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# Ellagic acid protects mitochondria from β-adrenergic agonist induced myocardial damage in rats; evidence from in vivo, in vitro and ultra structural study

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**ABSTRACT**

This study aimed to evaluate the protective role of ellagic acid against mitochondrial damage in beta adrenergic agonist (isoproterenol)-induced myocardial infarction in Wistar rats in order to provide a scientific basis for the use of ellagic acid in preventing myocardial infarction. Mitochondria were isolated from the heart tissue of rats from all the groups. Lipid profile, lipid peroxidation, antioxidant enzyme activities and mitochondrial enzyme activities were measured. The structure of mitochondria was analysed by transmission electron microscopy. Isoprotrerenol-induced rats showed a significant increase in mitochondrial lipid peroxidation products and alterations in mitochondrial lipid profile. Significant decreases in mitochondrial antioxidants, tricarboxylic acid cycle enzymes were also found in the heart mitochondria of isoproterenol-induced myocardial infarcted rats. The protective effect of ellagic acid on the heart mitochondrial structure was evidenced by transmission electron microscopic study. Free radical scavenging and metal chelating activities of ellagic acid which are confirmed by *in vitro* studies may be the mechanism responsible for the protective action against mitochondrial damage in myocardial infarction. The results are support the potential important of ellagic acid in regular diet to prevent myocardiasl infarction.

*Key words:* Ellagic acid, mitochondrial enzymes, lipids, antioxidants, transmission electron microscopy

**Abbreviation**

ATP: adenosine triphosphate; ROS: reactive oxygen species; FFA: free fatty acid FFA; TBARS: thiobarbituric acid reactive substances; LOOH: lipid hydroperoxides; SOD: superoxide dismutase; GPx: glutathione peroxidase; GRx: glutathione reductase; GST: glutathione-S-transferase; ICDH: isocitrate dehydrogenase; SDH: succinate dehydrogenase, MDH: malate dehydrogenase, α-KGDH: α-ketoglutarate dehydrogenase, TEM:Transmission electrom microscopic; DMPD: N, N-dimethyl-p-phenylene diamine dihydrochloride; FRAP: ferric reducing antioxidant power; TCA: tricarboxylic acid; NADPH: Nicotinamide adenine dinucleotide phosphate; PPP: pentose phosphate pathway.

## 1. Introduction

Cardiovascular diseases are the principal cause of death worldwide. Myocardial ischaemia, one of the important cardiovascular diseases is a condition that exists when there is reduced coronary blood flow, resulting in a decrease in the supply of oxygen and nutrients to the heart causing heart cells to die. The cardiomyocyte is a most energy demanding cell in the body and is totally dependent on oxidative phosphorylation to supply the large amount of ATP required for beat-by-beat contraction and relaxation (Halestrap et al. 2007). The energy metabolism of the heart relates essentially to the contractile mechanisms of the myofibrils and oxidative phosphorylation in the mitochondria (Mela-Riker et al. 1985). Mitochondria are mainly involved in the production and regulation of cellular bioenergetic supply in the form of ATP (Marín-García & Goldenthal, 2004) and as the main consumers of molecular oxygen in the cardiac cells, they play a central role in molecular events leading to tissue damage occurring in the condition ischaemia (Suchalatha et al. 2007). In cardiac cells, mitochondria are the major source of ROS, resulting in the incomplete metabolism of 1–3% of the consumed oxygen (Boveris & Chance, 1973; Nohl & Hegner 1978) excessive ROS production leads to damage electron transport complexes, impair respiration and dysfunction of cells. The role of mitochondria in energy metabolism, programmed cell death, $Ca^{2+}$ homeostasis, and cell signalling are the main reasons justifying the new therapeutic approaches that have focused on mitochondria as one of the targets for protecting the myocardium.

