Pharmacodynamics of Ellagic Acid on Cardiac Troponin-T, Lyosomal Enzymes and Membrane Bound ATPases: Mechanistic Clues from Biochemical, Cytokine and *In vitro* Studies

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Abstract

The anti lipid peroxidative and antioxidant effects of ellagic acid against isoproterenol-induced myocardial infarcted rats were reported previously. This study was designed to evaluate the protective effects of ellagic acid on the levels of cardiac troponin-T, lysosomal enzymes, and membrane bound ATPases along with the role of pro inflammatory cytokine. Male albino Wistar rats were pretreated with ellagic acid (7.5 mg/kg and 15 mg/kg body weight) daily for a period of 10 days. After the treatment period isoproterenol (100 mg/kg) was subcutaneously injected to rats twice at an interval of 24 h. The protective effects of pretreatment with ellagic acid were measured by biochemical analysis and reverse transcriptase polymerase chain reaction. Evidence of myocardial infarction in isoproterenol induced rats including significant increase in the serum level of cardiac troponin-T and decreased levels of creatine kinase and lactate dehydrogenase in heart tissue homogenate were observed. The pretreatment with ellagic acid restored the levels of cardiac markers in the serum and heart tissue homogenates. The activities of lysosomal enzymes (β-D-glucuronidase, β-N-acetyl glucosaminidase, β-galactosidase, cathepsin-D and acid phosphatase) were increased significantly in the serum and heart tissue of isoproterenol-induced rats. The activity of Na^+/K^+ATPase declined while the activities of Ca^{2+}ATPase and Mg^{2+}ATPase were increased significantly in the heart of isoproterenol-induced rats. Pretreatment with ellagic acid restored the activities of lysosomal enzymes and membrane bound ATPases. The higher expressions of pro-inflammatory cytokines such as interleukin-1β, interleukin-6 and tumour necrosis factor-α in the isoproterenol-induced rats were controlled by the pretreatment with ellagic acid. Our results imply that oral pretreatment with ellagic acid protects the heart lysosomal membrane against isoproterenol-induced cardiac damage. The observed effects might be due to the free radical scavenging, membrane stabilizing and anti-inflammatory properties of ellagic acid.

Key words: *Troponin-T, Lysosomal Enzymes, ATPases, Pro-inflammatory Cytokines*

1. Introduction

Worldwide, cardiovascular disease is estimated to be the leading cause of death and loss of disability-adjusted life years [1]. Although remarkable developments have been made in the treatment of cardiovascular disease, myocardial infarction (MI) remains an important health problem and receives ongoing attention from basic and clinical researchers and practising physicians. MI describes the process of myocardial cell death due to ischaemia, or perfusion imbalance between supply and demand within the coronary arteries.

Considerable attention has been focused on the role of lysosomal alterations in ischaemic cardiac damage. Lysosomal enzymes play an important role in the inflammatory process during MI. The intracellular release of lysosomal enzymes following myocardial ischaemia may directly, or through activation of complement pathways, result in cell injury and death [2]. There is a possibility of myocardial cell membranes stabilizing, particularly the lysosomal membranes, which improves the viability of ischaemic cardiac muscle and prevents MI [3].

ATPases of cardiac cells play an important role in the maintenance of normal ion levels within the heart and play a significant role in contraction and relaxation cycles of cardiac muscle [4]. They act as key messengers in signal transduction pathways in the heart and hence the regulation of cardiac contractility and hypertrophy. They offer potentially exciting opportunities as new therapeutic targets for heart failure [5].

The role of pro inflammatory cytokines in heart failure is an emerging area in cardiovascular research. Current therapeutic strategies that improve survival in heart failure patients have been shown to favourably alter both cytokine profile and interstitial fibrosis [6]. Therefore, we hypothesized that a therapeutic strategy which minimizes inflammatory cytokine releases is a possible innovative approach to the prevention of MI.

Isoproterenol (ISO), a sympathomimetic β-adrenergic receptor agonist, administered in high doses in animals, may deplete the energy reserve of cardiomyocytes and may thus result in biochemical and structural changes which are responsible for the development of irreversible damage of myocardium in experimental animals. The metabolic and morphologic abnormalities produced by ISO in the heart tissue of experimental animals are similar to those observed in human MI [7] and is widely used in preclinical research.

In recent years, significant emphasis is being placed on identifying cardio protective phyto-nutrients. Ellagic acid (EA) 4,4',5,5',6,6'-hexahydroxydiphenic acid 2,6,2',6'-dilactone is a naturally occurring polyphenol compound found in blackberry, strawberry, raspberry, bayberry, pineapple and pomegranate [8]. It is reported to have important biological properties such as anti-inflammatory, hepato protective and chemopreventive activities [9-11]. A recent study from our laboratory showed ellagic acid exhibit cardioprotective activity against experimentally induced MI [12]. In continuation of our research on the effect of ellegic acid against ISO-induced MI, we studied the role of ellagic acid on cardiac troponin-T, lysosomal enzymes, and membrane bound ATPases along with the role of pro inflammatory cytokine. We propose potential phyto-pharmaceutical strategies using EA to depress the deleterious effect of free radical induced oxidative stress during and immediately after acute coronary events. Our study reports the possible underlying mechanism of action of EA by its reducing potential and free radical scavenging effect.

