Original Article

Aegle marmelos fruit extract attenuates isoproterenol-induced oxidative stress in rats

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Myocardial infarction is a major public health concern and the leading cause of death throughout the world. The present study investigates the ability of *Aegle marmelos* fruit extract to prevent pathological changes and oxidative stress after isoproterenol induced myocardial infarction in rats. *In vitro* studies showed that *Aegle marmelos* fruit extract possesses antioxidant activity. Administration of isoproterenol (85 mg/kg body weight) to rats resulted in significantly elevated plasma transaminases, lactate dehydrogenase and creatine kinase, however, cardiac tissue analyses showed decreased activity of the above enzymes compared to experimental control rats. Further, isoproterenol administration significantly increased plasma and cardiac tissue thiobarbituric acid reactive substances and lowered the activities of cardiac tissue superoxide dismutase, catalase, reduced glutathione, glutathione peroxidase and glutathione-S-transferase when compared to control groups. Pretreatment with *Aegle marmelos* fruit extract at a dose of 150 mg/kg body weight for a period of 45 days significantly prevented the observed alterations. Our data suggest that *Aegle marmelos* fruit extract exerts its protective effect by decreasing thiobarbituric acid reactive substances and elevating antioxidants status in isoproterenol treated rats. Both biochemical and histopathological results in the isoproterenol-induced myocardial infarction model emphasize the beneficial action of *Aegle marmelos* fruit extract as a cardioprotective agent.

***Key Words*: *Aegle marmelos*, isoproterenol, myocardial infarction, oxidative stress**

Myocardial infarction (MI) is the major form of ischemic heart diseases (IHD) and is characterized by an imbalance of coronary blood supply and myocardial demand which results in ischemia and myocardial death.[1] Previous studies have shown that during ischemic injury, oxidative stress produced by the generation of reactive oxygen species (ROS) plays a key role in the development of MI.[2,3] To scavenge ROS, cells have several antioxidant enzymes, the protective action of these enzymes is usually due to the inhibition of free radical-induced chain reaction and the resultant prevention of peroxidative deterioration of structural lipids in membranous organelles.[4,5]

Isoproterenol (ISO), a synthetic β-adrenoceptor agonist induces cardiotoxicity in the form of MI in rats as a result of disturbance in physiological balance between production of free radicals and antioxidative defense system.[6] Various mechanisms have been proposed to explain the ISO-induced cardiac damage, generation of highly cytotoxic free radicals through the auto-oxidation of catecholamine has been implicated as one of the important causative factors.[7] A better understanding of the processes involved in the pathophysiology of MI has led the researchers to search for safer medication that can limit the extent of myocardial injury.[8] Previous studies have demonstrated that use of antioxidants as a preventive approach to attenuate myocardial dysfunction and limit the infarct size as well as slow down the progression and consequences of MI. Recently, there has been renewed interest in medicinal plants that have been found to have certain preventive measures in the treatment of cardiovascular diseases.[9] Many plant products are rich in polyphenolics including tannins and flavonoids, which are group of compounds with diverse in chemical structure, characteristics and widely recognized as naturally occurring antioxidants.[10] Reports revealed that compounds in their natural formulations are more active than isolated form, since they contain both dotes and antidotes.[11]

There is a growing interest in traditionally used medicinal plants which produce a variety of compounds having therapeutic properties.[12] One such medicinal plant used since ancient time is *Aegle marmelos* (L.), commonly known as Bael and belongs to the family Rutaceae. Medicinal properties of *Aegle marmelos* were well explained in Ayurveda, Indian traditional medicine.[13] *Aegle marmelos* fruit are reported to be contain several active principles like marmelosin, marmelide, Luvangetin, aurapten, psoralen, tannin.[14,15] The fruit extract of *Aegle marmelos* have demonstrated antidiabetic,[16] antihyperlipidemic,[17] gastroprotective and antidiarrhoeal,[18,19] radioprotective,[20] antimicrobial,[21] activities. Previous studies showed preventive role of *Aegle marmelos* leaf extract on isoprenaline-induced MI.[17] Though *Aegle marmelos* fruit has several therapeutic properties, it has not been tested against experimentally induced MI. Hence, the present study was planned to evaluate the preventive effect of AMFE on isoproterenol induced oxidative stress in MI of rats.