Isoproterenol, a sympathomimetic β-adrenergic agonist is used to evaluate the protective effect of ellagic acid on mitochondrial damage. The ability of oxidized metabolic products of isoproterenol to interact with sulphydryl groups of various proteins leads to the production of free radicals. Oxidative stress produced by the free radicals of isoproterenol

metabolites along with the positive inotropic effect of catecholamine may the main mechanisms through which isoproterenol exert its toxic effects. The rat model of isoproterenol-induced myocardial necrosis serves as a well accepted standardized model to evaluate several cardiac dysfunctions of the heart and its subcellular fractions and to study the efficacy of various cardioprotective agents (Devika & Stanely Mainzen Prince, 2008; Senthil Kumaran & Stanely Mainzen Prince, 2010).

The current knowledge about the beneficial effects of nutraceuticals represents a great impact on nutritional therapy and stimulated research on favorable properties of bioactive compounds-enriched nutraceuticals. Dietary modifications are one of the key elements in the management of cardiovascular abnormalities. For many years, polyphenolic phytochemicals were thought to protect cells against oxidative damage through scavenging of free radicals. Among all polyphenolics, ellagic acid has received most attention because of its wide array of biological properties. Epidemiological evidence indicates that consumption of ellagic acid-rich foods may be protective against certain chronic diseases in human (Landete 2011; Daly et al. 2010). The phenolic nature of EA makes itself a powerful antioxidant (Solon et al. 2000; Festa et al. 2001). Previous studies indicated that ellagic acid showed chemoprotective (Vattem & Shetty, 2005), anti inflammatory (Corbett et al. 2010) and anti-lipidperoxidation (Osawa et al. 1987) properties. Daly et al (2010) reported the ellagic acid mediated protection against $H_2O_2$-induced DNA damage. A recent study from our laboratory showed that ellagic acid exhibits cardioprotective activity against experimentally induced myocardial infarction (Mari Kannan & Darlin Quine, 2011). Based on its lipophilic nature and multiple pharmacological actions, we hypothesise that the beneficial effects of ellagic acid can be extended up to the intracellular fractions of the heart and prevent the pathological alterations induced by the overproduction of ROS.

Thus, the present study is an attempt to indicate the most therapeutical benefit of ellagic acid intake by studying it's protective activity against isoproterenol-induced mitochondrial damage in rats. Our research attempted to demonstrate the molecular mechanism of its therapeutic effect by studying the lipid fractions, antioxidant enzymes, and other biochemical markers in the mitochondrial fractions. Transmission electron microscopic study was carried out to confirm the effect of ellagic acid on mitochondria. Additionally, we evaluated the possible free radical scavenging activity by *in vitro* assays such as ferric reducing antioxidant power and N, N-dimethyl-p-phenylene diamine dihydrochloride free radical scavenging assay to confirm the mechanism of action of ellagic acid.

## 2. Materials and methods

### *2.1. Experimental animals*

All the experiments were done on male albino Wistar rats weighing 180–200 g (4–5 months old), obtained from the Srivenkateshwara Enterprises, Bangalore, Karnataka, India. They were housed in polypropylene cages (47 cm × 34 cm × 20 cm) lined with husk, renewed every 24 h under 12 h light/dark cycle at around 22 ºC. The rats had free access to tap water and food. The rats were fed on a standard pellet diet (Pranav Agro Industries Ltd., Pune, Maharashtra, India). The diet provided metabolizable energy of 3600 kcal. 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 Animal Ethical Committee of Jayamukhi College of Pharmacy (Approval No. 28; 2 January 2010).

### *2.2. Drug and chemicals*

Ellagic acid and isoproterenol hydrochloride were purchased from Sigma Chemical Company, St.Louis, MO, USA. Nitroblue tetrazolium, phenazine methosulphate, butylated hydroxy toluene, 1-chloro-2,4-dinitrobenzene, 2,4-dinitrophenylhydrazine, trisodium citrate, glutathione, potassium-α-ketoglutarate, thiamine pyrophosphate, sodium succinate, oxaloacetate, cytochrome-C, N, N-dimethyl-p-phenylene diamine dihydrochloride and 2,4,6-tri(2-pyridyl)-s-triazine were purchased from Himedia, India. All other chemicals used were of analytical grade.