2. Materials and methods

2.1. Experimental animals

All the experiments were carried out with male albino Wistar rats (*Rattus norvegicus*) weighing 180-200 g, purchased from Mahaveer Enterprises, Hyderabad, India. They were housed in polypropylene cages (47 × 34 × 20 cm) lined with husk, renewed every 24 h under a 12 h light/dark cycle at around 22 °C with 50% humidity. The rats had free access to water and food. The rats were fed on a standard pellet diet (Pranav Agro Industries Ltd., Pune, Maharashtra, India). The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Institutional Animal Ethical Committee of Jayamukhi College of Pharmacy (Approval No. 022; 28.2.2010) **[12].**

2.2. Drug and chemicals

Ellagic acid, Isoproterenol hydrochloride, p-nitrophenyl-N-acetyl-β-D-glucosaminide, and p-nitrophenyl-β-D-glucuronide were purchased from Sigma Chemical Co., St. Louis, MO, USA. p-Nitrophenyl β–D-N-acetyl glucosaminide, p-nitrophenol and tyrosine were purchased from Himedia Laboratories Private Limited, Mumbai, India. PCR master mix, DNA isolation kit, mRNA purification kit, oligo(dt), DNAse and lambda DNA/Hind III were purchased from Medox Biotech India Private limited, Chennai, India. Primers for reverse transcriptase polymerase chain reactions (RT-PCR) were purchased from Ocimum Bio solutions Limited, Hyderabad, India. All the other chemicals used were of an analytical grade.

2.3. Induction of experimental myocardial infarction

Isoproterenol (100 mg/kg body weight) was dissolved in saline and injected subcutaneously to rats at an interval of 24 h for 2 days [4].

2.4. Experimental design

We have used identical experimental animal in the previously published paper [12] and this paper. The animals were divided into six groups of six rats each. Group I: normal control rats were given 2 ml of saline orally by gastric intubation daily for a period of 10 days; Group II: normal rats were treated with EA (7.5 mg/kg) dissolved in 2 ml of saline orally by gastric intubation daily for a period of 10 days; Group III: normal rats were treated with EA (15 mg/kg) dissolved in 2 ml of saline orally by gastric intubation daily for a period of 10 days; Group IV: normal control rats were given 2 ml of saline orally by gastric intubation daily for a period of 10 days and then subcutaneously injected with ISO (100 mg/kg) in 2 ml of saline at an interval of 24 h for 2 days (on 11^{th} and 12^{th} day); Group V: rats were pretreated with EA (7.5 mg/kg) in 2 ml of saline orally by gastric intubation daily for a period of 10 days and then subcutaneously injected with ISO (100 mg/kg) at an interval of 24 h for 2 days (on 11^{th} and 12^{th} day); Group VI: rats were pretreated with EA (15 mg/kg) in 2 ml of saline orally by gastric intubation daily for a period of 10 days and then subcutaneously injected with ISO (100 mg/kg) once a day for 2 days (on 11^{th} and 12^{th} day). Twenty four hours after the second dose of ISO, the rats of all the groups were anesthetized and sacrificed by cervical decapitation. Blood was collected and the serum separated by centrifugation. The heart tissue sample was cut open and placed in isotonic saline to remove the blood. Then the heart tissue was rinsed in ice cold 0.25 M sucrose, blotted, weighed and minced. The enzyme extracts were prepared by homogenizing the tissue samples in 0.25 M sucrose at 4 °C. A portion of this preparation was used to determine the total activities of

enzymes. Another portion of the homogenate was subjected to differential centrifugation, and the lysosomal fractions were separated by the method of Wattiaux et al. [13]. Fresh heart tissue was homogenized in 0.25 M sucrose solution. The homogenate was filtered and centrifuged at 3000 ×g for 10 min in a refrigerated centrifuge. The pellet was removed and homogenized again and suspended as before. The supernatants were combined and centrifuged again at 15,000 ×g for 20 min. The lysosomal pellet was suspended in 1.15% potassium chloride and used for the assay of enzymes.

2.5. Assay of cardiac marker enzymes

The level of serum cardiac troponin-T (cTn-T) was estimated by chemiluminescence immunoassay, using the standard kit purchased from Roche Diagnostics, Switzerland. The activity of creatine kinase (CK) in the heart tissue homogenate was assayed by the method of Okinaka et al. [14]. The activity of the lactate dehydrogenase (LDH) was assayed in the heart tissue homogenate by the method of King [15].

2.6. Assay of lysosomal hydrolases

The activity of β-D-N-acetyl-glucosaminidase was assayed in the serum and heart tissue homogenate by the method of Moore and Morris [16]. The activity of β- D-galactosidase was assayed in the serum and heart tissue homogenate by the method of Conchie et al. [17]. The activity of acid phosphatase was assayed in the serum and heart homogenate by the method of King [18]. The activity of β-glucuronidase was assayed in the serum, heart homogenate, and lysosomal fractions of the heart tissue by the method of Kawai

and Anno [19]. The activity of cathepsin-D was determined in the serum, heart homogenate, and lysosomal fractions of the heart tissue by the method of Sapolsky et al. [20].

2.7. Assay of membrane-bound ATPases

The activity of sodium/potassium-dependent ATPase (Na^+/K^+ATPase) was assayed by the method Bonting [21]. The activity of calcium- dependent ATPase (Ca^{2+}ATPase) was assayed by the method of Hjertén and Pan [22]. The activity of magnesium-dependent ATPase (Mg^{2+}ATPase) was assayed in the homogenate of heart tissue by the method of Ohnishi et al. [23].

2.8. RNA Isolation and RT-PCR

Total cellular ribonucleic acid (RNA) was extracted from the heart tissue by Easy spin column kit as per the instructions given by the manufacturer. (Medox Biotech India Private Limited, Chennai, India). The isolated RNA was treated with RNase-free DNase I at 37 °C for 30 min for the removal of DNA. Samples were incubated at 30 °C for 60 min. Reactions were stopped by heating at 95 °C for 10 min.