Materials and Methods

Chemicals. ISO (D,L-4-(2-(isopropylamino)-1-hydroxyethyl) pyrocatechol) used to induce cardiac damage was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and were supplied by Sisco Research Laboratories (Mumbai, India).

Preparation of *Aegle marmelos* unripe fruit extract. Fresh *Aegle marmelos* unripe fruits were collected around Sri Krishnadevaraya University premises and verified with specimens available at the Botanical Herbarium, Dept of Botany, Sri Krishnadevaraya University, Anantapur, India. Fruits were air dried and powdered in an electric blender. The powder was boiled in distilled water. After filtration through whatmann No.40 filter paper the extract was evaporated to dryness by slow heating and

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continuous stirring in a water bath. The dark brown residue left behind was collected and was used for the study. The extract contains 26% total phenolic compounds including flavonoids and tannoids. The extract was dissolved in distilled water prior to administration.

Experimental design. Adult male albino Wistar rats weighing 150–180 g were divided in to four groups of six animals in each, procured from National Institute of Nutrition, Hyderabad, India. Atherogenesis was induced by s.c., injection of ISO 85 mg/kg body weight, dissolved in physiological saline, twice at an interval of 24 h for two consecutive days. AMFE was pretreated to rats orally at a dose of 150 mg/kg body weight/day for a period of 45 days, and then administered ISO at the dose of 85 mg/kg body weight. All the groups were fed with standard pellet diet (M/s. Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. The dose of AMFE used in the present work was fixed based on the acute oral toxicity study. The present study was approved by Sri Krishnadevaraya University institutional animal ethical committee. At the end of the experimental period, the rats in each group were fasted overnight and then sacrificed by cervical dislocation. Blood was collected with anticoagulant by cardiac puncture and plasma was separated for various biochemical estimations.

***In vitro* assays.** The effect of AMFE on various free radicals using standardized methods as described previously.[22] The ability to scavenge the stable free radical, DPPH (1,1-diphenyl-2-picryl hydrazyl) was measured as a decrease in absorbance at 517 nm and the percentage of inhibition was calculated. Nitric oxide ($NO^{\bullet}$) generated from sodium nitroprusside was measured by the Greiss reaction.[23] The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with napthylethylene diamine was read at 546 nm and percentage of inhibition was calculated. The hydroxyl radical ($OH^{\bullet}$) scavenging activity of AMFE was measured by the deoxyribose method.[24] The absorbance of the resulting solution was measured spectrophotometrically at 532 nm and percentage of inhibition was calculated. The ability to scavenge the super oxide radical ($O_2^{\bullet}$) of AMFE was measured.[25] Superoxide radical generated in PMS-NADH system by oxidation of NADH was assayed by the reduction of NBT at 560 nm. The total antioxidant capacity was assessed based on the ability of a compound to scavenge the stable ABTS radical (decolourisation assay).[26] $ABTS^{+}$ generated by oxidation of ABTS (1.8 mM) with potassium persulfate (2 mM). The reaction mixture contained ABTS (0.002 M), AMFE (50–1,000 μg) and buffer in a total volume of 3.5 ml. The absorbance was measured at 734 nm in a UV-visible spectrophotometer. IC_{50} value in the concentration of sample required scavenging 50% ABTS free radical.

Biochemical analysis of plasma and cardiac tissue. The heart was immediately washed with ice cold 0.9% saline and homogenate was prepared in 0.1 N Tris HCl buffer (pH 7.4). The homogenate was centrifuged and the clear supernatant was collected for the assay. The Serum and cardiac tissue levels of AST and ALT were assayed,[27] LDH enzyme activity was estimated by the method described previously[28] and CK-MB enzyme activity was determined as described.[29] Thiobarbituric acid reactive substances (TBARS) were determined by measuring the formation of malondialdehyde.[30] The plasma vitamin C level was estimated and the values are expressed as mg/dl.[31]

Cardiac antioxidant status. Superoxide dismutase (SOD) activity was determined.[32] A single unite of enzyme was expressed as 50% inhibition of NBT (nitro blue tetrazolium) reduction/min/mg protein while glutathione peroxidase (GPx) was assayed.[33] GPx activity was expressed μmol GSH oxidized/min/mg protein. Glutathione-S-transferase (GST) activity was measured as described previously.[34] GST activity expressed as μmoL CDNB utilized/min/mg protein. Total reduced glutathione (GSH) content was measured by the method described.[35] Catalase (CAT) was assayed as described.[36] The CAT activity was expressed as nmoL H_2O_2 decreased/mg protein/min.