*2.3. Induction of experimental myocardial infarction*

Isoproterenol (100 mg/kg) was dissolved in saline and subcutaneously injected to rats at intervals of 24 h for 2 days (Devika & Stanely Mainzen Prince, 2008).

*2.4. Experimental design*

The animals were randomly divided into six groups of eight rats each. Two rats from each group were used for transmission electron microscopic study. 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 ellagic acid (7.5 mg/kg) in 2 ml of saline orally by gastric intubation daily for a period of 10 days; Group III: normal rats were treated with ellagic acid (15 mg/kg) in 2 ml of saline orally by gastric intubation daily for a period of 10 days, Group IV: rats were subcutaneously injected with isoproterenol (100 mg/kg) in 2 ml of saline twice at an interval of 24 h (on the 11th and 12th days); Group V: rats were pretreated with ellagic acid (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 isoproterenol (100 mg/kg) twice at an interval of 24 h (on the 11th and 12th days); Group VI: rats were pretreated with ellagic acid (15 mg/kg) in 2 ml of saline orally by gastric intubation daily for a period of 10 days and then subcutaneously injected with isoproterenol (100 mg/kg) twice at an interval of 24 h (on the 11th and 12th days). Twelve hours after the second dose of isoproterenol injection, all the rats were anesthetized and sacrificed by cervical decapitation. Heart tissues were excised immediately and rinsed in ice-chilled saline. They were blotted free of blood and tissue fluids. Then they were weighed and stored at −80 °C till further use for the analysis.

### *2.5. Isolation of heart mitochondria*

Heart mitochondria were isolated by the method of Takasawa et al. (1993).

### *2.6. Biochemical analysis*

#### *2.6.1. Estimation of mitochondrial lipids*

From the mitochondrial fraction, the lipids were extracted by the method of Folch et al. (1957). The concentration of cholesterol in the mitochondrial lipid fraction was estimated by the method of Zlatkis et al. (1953). The concentration of free fatty acid (FFA) in the mitochondrial lipid fraction was estimated by the method of Falholt et al. (1973). The levels of triglycerides in the mitochondrial lipid fraction were estimated by a reagent kit from Accurex Bio Pvt. Ltd, Mumbai. Phospholipid content in the mitochondrial lipid fraction was estimated by the method of Zilversmit et al. (1950).

*2.6.2. Estimation of lipid peroxidation products*

The levels of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) in the heart mitochondria were estimated by the methods of Fraga et al. (1988) and Jiang et al. (1992), respectively.

*2.6.3. Assay of antioxidant enzymes in heart mitochondrial fraction*

The activities of mitochondrial antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRx) and glutathione-S-transferase (GST) were assayed by the methods of Kakkar et al. (1984), Rotruck et al. (1973), Horn & Burns (1978) and Habig & Jakoby (1981), respectively. The concentration of reduced glutathione (GSH) was estimated by the method of Ellman (1959).

*2.6.4. Assay of heart mitochondrial enzymes*

The activities of isocitrate dehydrogenase (ICDH), succinate dehydrogenase (SDH), malate dehydrogenase (MDH), α-ketoglutarate dehydrogenase (α-KGDH), were estimated according to the standard procedures of King (1965), Slater & Bonner (1952), Mehler et al. (1948), Reed & Mukherjee (1969), respectively.

*2.6.5. Estimation of $Ca^{2+}$ in heart mitochondrial fraction*

The concentration of mitochondrial $Ca^{2+}$ was measured by the O-cresolphthalein complexone (OCPC) method using a reagent kit (Span Diagnostic Limited, India).

*2.7. Transmission electron microscopic (TEM) analysis*

The specimen cut into small bits was fixed in 2.5% buffered glutaraldehyde and post fixed in 1% buffered osmium tetroxide. The specimen bits were then treated with graded series of acetone followed by propylene oxide. Then they were infiltrated with EPON 812 epoxide resin and embedded with the same resin. The semi thin sections cut from the resin blocks using the LEICA ULTRACUT R ultramicrotome with diamond knife were stained with toluidine blue and the ultra thin sections collected on copper grids were stained with uranyl acetate followed by lead citrate and finally screened in the Philips 301 TEM at an accelerating voltage of 60 kV.