The cytokine mRNAs, interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) along with mRNA for the cellular enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control were studied by reverse transcriptase polymerase chain reaction study. Samples were incubated at 30 °C for 60 min. Reactions were stopped by heating at 95 °C for 10 min. Reverse transcription was carried out using 2 μg of the total RNA and 200 units of M-MuLV reverse transcriptase. Amplification was performed by the Medox PCR Master Mix in a volume of 25 μl. PCR was carried out,

using a hot start method by adding 2 µl of cDNA product to 18 µl of PCR buffer containing 67 mM Tris (pH8.8), 1.5 mM magnesium chloride, 16.6 mM ammonium sulphate, mixed deoxynucleoside triphosphates (dNTPs) at 200 µM, 125 U ml^{-1} Taq polymerase (Gibco/BRL), and 0.3 µM of sense and anti-sense primers. The primers were used as rat sense and anti-sense primers, respectively. IL-1β: 5′-ATG GCA ACT GTC CCT GAA CTC AAC T-3′; and 5′-CAG GAC AGG TAT AGA TTC AAC CCC TT-3′; IL-6 : 5′-CCA GTT GCC TTC TTG GGA CTG ATG-3′; and 5′-ATT TTC TGA CCA CAG TGA GGA ATG-3′; TNF-α :5′-ATG AGC ACG GAA AGC ATG ATC CGA-3′; and 5′-CCA AAG TAG ACC TGC CCG GAC TC-3′; GADPH :5′ TTC TTG TGC AGT GCC AGC CTC GTC-3′and 5′ TAG GAA CAC GGA AGG CCA TGC CAG -3′. The RT-PCR products were electrophoresed on a 3% agarose gel and visualized by staining with ethidium bromide.

2.9. In vitro analysis

Total reducing power of EA was determined according to the method of Oyaizu [24]. The antioxidant activity of the EA was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity by the method of Braca et al. [25].

2.10. Statistical analysis

Results are expressed as mean ± S.D. One way analysis of variance (ANOVA) was carried out, and the statistical comparisons among the groups were performed with Duncan's multiple range test (DMRT) using a statistical package for the social science (SPSS) software version 16.00. Throughout the report, wherever we have used the word “significant” to

describe results, we mean “statistically significant at an alpha level of 0.05 (p<0.05) for the two-sided alternative hypothesis.

3. Results

The effect of EA on the levels of cardfiac markers in the normal and ISO-induced myocardial infarcted rats was measured (Table 1). In the ISO-induced rats, the level of cTn-T significantly increased in the serum. Oral pretreatment with EA (7.5 mg/kg and 15 mg/kg) significantly decreased the level of cTn-T in the serum of the ISO-induced rats. CK and LDH are among the classical biochemical markers of myocardial damage and are closely correlated with mortality. We studied the activities of these enzymes in the cardiac tissue homogenate of normal and experimental rats. In the ISO-induced rats, the activities of CK and LDH significantly declined in the heart tissue. Oral pretreatment with EA (7.5 mg/kg and 15 mg/kg) significantly increased the activity of CK and LDH in the heart tissue of the ISO-induced rats.

Table 2 and 3 show the activities of lysosomal enzymes in the serum and heart of normal and experimental rats respectively. Our results showed significantly increased activity of β-D-N-acetyl glucosaminidase, β-D-glucuronidase, β-galactosidase, cathepsin-D, β-glucosidase and acid phosphatase in the serum and heart tissue homogenate of the rats induced with ISO, compared to the normal control rats. Oral pretreatment with EA (7.5 mg/kg and 15 mg/kg) significantly decreased the activities of these enzymes in the serum and heart tissue homogenate, compared to those of the ISO-induced rats.

The activities of β-D glucuronidase and cathepsin-D were studied in the lysosomal fractions of normal and experimental rats. The activities of these enzymes significantly declined in the lysosomal fractions of the heart, compared to the normal control rats. Oral

pretreatment with EA (7.5 mg/kg and 15 mg/kg) showed a significant increase in the activities of these enzymes in the lysosomal fractions of the heart, compared to those of the ISO-induced rats (Fig. 1).

A growing body of evidence on membrane bound ATPases in the myocardium redirected our attention from their limited role as ion transporters to a more dominant role in cellular signaling pathways. Thus, we studied the activities of Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in the heart tissue homogenate. The activity of Na^+/K^+ATPase significantly decreased and the activities of Ca^{2+}ATPase and Mg^{2+}ATPase significantly increased in the heart of the ISO-induced rats, compared to the normal control rats. Oral pretreatment with EA (7.5 mg/kg and 15 mg/kg) for a period of 10 days significantly increased the activity of Na^+/K^+ATPase and significantly decreased the activities of Ca^{2+}ATPase and Mg^{2+}ATPase in the heart MI induced rats, compared to those of the ISO-induced myocardial infarcted rats (Fig. 2).

Fig. 3 shows the expression of RT-PCR products of proinflammatory cytokines (IL-1β, IL-6 and TNF-α,) in the normal and experimental rats. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as the normalization control. The higher expressions of IL-1β, IL-6 and TNF-α in the ISO-induced rats demonstrate its positive correlation with MI. The oral pretreatment of EA reduced the expression of the proinflammatory markers, explaining its involvement in the reduction of the inflammatory process.

For all the biochemical parameters studied, EA at a dose of 15 mg/kg showed a higher significant effect than the lower dose (7.5mg/kg), and the oral administration of EA (7.5 mg/kg and 15mg/kg) for a period of 10 days in normal control rats had no appreciable effect.