Histopathological examination. The hearts were fixed in 10% formalin solution and embedded in paraffin. The fixed tissues were processed routinely, and were then embedded in paraffin, sections measuring 4 μm thickness were made and deparaffinized and rehydrated using standard techniques. The sections stained with hematoxylin and eosin (H&E) and observed microscopically. The severity and extent of cardiac damage were noted for each specimen.

Statistical analysis. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Results were expressed as mean ± SEM of six rats in each group. p values <0.05 was considered significant.

Results

Fig. 1 shows the percentage scavenging action of AMFE on free radical generation. AMFE scavenges DPPH radicals in a dose-dependent manner (50–1,000 μg). a) DPPH radical radical scavenging, b) $O_2^{\bullet}$, c) $NO^{\bullet}$, d) $OH^{\bullet}$ and e) $ABTS^{+}$ radical scavenging activities were followed on dose-dependent (50–1,000 μg). At lower doses there was poor effect has been traced. However, at higher dose AMFE showed significant free radical scavenging property.

Table 1 depicts the levels of plasma marker enzymes such as AST, ALT, LDH and CK-MB in control and different experimental groups. In ISO administered rats the levels of these marker enzymes were increased significantly compared with control rats. The rats pretreated with AMFE showed significant decrease in the same when compared to ISO administered rats.

The cardiac marker enzymes *viz.* AST, ALT, LDH and CK-MB of control and different experimental groups is presented in Table 2. In ISO administered group their levels were significantly decreased compared to control. Rats pretreated with AMFE showed significant increase and maintained to near normal levels compared to ISO alone administered rats.

As reported in Table 3, there was significant decrease in GSH, GPx, and SOD levels in the heart tissue of ISO administered rats, compared to normal rats. Pretreatment with AMFE resulted in marked improvement in these indices at the end of the experiment and were reverted back to normal level, compared to ISO-administered rats. In addition, the significantly increased activity of CAT observed in rats pretreated with AMFE, compared to ISO-administered rats where as CAT activity is decreased significantly in ISO administered rats compared to control.

The vitamin C levels in plasma of control and experimental animals were showed in Fig. 2(a). The levels of vitamin C decreased partially in ISO administered rats, compared to control rats, AMFE pretreatment brought vitamin C levels to normal in ISO treated rats. Fig. 2 shows the levels of TBARS in plasma (b) and cardiac tissue (c) of control and experimental animals. The TBARS levels were significantly higher in ISO administered rats compared to controls where as in AMFE pretreated rats the same was decreased significantly when compared to ISO alone administered rats.

Histological changes of heart tissue sections of all groups of rats were carried out by light microscopy study and showed in Fig. 3 (group I–IV). Cardiac sections of the ISO alone administered rat showed edema, focal hemorrhage and leukocyte infiltration with fragmentation of muscle fibers suggesting of necrosis (group II). The AMFE pretreated rat heart showed a normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils when compared to ISO administered group heart sections (group III). The AMFE treated rats showed a well-preserved normal morphology of cardiac muscle with no evidence of focal necrosis when compared to the ISO administered rat heart (group IV). Hence, from the histological evidence it is clear that