*2.8. In vitro studies*

The antioxidant activity of ellagic acid was determined using N, N-dimethyl-p-phenylene diamine dihydrochloride (DMPD) according to the method described by Fogliano et al. (1999). The ferric reducing antioxidant power (FRAP) assay of ellagic acid was performed according to the method of Benzie et al. (1999).

*2.9. Statistical analysis*

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 16.00. Results were expressed as mean ± standard

deviation for six rats in each group. $p$ values <0.05 were considered significant. 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**

Free radical mediated lipid peroxidation and alterations in the lipid profile are related to the membrane integrity and permeability of mitochondria. Isoproterenol-induced myocardial infarcted rats showed significant increase in the levels of TBARS and LOOH in the heart mitochondrial fraction, compared to normal control rats. Pretreatment with ellagic acid (7.5 and 15 mg/kg) daily for a period of 10 days significantly reduced the levels of TBARS and LOOH in the mitochondrial fraction of isoproterenol-induced rats (Table 1).

Rats induced with isoproterenol showed significant decrease in the activities of peroxidase enzyme (SOD) and other endogenous antioxidant enzymes (GPx, GR, GST) in the heart mitochondria, when compared to normal control rats. The levels of GSH also reduced in the heart mitochondria. Oral pretreatment with ellagic acid (7.5 and 15 mg/kg) to isoproterenol-induced rats daily for a period of 10 days significantly increased the activity of SOD in the heart mitochondria, when compared with isoproterenol-induced untreated rats. The activities of endogenous antioxidant enzymes were significantly increased in the heart mitochondria the levels of glutathione also increased significantly in the heart mitochondria of isoproterenol-induced rats (Table 1).

The levels of mitochondrial cholesterol, FFA and triglycerides in isoproterenol-induced rats were significantly increased and the level of phospholipids in the heart mitochondria was significantly decreased, compared with normal control rats. Pretreatment with ellagic acid

(7.5 and 15 mg/kg) daily for a period of 10 days significantly decreased the levels of cholesterol, FFA, triglycerides and significantly increased the levels of phospholipids in the heart mitochondrial fractions of isoproterenol-induced rats when compared with isoproterenol-induced untreated rats (Table 2).

The activities of tricarboxylic acid (TCA) cycle enzymes were measured to analyse the role of ellagic acid on energy metabolism (Table 3). The activities of ICDH, SDH, MDH and α-KGDH were decreased significantly in the heart mitochondria of isoproterenol-induced rats, when compared to normal control rats. The results confirmed the deleterious effect of isoproterenol on TCA cycle enzymes. Pretreatment with ellagic acid (7.5 and 15 mg/kg) daily for a period of 10 days significantly increased the activities of these enzymes in isoproterenol-induced rats, when compared to isopreoterenol-induced untreated rats.

The levels of calcium in the heart mitochondria were measured in the mitochondrial fractions of normal and experimental rats. Isoproterenol-induced myocardial infarcted rats showed significantly increased level of calcium in the heart mitochondrial fraction, compared to normal control rats. Pretreatment with ellagic acid (7.5 and 15 mg/kg) daily for a period of 10 days significantly reduced the elevated level of calcium in the mitochondrial fraction, compared to those of the isoproterenol-induced rats (Fig. 1).

The TEM images of the heart mitochondria showed regular pattern of myofibrils and mitochondrial cristae with normal shape in normal control rats (Fig. 2 a). Normal rats treated with ellagic acid (7.5 and 15 mg/kg) also showed normal architecture without any pathological changes (Fig. 2 b and c). In isoproterenol-induced rats, significant changes in the mitochondrial ultra structure in the heart tissue were observed. The mitochondrial structure was observed with thickened membranes and reduction in the number of cristae. Some of the mitochondria suffered high-amplitude swelling and the cristae of mitochondria were sparse with vacuolation (Fig. 2 d). Marked increase in mitochondrial size (giantism) and decreases

in the density of their inner compartment were also observed in isoproterenol-induced rats. Pretreatment of isoproterenol-induced rats with ellagic acid (7.5 mg/kg) showed that the improved structure of cristae, with less vacuolar degeneration and less swelling in the mitochondria (Fig.2 e). Reduction in average size of the mitochondria and improved density of their inner compartment were observed. Pretreatment of isoproterenol-induced rats with ellagic acid (15 mg/kg) showed that the mild separation of cristae without swelling and vacuolation (Fig. 2 f). Numbers of cristae are more and the size of the mitochondria was almost same as normal control rats.