The reducing power of EA at various concentrations (10, 20, 30, 40 and 50 μM) was studied and ascorbic acid served as standard. The reducing power of EA increases with increasing concentration. The reducing power of EA at different concentrations (10, 20, 30,

40 and 50 µM) was found to be 18.51, 37.84, 61.22, 79.31 and 85.54% respectively. The reducing ability of EA is more than the standard compound ascorbic acid in all concentrations.

The free radical scavenging activity of EA on DPPH• free radicals was observed. EA scavenges free radicals dose dependently. The percentage scavenging effects of EA on DPPH• at different concentrations (10, 20, 30, 40 and 50µM) were found to be 13.56, 23.8, 50.84, 68.65 and 87.43% respectively. The EC_{50} value of EA is 28.5 µM indicates the stronger ability of the EA to act as DPPH scavenger in lower doses.

4. Discussion

In our previous study, we evaluated the cardioprotective effect of EA on electrocardiographical changes, blood pressure, serum cardiac troponin-I, serum cardiac troponin, serum lactate dehydrogenase, C-reactive protein, plasma homocystein, lipid peroxidation, antioxidant system and histopathology of heart in ISO-induced myocardial infarction [12].

The excess metabolites produced by a supramaximal dose of ISO in phase-I (hydroxylation) and phase II (methylation, glucuronidation and sulphation) metabolism can cause oxidative stress by increased production of superoxide free radical and hydroxyl free radicals followed by myocardial necrosis. The damage to cardiomyocyte by excessive free radicals produced in ISO metabolism affects its contractility and results in cardiac failure.

Cardiac troponins are regulatory proteins that control the calcium-mediated interaction of actin and myosin, which results in contraction and relaxation of striated muscle. An elevated troponin level predicts the risk of both cardiac death and subsequent infarction. The release of cTn-T after MI has been correlated with the severity of infarction in

experimental animal models and it can be used for the early detection of cardiotoxic and/or cardiodegenerative effects in animals [26, 27]. In this study, increased cTn-T levels were observed in the serum of ISO-induced myocardial infarcted rats. Cytotoxic free radicals produced by ISO may involve damage to myocytes and the release of cTn-T into the serum. Oral pretreatment with EA significantly reduced the level of cTn-T in the serum of ISO-induced myocardial infarcted rats, demonstrating its capability to control the free radical attack and protect the myocardium from ischaemia and necrosis.

In this study we observed the beneficial effect of EA by studying the level of CK and LDH in cardiac tissue. CK and LDH are the diagnostic markers generally used to determine cardiac damage. The levels of marker enzymes in the heart tissue homogenates decreased significantly in ISO-induced rats, compared to normal control rats. This was due to the enzymes leaking out of the damaged tissues when the cell membrane became permeable or was ruptured during ISO-induced necrotic damage of the myocardial membrane [28]. Oral pretreatment with EA near normalized the levels of these enzymes in the heart tissue of the ISO-induced rats, suggesting its ability to maintain membrane integrity, thereby restricting the outflow of these enzymes from the heart tissue into the circulatory system of myocardial infarcted rats. EA contains four hydroxyl groups and two lactone groups. The former are known to increase antioxidant activity in lipid peroxidation and protect cells from oxidative damage [29]. EA inhibits generation of superoxide and hydroxyl free radicals in both enzymatic and nonenzymatic systems by means of its metal-chelating property, thus providing protection against lipid peroxidation [30].

Lysosomes, acidic vacuolar organelles possessing several dozen hydrolytic enzymes, perhaps play the most important role in the degradation of damaged cellular components [31, 32]. The stability and integrity of lysosomal membrane is vital to maintain normal levels of lysosomal enzymes in tissues and body fluids [4]. Brunk et al. [33, 34] established that the

extent of lysosomal damage determines the cells' fate. Limited lysosomal release results in cell death by apoptosis, while massive lysosomal breakdown leads to necrosis. We observed increased activities of lysosomal enzymes in the serum and heart tissue homogenates of ISO-induced rats. The toxic free radicals produced by a supraphysiologic dose of ISO acted on cell membranes, possibly causing alterations in the lysosomal structure. Oxidative stress exceeding a certain limit causes lysosomal rupture due to intralysosomal iron-catalyzed reactions [35]. Release of hydrolytic enzymes from lysosomes after coronary occlusion may be a causative factor for the development of MI [36]. We observed significantly decreased levels of β-D-glucuronidase and cathepsin-D in the lysosomal fractions of the ISO-induced myocardial infarcted rats, compared to normal control rats. The decreased activities of these enzymes in the lysosomal fractions confirmed the abnormal release of lysosomal enzymes involved in myocardial cell death. Lysosomal destabilization may be prevented either by inhibition of cellular peroxidation or prevention of iron catalyzed oxidative reactions, which involve peroxidation of cellular membranes, energy depletion and leakage of lysosomal content [37]. Oral pretreatment with EA decreased the activities of lysosomal enzymes (β-glucuronidase, β-N-acetyl glucosaminidase, β-galactosidase, cathepsin-D and acid phosphatase) in the serum and heart tissue homogenate of ISO-induced rats and increased the activities of β-glucuronidase and cathepsin-D enzymes in the lysosomal fraction by inhibiting the release of these enzymes and enhancing the stability of the lysosomes. Gil et al., [38] suggest that phenolic compounds can act as free radical scavengers by virtue of their hydrogen donating ability, forming phenoxy radicals that can rearrange to form quinone methide radical intermediate, which is excreted via the bile. The free radical scavenging ability of EA may protect cell membranes and reduce lysosomal destabilization and leakage of lysosomal content.