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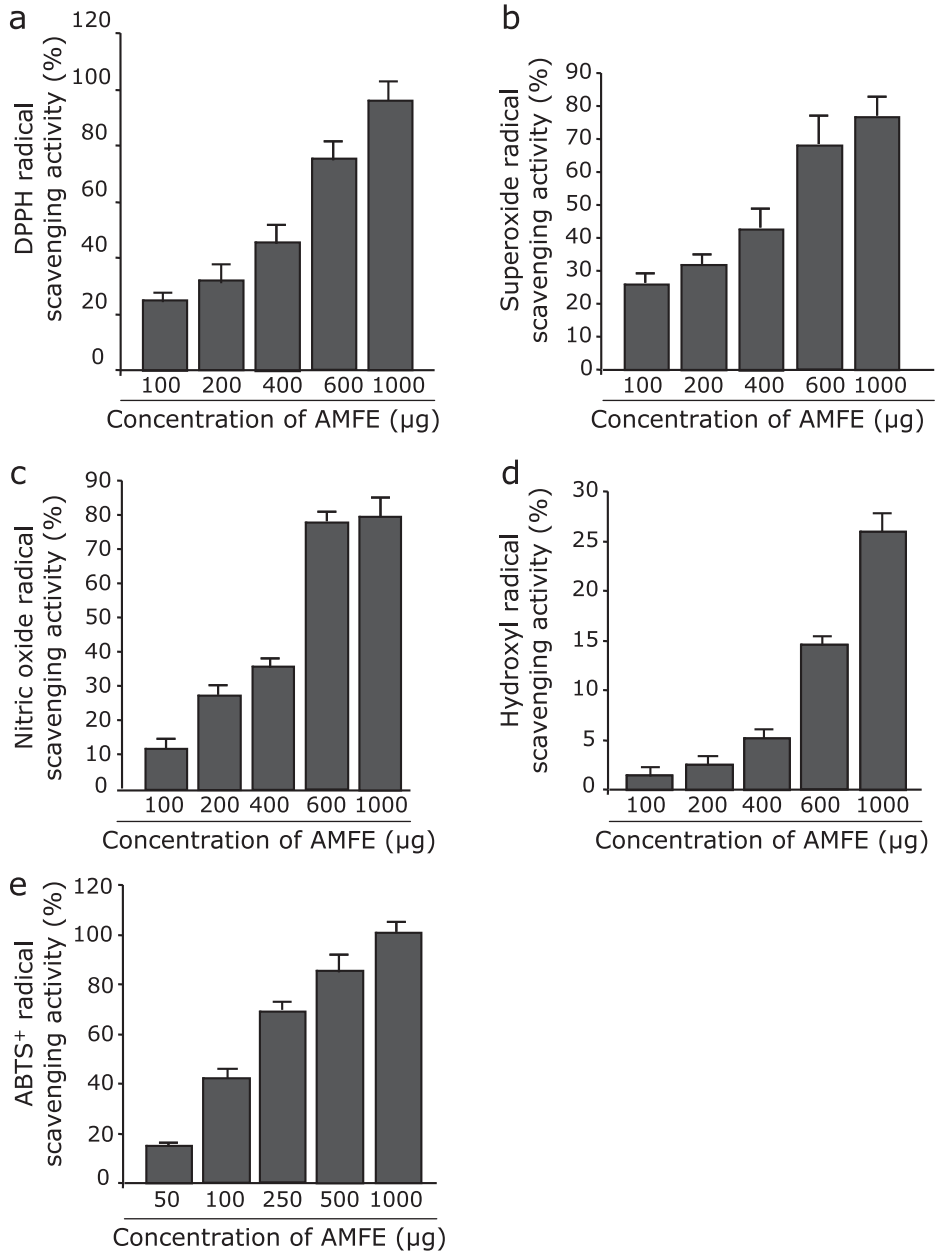

Fig. 1. Effect of various concentrations of AMFE on (a) scavenging of 1,1-diphenyl-2-picryl hydrazyl (DPPH) radicals, (b) super oxide radical scavenging activity, (c) nitric oxide (NO) radicals, (d) hydroxyl radical scavenging activity, (e) $ABTS^{+}$ radical scavenging activity. The values are expressed as % scavenging activity. Values are mean ± SEM of three individual experiments.

Table 1. Effect of AMFE on plasma AST, ALT, LDH and CK-MB enzyme activities in ISO treated rats

Parameter	Control	ISO	AMFE	AMFE + ISO
AST	27.6 ± 1.04^{a}	78.6 ± 0.70^{b}	28.3 ± 0.21^{a}	32.7 ± 0.88^{a}
ALT	28 ± 0.79^{a}	43.2 ± 1.01^{b}	28.5 ± 0.76^{a}	28.2 ± 0.72^{a}
LDH	91.8 ± 1.11^{a}	164.1 ± 3.41^{c}	88.8 ± 1.58^{a}	105.5 ± 1.24^{b}
CK-MB	230 ± 4.91^{a}	331.9 ± 3.19^{c}	232.5 ± 3.90^{a}	243.9 ± 3.76^{b}

Values are mean ± SEM of six rats in each group. ^{a,b,c}Means in the same row not sharing a common superscript are significantly different ($p<0.05$) among groups. All values are expressed as IU/L.