The $DMPD^{\bullet+}$ scavenging activity of ellagic acid at various concentrations (10-50 μM) were measured (Fig. 3). Our results demonstrated marked $DMPD^{\bullet+}$ scavenging activity. The reducing potentials of antioxidants are associated with their electron donating abilities to break the free radical chain reactions. In the present study, FRAP value was applied to evaluate the antioxidant power of ellagic acid. Different concentrations of ellagic acid (10-50 μM) showed marked increase in the FRAP values with higher concentrations, suggesting that the antioxidant power of ellagic acid was much stronger with increasing concentrations (Fig. 4). The results indicated that ellagic acid might act as electron donors and react with free radicals to convert them into more stable products to terminate the free radical chain reactions.

**4. Discussion**

The current knowledge about the beneficial effects of nutraceuticals represents a great impact on nutritional therapy and stimulated the research on favorable properties of bioactive compounds-enriched nutraceuticals (Andlauer & Fürst, 2002). 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 isoproterenol-induced myocardial infarction (Mari Kannan & Darlin Quine, 2011).

An excessive β-adrenergic stimulation by isoproterenol produce more cyclic adenosine monophosphate that lead to enhanced release of calcium by the sarcoplasmic reticulum in the heart. Subsequent increase in inotropy and generation of lipid peroxides during ischaemia could damage the mitochondrial membrane and affect their functions. Increased lipid peroxidation in the mitochondria of the heart was indicated by increased activities of TBARS and LOOH in the isoproterenol-induced myocardial infarcted rats. Pretreatment with ellagic acid significantly decreased the activities of TBARS and LOOH in the mitochondria of isoproterenol-induced myocardial infarcted rat heart. Ellagic acid 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 (Pari & Sivasankari 2008). Our results confirmed the anti lipid peroxidative action of ellagic acid against isoproterenol-induced mitochondrial damage in myocardium.

We have addressed the question of how isoproterenol affected the lipid levels in the mitochondrial fraction and the effect of pretreatment with ellagic acid on cholesterol, free fatty acids and triglycerides. We observed an increased level of cholesterol in the mitochondria of heart tissue in isoproterenol-induced rats which suggests redistribution of cholesterol in the mitochondria of ischaemic cells. Increased cholesterol levels in the mitochondrial membrane affect the permeability of ions and the fluidity. The metabolic products of isoproterenol produced more free radicals and the phospholipid rich mitochondrial membrane is vulnerable to free radical attack. This may be the reason for decreased levels of phospholipids in mitochondrial fractions of the heart of isoproterenol-induced rats. Pretreatment with ellagic acid significantly increased the level of phospholipids

in the isoproterenol-induced rats. Ellagic acid's ability to counter free radical attack may protect phospholipids in mitochondria. In addition, we observed an increased level of triglycerides in the isoproterenol-induced rats. Previous reports (Golfman et al. 2005; Yang et al. 2007) suggested that the decreased delivery of fatty acids from the cardiomyocytes reverses triglyceride accumulation and leads to contractile dysfunction. Pretreatment with ellagic acid reduced the levels of triglycerides in isoproterenol-induced rats. Ellagic acid may channel fatty acids to triacylglycerol synthesis and divert lipids from toxic metabolic pathways. For example, de novo ceramide synthesis may protect mitochondria against changes in lipid metabolism.