Membrane proteins are in general regarded as extremely potent drug targets due to their role as transporters and mediators in the interaction of cells with the surrounding environment, between cells, and between cellular compartments. Maintenance of the proper gradients for essential ions across cellular membranes makes P-type ATPases crucial for cell survival [39]. Na^+/K^+ATPase, an extensively studied member of the P-type ATPase family, is involved in the regulation of cell volume, development of membrane potential and transport of nutrients in animal tissues [40]. The Na^+/Ca^{2+}-exchanger removes cytosolic Ca^{2+} rapidly in cardiomyocytes [41]. Inappropriate regulation of Na^+/K^+ATPase activity, which leads to excessive loss of K^+ from cells and the gain of Na^+ by cells can result in disturbances in Ca^{2+} signalling and contractility [42]. Significantly decreased activity of Na^+/K^+ATPase was observed in the heart tissue homogenate of ISO-induced myocardial infarcted rats. Our results also showed significantly increased activity of Ca^{2+}ATPase and Mg^{2+}ATPase in the heart tissue homogenates of ISO-induced rats. ISO a non competitive β-adrenergic stimulator that posphorylates cyclic adenosine monophosphate at several sites on the C-terminal chains of the Ca^{2+}ATPase and increases the probability of intracellular Ca^{2+} in myocardial tissue, which subsequently brings about extended relaxation in the heart tissue of ISO-induced rats. Mg^{2+}ATPase is believed to be responsible for the aminophospholid translocase activity of the plasma membrane [43], which could affect the activity of the membrane Ca^{2+}ATPase [44]. The oral pretreatment of EA (7.5mg/kgand 15mg/kg) significantly increased the activity of Na^+/K^+ATPase and decreased the levels of Ca^{2+}ATPase and Mg^{2+}ATPase enzymes in the heart tissue homogenate of ISO-induced rats. The activation of the Na^+/K^+ATPase by EA may trigger cytosolic signalling pathways, which in turn, may result in cardio protection. The decreased activities of Ca^{2+}ATPase and Mg^{2+}ATPase by EA further evidenced its cardioprotective effect.

Pro-inflammatory cytokines, namely IL-1β, IL-6 and TNF-α are elevated in acute myocardial injury and infarction. IL-1β is interesting because its elevated levels are correlated with disease severity [45], and prolonged IL-1β stimulation regulates the adenylyl cyclase cascade in several cell systems. IL-1β reduced cAMP production in response to ISO in human airway smooth muscle cells [46]. The importance of IL-6 in the pathophysiology of coronary artery diseases has been documented in recent studies [47, 48]. TNF-α is a relevant cytokine in the course of the inflammation process in coronary heart disease and systemically affects a number of mediators of the pathological process involved in MI. In fact, cardio myocytes have recently been shown to be a main source of TNF-α production in conditions such as MI and heart failure. It inhibits myocardial contractility, induces apoptosis of cardio myocytes and acts directly on the vascular endothelium as well as on cardio myocytes to increase the adhesion of leukocytes during inflammation [49]. Furthermore, pro-inflammatory cytokines alter cell proliferation, production of the collagen extra-cellular matrix, and generation of mediator substances by the cardiac fibroblast [50]. In chronic heart failure, pro-inflammatory cytokines have been implicated as mediators of pathological cardiac remodelling, with increasing evidence linking these molecules to heart failure progression [51].

We observed higher expressions of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the ISO-induced myocardial infarcted rats. Murray et al. [52] confirm that constant β-adrenergic stimulation is a stimulus for local myocardial expression of IL-1β, IL-6 and TNF-α in experimental rats, and the result of the study supports our findings. Chandrasekar et al. [53] also demonstrate that an abnormal dose of ISO can induce the activation of the nuclear factor kappa B (NF-**κ**B) in a β_2 adrenoceptor-dependent manner, suggesting that the activation of β-adrenoceptors contributes to a pro-inflammatory state of the cardiovascular system. Elevated levels of these cytokines probably contribute to a catabolic environment,

which, over time, may gradually reduce cardiac muscle function [54]. Previous report from our laboratory explained that the excessive dose of ISO increased the C-reactive protein level in the systemic circulation which indicates the involvement of inflammatory process in ISO-induced myocardial damage [12]. Oral pretreatment of EA decreases the expression of these cytokines in the ISO-induced myocardial infarcted rats. In recent years reports of natural compounds in its anti-inflammatory activity have emerged. The polyphenolic phytonutrients act through the modulation of pro-inflammatory gene expressions, such as cyclooxygenase, lipoxygenase, nitric oxide synthases and several pivotal cytokines, mainly by acting through the NF-**κ**B and mitogen-activated protein kinase signaling [55]. Principally, polyphenols exhibit inhibitory action against the inducible nitric oxide synthase, strengthen cellular protection against oxidative stress, reduce the DNA damage associated with NO [56] and inhibit pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α [57, 58]. Research conducted by Rogerio et al. [59] suggests that EA may be involved in the inhibition of the cyclooxygenase enzyme. Pretreatment with EA significantly decreased the C-reactive protein level in ISO-induced MI was reported earlier from our laboratory [12]. The present study confirmed that pretreatment of EA results in a significant decrease in the expression of pro-inflammatory cytokines in the heart tissue of ISO-induced rats. The anti-inflammatory activity of EA may be the possible mechanism of action for reduced pro-inflammatory cytokines in ISO-induced rats.