Table 2. Effect of AMFE on cardiac tissue AST, ALT, LDH and CK-MB enzyme activities in ISO treated rats

Parameter	Control	ISO	AMFE	AMFE + ISO
AST	48.3 ± 0.78^{a}	33.6 ± 0.65^{b}	48.9 ± 0.84^{a}	49.4 ± 0.74^{a}
ALT	33.7 ± 0.74^{a}	23.1 ± 0.72^{b}	33.4 ± 0.60^{a}	34.9 ± 0.82^{a}
LDH	159.4 ± 1.69^{a}	93.1 ± 0.98^{b}	33.4 ± 0.60^{a}	157.4 ± 2.71^{a}
CK-MB	19.40 ± 0.32^{a}	13.7 ± 0.50^{b}	19.6 ± 0.37^{a}	20.6 ± 0.60^{a}

Values are mean ± SEM of six rats in each group. ^{a,b,c}Means in the same row not sharing a common superscript are significantly different ($p<0.05$) among groups. All values are expressed as IU/L.

Table 3. Effect of AMFE on cardiac tissue antioxidant enzyme levels in ISO treated rats

Parameter	Control	ISO	AMFE	AMFE + ISO
GSH (μmoL/mg protein)	7.40 ± 0.80^{a}	4.15 ± 0.34^{b}	7.45 ± 0.42^{a}	7.37 ± 0.77^{a}
GPx (μmol of GSH consumed/min/mg protein)	3.42 ± 0.17^{a}	1.81 ± 0.13^{b}	3.52 ± 0.22^{a}	3.54 ± 0.16^{a}
GST (μmol of GSH-CDNB conjugate formed/min/mg protein)	18.49 ± 1.99^{a}	9.85 ± 2.24^{b}	18.56 ± 2.19^{a}	18.61 ± 1.20^{a}
SOD (U/mg protein/min)	7.41 ± 0.58^{a}	4.30 ± 0.32^{b}	7.48 ± 0.61^{a}	7.30 ± 0.55^{a}
CAT (H202 decomposed/mg protein/min)	47.23 ± 2.71^{a}	25.96 ± 1.02^{c}	46.13 ± 1.76^{a}	49.33 ± 2.38^{b}

Values are mean ± SEM of six rats in each group. ^{a,b,c}Means in the same row not sharing a common superscript are significantly different ($p<0.05$) among groups.