Since mitochondria are known to be one of the major players in oxidative stress, a drug which can act on the mitochondrial antioxidant defense system should ameliorate cardiac damage in pathological conditions. We hypothesised that part of the protective actions of ellagic acid against lipid peroxidation and mitochondrial lipid regulating action must occur via another mechanism, such as antioxidant activity. Acute β-adrenergic receptor stimulation not only rapidly generates ROS, it also depresses total cellular antioxidant capacity, down-regulates copper–zinc superoxide dismutase enzyme activity, protein and mRNA. Furthermore, it reduces glutathione levels, leading to the loss of membrane integrity and inducing heart contractile dysfunction and myocyte toxicity, finally producing myocardial necrosis (Rathore et al.1998; Srivastava et al. 2007). A significant decrease in the activities of anti-peroxidative enzyme SOD was observed in the mitochondrial fractions of isoproterenol-induced rats. GSH is an important antioxidant in response to the oxidative stress that protects the mitochondrial membrane from the damaging action of lipid peroxidation (Senthil Kumaran & Stanely Mainzen Prince, 2010). It can act as the electron donor for GPx in animal cells, and also directly reacts with ROS. In addition, oxidized product of GSH (GSSG) can be reduced to GSH by the NADPH-dependent reaction

catalyzed by glutathione reductase (Izawa et al.1998). GPx is the most important peroxidase for $H_2O_2$ removal in mammals. Further, it is present in relatively large amounts within the heart, especially in the cytosolic and mitochondrial compartments (Le et al. 1993). GST are phase II detoxification enzymes that catalyze the conjugation of reduced GSH with a variety of xenobiotic and endogenous electrophiles (Hayes & Pulford, 1995). In mitochondria, the decreased GSH level and increased oxidized GSH, inactivate GSH-dependent enzymes, such as GPx and GST in isoproterenol-induced myocardial infarcted rats. Thus, maintaining intracellular GSH content in mitochondria can protect cardiomyocytes from cellular damage. Pretreatment with ellagic acid significantly increased the levels of GSH along with GSH-dependent enzymes in the mitochondrial fraction of isoproterenol-induced rats. Ellagic acid may act as an antioxidant by scavenging ROS and also improving the endogenous antioxidant system in isoproterenol-induced rats (Mari Kannan & Darling Quine 2011). In addition, ellagic acid, which is partially hydrophobic in nature, can be a perfect candidate for the activity of signalling pathways responsible for stimulation of the pentose phosphate pathway (PPP) (Chen et al. 2000; Owuor & Kong, 2002). The stimulation of the PPP may be closely related to the cellular antioxidant response pathway (Shetty & Wahlqvist, 2004).

The mitochondrial enzymes (ICDH, SDH, MDH and α-KGDH) catalyse the oxidation of several substrates through the tricarboxylic acid (TCA) cycle, yielding reducing equivalents, which are channelled through the respiratory chain for the synthesis of ATP by oxidative phosphorylation. ICDH is the predominant enzyme in the heart (Plaut et al. 1983) and it is the only NADPH-producing enzyme for the regeneration of glutathione in mitochondria. The high activity of ICDH can contribute to the regeneration of reduced glutathione in heart mitochondria, resulting in a markedly high resistance of the heart enzymes against oxidative stress. SDH is one of the important enzymes that regulate the production of ATP in mitochondria. It is a typical thiol (SH) group enzyme and sensitive to

free radicals. MDH catalyzes a reversible $NAD^+$-dependent-dehydrogenase reaction involved in central metabolism and redox homeostasis between organelle compartments. MDH is located in the outer membrane of mitochondria and vulnerable to free radical attack (Yogeeta et al. 2006). The α-ketoglutarate dehydrogenase catalyzes the conversion of α-ketoglutarate to succinyl-CoA and NADH in mitochondria. isoproterenol administration to rats significantly decreased the activities of TCA cycle enzymes, such as ICDH, SDH, MDH and α-KGDH. These dehydrogenases are located in the outer membrane of the mitochondria and are affected by increased levels of free radicals produced following isoproterenol administration (Prabhu et al. 2006). The reduced activities of these important enzymes decrease the fatty acid and carbohydrate metabolism. Pretreatment with ellagic acid showed significantly improved activities of TCA cycle enzymes in isoproterenol-induced rats. Lee et al. (2010) reported that ellagic acid inhibited NAPDH and reduced the overproduction of superoxide and down-regulating inducible nitric oxide synthase thereby suppressing the overproduction of nitric oxide. Therefore, ellagic acid can protect TCA cycle enzymes from free radical attack and helps to maintain the energy levels of mitochondria.