Interestingly, the increased expression of pro-inflammatory cytokines in the ISO-induced myocardial infarcted rats parallels the increased leakage of lysosomal enzymes altered membrane bound ATPase and increased levels of cTn-T in the ISO-induced rats. The protective effect of EA also showed a positive correlation with all the parameters. The oral pretreatment with EA significantly decreased the expression of proinflammatory cytokines, reduced the activity of lysososmal enzymes in the serum and heart tissue, increased activity

of lysososmal enzymes in lysosomal fraction, maintained membrane bound ATPases, reduced the level of cTn-T in the serum and increased the levels of CK and LDH in the heart tissue homogenate.

The protective action of EA may be due to its antioxidant effect. The chemical structure of EA contains four hydroxyl groups on the benzene moiety that may enhance the free radical scavenging effect. This could be one of the reasons for its membrane stabilizing and the cardioprotective effects. For the quantitative investigation of hydrogen-radical donation, stable radicals have the advantage that their concentrations are readily and directly measurable. The *in vitro* studies on EA have shown its reducing power and DPPH free radical scavenging activity. Since the reducing capacity of a compound and free radical scavenging activities serve as a significant indicator of its potential antioxidant ability, EA can be considered a potent source of natural antioxidants and the antioxidant mechanism of EA may be the reason for its protective effect against ISO-induced alterations in the cardiac tissue, lysososmal membrane, membrane bound enzymes and proinflammatory cytokines.

In conclusion, our study provided evidence of the cardioprotective effect of EA through its membrane stabilizing effect on lysosomes and membrane bound ATPases. EA attenuates the increase in the level of proinflammatory cytokines after ISO-induced oxidative stress. *In vitro* studies support the theory that the antioxidant capacity of EA may be the reason for the protective activities. Based on the pharmacodynamics of EA, our research suggests that EA may be projected as a possible candidate in the research or therapeutic modulation of oxidative stress related cardiovascular diseases. A greater understanding of the ways in which EA may protect the heart from MI could help to establish effective therapeutic interventions with EA-rich foods.

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Figure Captions

Fig. 1: Effect of ellagic acid on the activities of β-D-glucuronidase and Cathepsin-D in lysosomal fraction of the heart in normal and isoproterenol-induced myocardial infarcted rats. Group I: Normal control rats; Group II: Normal rats + EA (7.5 mg/kg); Group III: Normal rats + EA (15 mg/kg); Group IV: ISO-induced rats (100mg/kg); Group V: EA (7.5 mg/kg) + ISO; Group VI: EA (15 mg/kg) + ISO. Each column is mean ± S.D. for six rats in each group. For β-D-glucuronidase, columns not sharing a common letter (a, b, c, d) differ significantly with each other. For cathepsin-D, columns not sharing a common letter (w, x, y, z) differ significantly with each other. Units*: β-D-glucuronidase: μmol of p-nitrophenol; Cathepsin D: μmol of tyrosine liberated/min/mg protein.

Fig. 2: Effect of ellagic acid on Sodium potassium dependent ATPase, (Na^+/K^+ATPase), Calcium dependent ATPase (Ca^{2+}ATPase) and Magnesium dependent ATPase (Mg^{2+}ATPase) in the heart of normal and isoproterenol-induced myocardial infarcted rats. Group I: Normal control rats; Group II: Normal rats + EA (7.5 mg/kg); Group III: Normal rats + EA (15 mg/kg); Group IV: ISO-induced rats (100mg/kg); Group V: EA (7.5 mg/kg) + ISO; Group VI: EA (15 mg/kg) + ISO. Each column is mean ± S.D. for six rats in each group. For Na^+/K^+ATPase, columns not sharing a common letter (a, b, c, d) differ

significantly with each other. For Ca^{2+}ATPase, columns not sharing a common letter (w, x, y, z) differ significantly with each other. For Mg^{2+}ATPase, columns not sharing a common letter (s, t, u, v) differ significantly with each other. Units*: Na^{+}/K^{+} ATPase: value x 10^{1} µ mol of phosphorous liberated/min/mg protein, Ca^{2+} ATPase and Mg^{+} ATPase: µ mol of phosphorous liberated/min/mg protein.

Fig. 3. Effect of ellagic acid on the RT-PCR products of IL-1β, IL-6 and TNF-α in an ethidium bromide stained agarose gel. GAPDH were used as normalization control.

Group I: Normal control rats; Group II: Normal rats + EA (7.5 mg/kg); Group III: Normal rats + EA (15 mg/kg); Group IV: ISO-induced rats (100mg/kg); Group V: EA (7.5 mg/kg) + ISO; Group VI: EA (15 mg/kg) + ISO.

Groups	Serum Troponin-T (ng/ ml)	Heart tissue Creatine kinase (IU/L)	Heart tissue Lactate dehydrogenase (IU/L)
Normal control	0.49 ± 0.04^{a}	14.17 ± 1.03^{a}	113.99 ± 11.70^{a}
Normal+ ellagic acid (7.5 mg/kg)	0.53 ± 0.03^{a}	14.71 ± 1.35^{a}	115.07 ± 11.62^{a}
Normal+ ellagic acid (15mg/kg)	0.53 ± 0.04^{a}	14.16 ± 1.45^{a}	114.62 ± 11.12^{a}
Isoproterenol-alone (100mg/kg)	1.72 ± 0.13^{b}	6.672 ± 0.61^{b}	81.23 ± 7.36^{b}
Ellagic acid (7.5 mg/kg)+ isoproterenol (100mg/kg)	0.96 ± 0.09^{c}	9.78 ± 0.85^{c}	91.43 ± 8.04^{c}
Ellagic acid (15 mg/kg) + isoproterenol (100mg/kg)	0.66 ± 0.05^{d}	12.24 ± 1.27^{d}	106.07 ± 10.71^{d}

Table 1: Effect of ellagic acid on cardiac markers

each value is mean ± standard deviation for six rats in each group, values not sharing a common superscript (a,b,c,d) differ significantly with each other ($p < 0.05$, duncan's multiple range test).