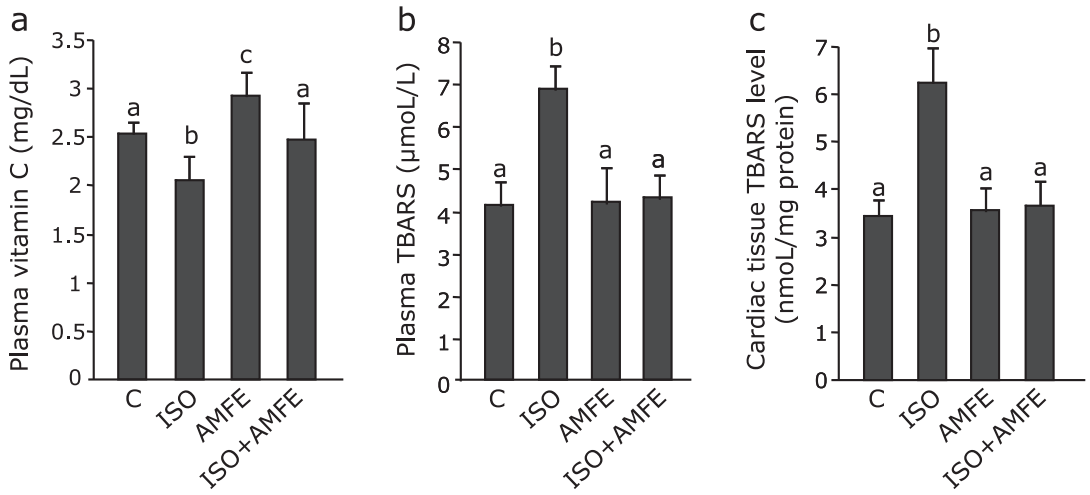

Fig. 2. Effect of AMFE administration on (a) plasma vitamin C, (b) plasma TBARS and (c) cardiac tissue TBARS levels in ISO treated rats. Values are mean ± SEM of six rats in each group. The means sharing a common letter are not significantly different among groups.

AMFE has protective nature against ISO-induced myocardial injury.

Discussion

Therapeutic interventions could improve impaired antioxidant defense mechanisms or diminish free radical production in ischemic myocardium has been of great interest.[37] Generally antioxidants, preferably from natural sources, have been considered as effective treatments.[38,39] Reactive nitrogen species (RNS) are an important mediator of many physiological functions, and its role in the pathogenesis of cardiovascular disease (CVD) is gaining recognition. Higher concentration of NO has been reported to be injurious to the tissue.[40] Our results in the present study showed that AMFE scavenges hydroxyl radical, nitric oxide radical and superoxide radical scavenging activity which are dose dependent. In the present study, AMFE also showed total antioxidant activity on dose dependent manner. The ability to retard lipid oxidation is attributable to the phenolic compounds present in AMFE which can quench ROS and RNS.[41]

The microscopic histopathological observations of heart sections of ISO treated rats showed hemorrhage in myocardium and epicardium as well as demarcated lesions particularly in the apical portion of the heart. In animals ISO develop myocardial infarction like lesions through β-adrenergic receptors, stimulating calcium intracellular influx-increasing cAMP levels and exhausting high-energy phosphates.[42] These lesions are morphologically similar to myofibrillar degeneration, myocardial hypertrophy, myocyte damage and cardiomyopathy including MI.[7] AMFE pretreated rats showed normal myofibrillar structures with striations, branched appearance and continuity with adjacent myofibrils. AMFE effectively prevented the adverse effects caused by ISO treatment and offered protection.

Further analysis of tissue damage marker enzymes in serum namely AST, ALT, LDH and CK-MB showed increased activities following ISO injection. Increase in AST/ALT ratio has clearly

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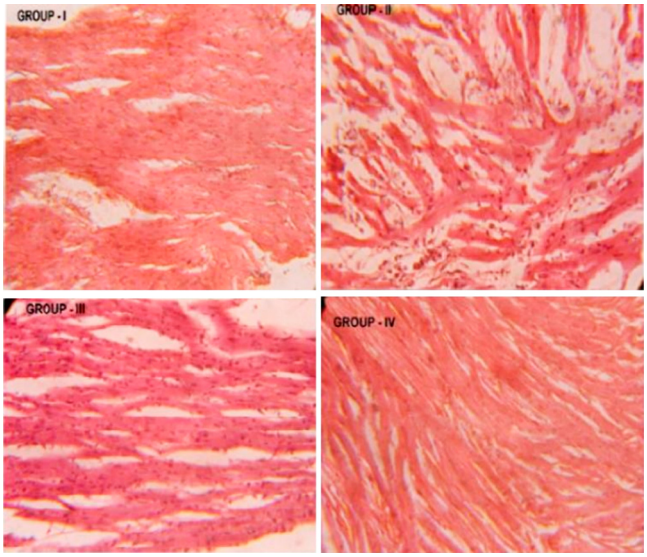

Fig. 3. Representative photomicrograph shows normal architecture of heart of the control group. Endocardium and pericardium are seen within normal limits with no infiltration of inflammatory cells (group I). Photomicrograph of a rat heart subjected to ISO induced focal loss of myofibers, myonecrosis, marked edema, and infiltration of chronic inflammatory cells. In subendocardium vacuolar changes and prominent edema along with chronic inflammatory cells are clearly visible (group II). Photomicrograph of the heart of an AMFE treated rats showed prevention of myonecrosis as evidenced by significant reduction in focal loss of myofibers and infiltration of inflammatory cells. Degree of edema also was markedly reduced with AMFE treatment (group IV). AMFE alone administered rat cardiomyocytes showed control rat signs (group III), original magnification, ×100.

