.05; **p< 0.01; ***p < 0.001; (one-way ANOVA followed by Dunnett's test).

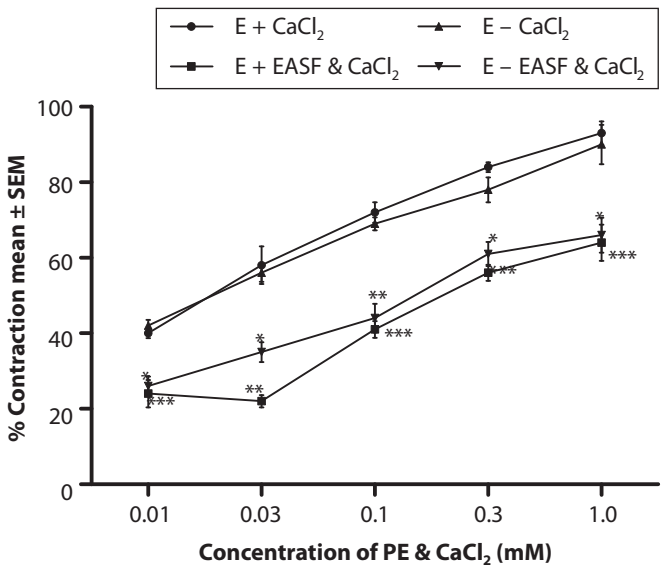

Fig.7b. Effect of EASF of *M. alba* on $CaCl_2$ on PE pre-contracted rat aortas in LRA-induced hypertension

Values represents mean ± SEM (n = 5).

Group E + EASF and $CaCl_2$ compared to Group E + $CaCl_2$.

Group E – EASF and $CaCl_2$ compared to Group E – $CaCl_2$.

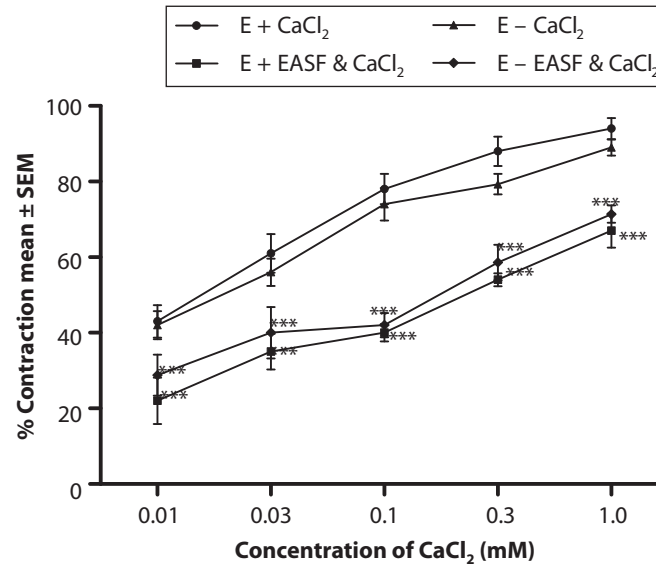

Fig. 7c. Effect of EASF of *M. alba* on $CaCl_2$-induced contractions in endothelium-intact and endothelium-denuded rat aortas in LRA-induced hypertension

Values represents mean ± SEM (n = 5).

Group E + EASF and $CaCl_2$ compared to Group E + $CaCl_2$.

Group E – EASF and $CaCl_2$ compared to Group E – $CaCl_2$.

increase in the weight of heart after ISO administration might be due to the increased permeability of vessel walls; the local muscle tissue becomes edematous, and the cardiac muscle cells begin to swell because of diminished cellular metabolism. ISO by its positive inotropic and chronotropic action increases blood pressure and leads to increase in the myocardial oxygen demand resulted into ischemic necrosis of myocardium in rats similar to that seen in human myocardial infarction. Pressure-rate index (PRI) was considered as an index of oxygen demand [25].

The results of the present study showed an elevation of ST segment and increase in heart rate, heart weight, and pressure-rate index after treatment with isoproterenol. Pretreatment with EASF (25, 50, and 100 mg/kg) markedly inhibited ISO-induced elevation of ST segment and heart rate, significantly reduced weight of the heart, and decreased PRI indicating protective effect of EASF on myocardial membrane.

The main finding of the study was that the administration of isoproterenol induces severe oxidative stress and results in necrotic lesions in the myocardium of rats. The increased generation of reactive oxygen species and/or depletion of the antioxidants in the defense system may contribute to oxidative stress and affect the pathogenesis of myocardial infarction. The increased levels of MDA indicate excessive formation of free radicals by auto-oxidation of ISO and activation of the lipid peroxidative process, resulting in irreversible damage to heart in animals subjected to ISO stress [27]. The SOD, CAT, and GSH levels were significantly depleted by ISO, whereas significant increase in LPO was observed. The administration of EASF of *M. alba* attenuated the oxidative stress and the biochemical changes associated with ISO-induced myocardial damage. The mechanism of such protection of EASF may be due to the augmentation of cellular antioxidants, such as GSH, SOD, and CAT as well as reduction of LPO. This finding supports the earlier observation in which the antioxidant potency of some phenolic compounds from *M. alba* has been reported. The naturally occurring flavonoids, especially those of the *M. alba,* have shown antioxidant activity in different model systems [12, 8].