Table 2: Effect of ellagic acid on lysosomal enzymes in the serum

Groups	β-D-N-acetyl glucosaminidase (µmol of p-nitro phenol liberated/ min/mg protein)	β-D-glucuronidase (µmol of p-nitrophenol liberated/ min/ mg protein)	β-galactosidase (µmol of p-nitrophenol liberated/ min/mg protein)	Cathepsin-D (µmol of tyrosine liberated/h/ 100mg protein)	β-glucosidase (µmol of p-nitrophenol liberated/min/mg protein)	Acid phosphatase (µmol of phenol released/h/ mg protein)
Normal control	12.73 ± 1.19[a]	8.20 ± 0.60[a]	19.26 ± 1.78[a]	10.01 ± 0.73[a]	23.15 ± 2.02[a]	57.64 ± 4.23[a]
Normal+ ellagic acid (7.5 mg/kg)	12.58 ± 1.12[a]	8.17 ± 0.58[a]	18.74 ± 1.82[a]	9.89 ± 0.76[a]	22.93 ± 2.12[a]	56.01 ± 4.28[a]
Normal+ ellagic acid (15mg/kg)	12.47 ± 1.22[a]	8.03 ± 0.65[a]	18.49 ± 1.66[a]	9.17 ± 0.65[a]	22.75 ± 2.02[a]	54.71 ± 4.36[a]
Isoproterenol-alone (100mg/kg)	22.59 ± 1.81[b]	15.73 ± 1.46[b]	28.00 ± 1.98[b]	18.01 ± 1.56[b]	49.37 ± 4.66[b]	85.65 ± 7.82[b]
Ellagic acid (7.5 mg/kg)+ isoproterenol (100mg/kg)	17.66 ± 1.56[c]	12.59 ± 1.06[c]	22.90 ± 2.09[c]	14.39 ± 0.88[c]	34.66 ± 3.35[c]	65.72 ± 6.32[c]
Ellagic acid (15 mg/kg) + isoproterenol (100mg/kg)	13.35 ± 1.11[d]	9.37 ± 0.75[d]	19.85 ± 1.48[a]	11.14 ± 0.77[a,d]	24.55 ± 2.30[a]	56.83 ± 4.86[a]

each value is mean ± standard deviation for six rats in each group, values not sharing a common superscript (a,b,c,d) differ significantly with each other ($p < 0.05$, duncan's multiple range test).

Table 3: Effect of ellagic acid on lysosomal enzymes in the heart

Groups	β-D-N-acetyl glucosaminidase (µmol of p-nitro phenol liberated/ min/mg protein)	β-D-glucuronidase (µmol of p-nitrophenol liberated/ min/ mg protein)	β-galactosidase (µmol of p-nitrophenol liberated/ min/mg protein)	Cathepsin-D (µmol of tyrosine liberated/h/100mg protein)	β-glucosidase (µmol of p-nitrophenol liberated/min/mg protein)	Acid phosphatase (µmol of phenol released/h/ mg protein)
Normal control	33.08 ± 2.84[a]	28.19 ± 2.68[a]	32.89 ± 1.67[a]	18.42 ± 1.85[a]	27.24 ± 2.19[a]	102.68 ± 7.15[a]
Normal+ ellagic acid (7.5 mg/kg)	32.70 ± 2.57[a]	27.28 ± 2.70[a]	32.48 ± 2.54[a]	18.26 ± 1.73[a]	26.41 ± 2.31[a]	102.27 ± 7.32[a]
Normal+ ellagic acid (15mg/kg)	32.65 ± 2.60a	27.14 ± 2.59[a]	32.92 ± 2.06[a]	18.19 ± 1.72[a]	25.79 ± 2.33[a]	100.72 ± 7.39[a]
Isoproterenol-alone (100mg/kg)	47.03 ± 4.17[b]	39.98 ± 3.96[b]	48.26 ± 5.65[b]	28.67 ± 2.49[b]	57.07 ± 4.92[b]	156.92 ± 14.36[b]
Ellagic acid (7.5 mg/kg)+ isoproterenol (100mg/kg)	42.14 ± 4.00[c]	32.02 ± 2.46[c]	37.83 ± 1.49[c]	20.06 ± 2.02[c]	41.47 ± 4.13[c]	127.19 ± 10.01[c]
Ellagic acid (15 mg/kg) + isoproterenol (100mg/kg)	35.37 ± 3.11[d]	28.13 ± 2.46[a]	34.08 ± 1.90[a]	19.15 ± 1.72[d,a]	28.22 ± 2.42[d]	105.91 ± 8.62[d]

each value is mean ± standard deviation for six rats in each group, values not sharing a common superscript (a,b,c,d) differ significantly with each other ($p < 0.05$, duncan's multiple range test).