showed damage and confirmed on set of MI. When myocardial cells are damaged, the cell membrane becomes permeable, which results in the leakage of these products. This might be the reason for increased activities of cardiac marker enzymes in serum and cardiac tissue of ISO-administered rats. Pretreatment with AMFE lowered the elevated activities of these enzymes in serum and maintained near to normal activities in cardiac tissue comparable to the ISO alone administered rats. This is an indication of the protective action of AMFE in reversing cardiac cell damage and membrane permeability due to ISO.

Increased plasma levels of ascorbic acid in AMFE treated rats, indicated its beneficial effect in the prevention of MI. Ascorbic acid is a biologically active reductant, an excellent hydrophilic antioxidant in plasma and disappear faster than other antioxidants when plasma is exposed to ROS.[43] The decrease in plasma ascorbic acid in ISO administered rats may be due to increased utilization as an antioxidant defense against increased ROS or due to a decrease in GSH level, since GSH is required for the recycling of ascorbic acid.[44] The rats pretreated with AMFE restored the plasma ascorbic acid level near to those of control group. The restoration of ascorbic acid levels could be due to improvement in endogenous antioxidant levels by free radical scavenging property of AMFE.

Lipid peroxidation is an important pathogenic event that has been linked to altered membrane structure and enzyme inactivation in myocardial infarction.[8] The levels of TBARS were significantly elevated in the plasma and heart tissue of ISO-administered rats. The elevated levels of TBARS observed in ISO administered rats might be due to comparatively low levels of antioxidant enzymes and increased oxidative stress.[45] The rats pretreated with AMFE showed reduced levels of lipid peroxidation when compared to ISO administered rats. Polyphenols present in AMFE might be responsible for antilipoperoxidative effect. Antioxidants are substances which can prevent or retard the oxidation of biomolecules such as lipids, proteins and DNA.[46] Our results showed lower levels of reduced glutathione content and a decrease in the activities of CAT, SOD, GST and GPx in the heart tissue of ISO administered rats as compared to control group. It could be due to the increased generation of ROS such as superoxide and hydrogen peroxide, which in turn leads to the inhibition of activities of these enzymes. The rats pretreated with AMFE showed an increase in GSH and significant elevation in the activities of all the above antioxidant enzymes when compared to ISO alone administered group. The data obtained in the present study indicated the protective nature of the AMFE against oxidative stress induced injury to cardiac tissue. Our lab reports also confirmed that besides AMFE antioxidant activity it showed hypolipidemic activity.[47] The increase in the levels of these enzymes might be due to the supply of exogenous antioxidants in the form of AMFE. The antioxidant properties of plant extracts have been attributed to their phenolic compound contents. Several epidemiological studies have been clearly indicated the beneficial effects of phenolic compounds as natural antioxidants.[48] The multi hydroxyl groups in the chemical structure of polyphenols make them ideal for free radical-scavenging reactions and as metal chelating agents.

In conclusion, the present study clearly demonstrated the antioxidant activity and membrane stabilizing property of phenolic compounds present in AMFE could ameliorate the occurrence of heart related diseases which is supported by *in vitro* assays and histopathological observations. The mechanism of action of the phenolic compounds present in AMFE to attenuate cardiac tissue damage needs further in-depth study.

Conflicts of Interest

None.

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Abbreviations

ALT	alanine transferase
AMFE	*Aegle marmelos* fruit extract
ANOVA	analysis of variance
AST	aspartate transferase
CAT	catalase
CK-MB	creatinine kinase
CVD	cardiovascular disease
DMRT	Duncan's multiple range test
DPPH	1,1-diphenyl-2-picryl hydrazyl
GPx	glutathione peroxidase
GSH	reduced glutathione
GST	glutathione-S-transferase
IHD	ischemic heart diseases
ISO	isoproterenol
LDH	lactate dehydrogenase
MI	myocardial infarction
NBT	nitro blue tetrazolium
NO	nitric oxide
$O_2^{\bullet}$	super oxide radical
$OH^{\bullet}$	hydroxyl radical
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances

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