Myocardium contains an abundant concentration of diagnostic marker enzymes. When myocardium gets damaged, it releases its contents into the extracellular fluid. In ISO-induced myocardial infarcted rats, the marker enzymes, such as lactate dehydrogenase (LDH), creatine kinase (CK-MB), and serum glutamate oxaloacetate transaminase (SGOT) were significantly increased [16]. The prior administrations of EASF significantly lower the ISO-induced elevation in the activities of diagnostic marker enzymes. This could be due to its action of maintaining membrane integrity, thereby restricting the leakage of these enzymes.

In the present study, the vascular reactivity to various catecholamines like adrenaline, noradrenaline, and phenylephrine was tested. The increased vascular sensitivity to these catecholamines was observed after administration of ISO; this may be due to altered sympathetic activity. The pretreatment with EASF significantly normalise the sensitivity to all catecholamines, indicating decreased sympathetic activity.

In histopathological studies, ISO-treated group showed focal myonecrosis and infiltration of inflammatory cells. The administration of EASF demonstrated reversal of myonecrosis and lymphatic infiltration (myocarditis). The inflammatory cells were seen in reduced density in the EASF-treated group as compared to ISO, confirming cardioprotective activity of EASF of *M. alba.*

The pathophysiological mechanism underlying renal hypertension involves activation of the renin–angiotensin–aldosterone system. Left renal artery (LRA) ligation produces renal ischemia initiating hypersecretion of renin and subsequent activation of renin–angiotensin–aldosterone system. Angiotensin-II-mediated vasoconstriction and aldosterone-induced sodium and water retention cause a rise in BP. The main actions of angiotensin-II include generalised vasoconstriction, increased release of noradrenaline from sympathetic nerve terminals, stimulation of proximal tubular reabsorption of sodium and alteration in cardiovascular structures [28]. The endothelium-dependent vasodilation and smooth muscle cell hyperpolarisation are impaired in aortic segments in LRA-induced hypertensive rats. However, no predisposing deficiency in NO has been demonstrated in renal hypertension. Therefore, reduced vasodilation in renal hypertension may also be related to an abnormal function of vascular smooth muscle cells. Most of the cellular mechanisms of NO-induced vasodilatation are mediated through the activation of soluble guanylyl-cyclase, which catalyses the conversion of guanosine 5-trisphosphate (GTP) to cGMP, and membrane hyperpolarisation through K^+ channel activation. When K^+ channels open in the vascular smooth muscle cell membrane, K^+ efflux increases, causing membrane hyperpolarisation, decreased Ca^{2+} entry (through voltage-operated Ca^{2+} channels), and vasodilatation [29, 30].

The results of study clearly indicated that there is a significant rise in blood pressure in renal hypertensive rats. Administration of EASF for six weeks significantly reduced blood pressure in renal hypertensive rats. This action of EASF may be due to the decreased sympathetic activity or increased vasodilating substances, which is further supported by in vitro study.

In in vitro study, the control group showed normal response to $CaCl_2$ on isolated rat aorta pre-contracted with phenylephrine, whereas ISO-treated group shifted the cumulative concentration response curve (CCRC) of $CaCl_2$ to the left. The incubation of aortic rings of EASF (6 mg/ml) for 30 min before PE significantly shifted the CCRC of $CaCl_2$ to the right with suppression of maxima as compared to CCRC of LRA group. Thus, EASF significantly decreased the contraction due to $CaCl_2$ on phenylephrine (PE) pre-contracted aorta in renal hypertensive rats. PE, an α-adrenoreceptor agonist, causes aortic contraction by Ca^{2+} influx through receptor-operated Ca^{2+} channels (ROCCs) and by release of Ca^{2+} from the sarcoplasmic reticulum. Thus, the decrease in blood pressure due to EASF in renal hypertension, reduced the aortic contraction when PE produced a steady contraction followed by gradual Ca^{2+} input in a Ca^{2+}-free solution, indicated that decreased influx of extracellular Ca^{2+} may be a critical mechanism in relaxing the aorta or due to increase in vasodilating substances such as NO which is responsible for vasodilation. This is further clarified by another study.

In the second set of experiment, $CaCl_2$ on isolated rat aortic rings pre-contracted with PE produced dose-dependant contractions in both endothelium-intact and endothelium-denuded rat aortas. $CaCl_2$ alone also produced dose-dependant contractions in both endothelium-intact and endothelium-denuded rat aortas. The effect of EASF on endothelium-intact and endothelium-denuded was similar. EASF antagonise the contraction produced by PE and $CaCl_2$ regardless of the presence or absence of endothelium. This suggests that the action of EASF was directly on vascular smooth muscle cells to induce relaxation and not by endothelium-derived vasodilator factors such as NO.

Vascular smooth muscle contraction caused by increased intracellular Ca^{2+} is due to influx of extracellular Ca^{2+} and release of Ca^{2+} from sarcoplasmic reticulum by activation of IP_3 and ryanodine receptors. Aortic contraction caused by PE is due to Ca^{2+} influx and release of Ca^{2+} from sarcoplasmic reticulum by stimulating phospholipase C to produce diacyl glycerol (DAG) and inositol triphosphate (IP_3), and subsequently DAG activates the light chain of myosin through activation of protein kinase C (PKC) and IP_3 receptor [29]. Thus, EASF showed inhibitory effect on Ca^{2+} and PE by inhibiting Ca^{2+} influx.

5. Conclusion

Our findings showed cardioprotective and antihypertensive activities of the EASF of *M. alba which* might be due to the regulation of antioxidant defensive mechanisms and blockade of calcium channels, respectively. Thus, this study indicates the beneficial role of *M. alba* in the treatment of cardiovascular complications.

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