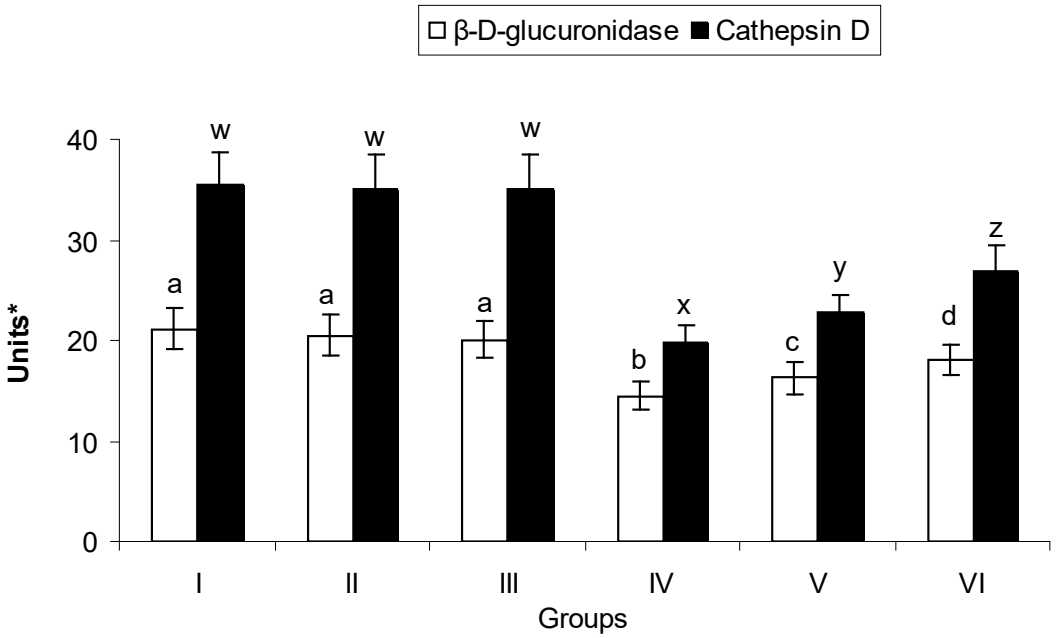

Figure 1

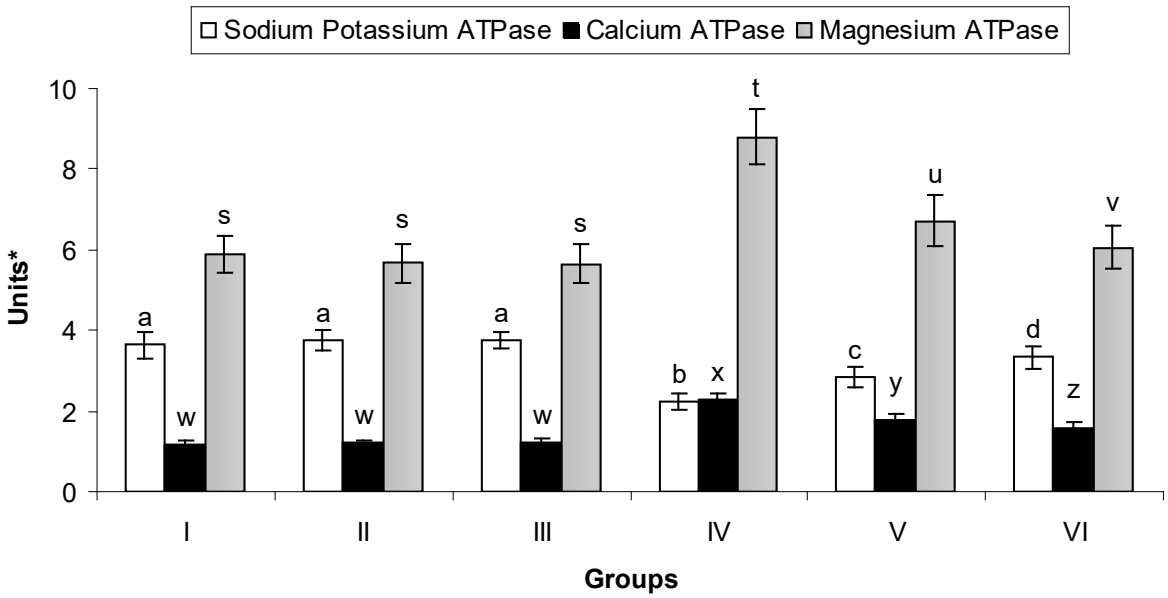

Figure 2

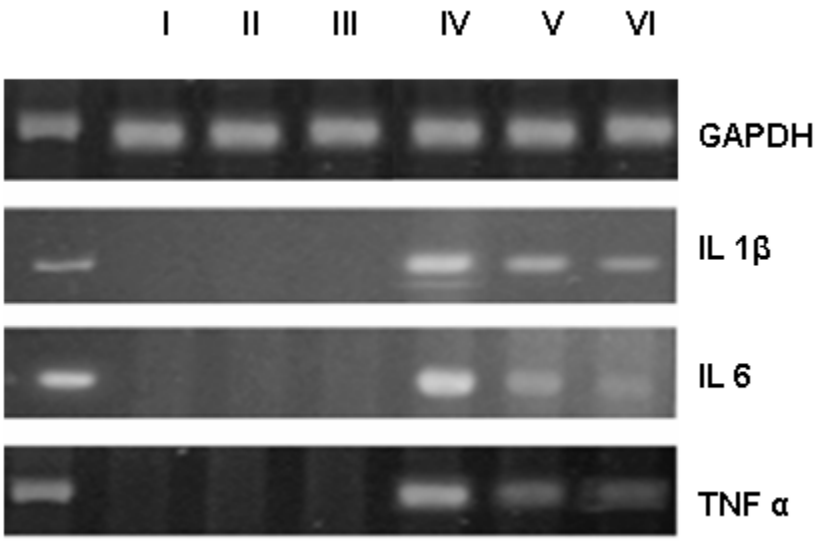

Figure 3