$Ca^{2+}$ is a key second messenger for the regulation of mitochondrial functions. Mitochondrial $Ca^{2+}$ uptake stimulates mitochondrial nitric oxide (NO) production via activation of mtNOS. NO modulates mitochondrial $O_2$ uptake, ROS generation and ATP production, and thus represents a crucial link for excitation-metabolism as well as excitation-contraction coupling in cardiac cells (Dedkova & Blatter, 2008). We observed an increased concentration of mitochondrial free calcium in isoproterenol-induced rats. The elevation of intracellular calcium levels is recognized as a cause of cytotoxicity induced by isoproterenol due to its increased production of ROS. Therefore, the blockage of isoproterenol-induced calcium influx might provide mitochondrial protection against isoproterenol-induced mitochondrial damage. In this study, pretreatment of isoproterenol-induced rats, with ellagic

acid significantly decreased the intracellular calcium levels compared to the isoproterenol-induced myocardial infarcted rats. The ability of phenolic acid to chelate with ions such as calcium in the extracellular matrix or in the cytosol and to alter the net concentration of free calcium was reported by Hider et al. (2001). Ellagic acid can interact with cell surface receptors and regulate ion pumps as there are structural similarities between ellagic acid and many biological signalling molecules. Based on our findings, the effect of ellagic acid is due to its antioxidant activity by inhibiting ROS, which, in turn, represses the release of the endothelial free calcium ion concentration and stabilizes the mitochondrial membrane.

Change in the mitochondrial morphology is a key indicator of cellular pathology. In the present study, transmission electron microscopic analysis of mitochondria of myocardial tissue demonstrated the degenerative changes due to isoproterenol administration. The marked increase in mitochondrial size is probably the result of a twofold process: growth of individual organelles or fusion of abutting mitochondria, or a combination of the two (Hoppel et al. 2009). Previous studies indicate that an excessive dose of isoproterenol damages the mitochondria of the heart and explain that the increased lipid peroxidation and decreased antioxidant system within the mitochondria are the reason (Senthil Kumaran & Stanely Mainzen Prince 2010; Stanely Mainzen Prince& Sathya 2010). Our results are in agreement with previous studies. A recent study from our laboratory indicated that an excessive dose of isoproterenol produces significant changes in C-reactive protein and plasma homocystein, implying that the injury is mediated through other mechanisms such as inflammation (Mari Kannan & Darling Quine 2011). Further studies are required to understand the mechanisms involved. Our results showed that the degenerative changes due to isoproterenol administration are reversed in ellagic acid **(**7.5 and 15 mg/kg) pretreated rats in a dose-dependent manner. A previous report from our laboratory explaines the antioxidant and anti lipid peroxidation potentials of ellagic acid against isoproterenol-induced myocardial

infarction (Mari Kannan & Darling Quine 2011). The mitochondrial membrane stabilizing capacity of ellagic acid was reported by Ou et al. (2010). These mechanisms may contribute to ellagic acid's beneficial effects on mitochondrial damage in cardiotoxicity.

The results of *in vitro* studies can be helpful in screening early formulations in preparation for initial clinical trials. The role of ROS in the cardiovascular pathopysiology stimulated the interest in the therapeutic consideration of antioxdants. Thus, the antioxidant activity of ellagic acid was analysed by *in vitro* methods. *In vitro* studies show that ellagic acid, at concentrations in the range 10-50 μM , show antioxidant effects. DMPD assay showed the ability of ellagic acid to reduce free radicals in a dose-dependent manner. This antioxidant activity allows ellagic acid to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. The antioxidant efficiency of EA is directly correlated with their degree of hydroxylation (Landete, 2011). The FRAP assay gives an indication of the reducing ability of ellagic acid. The FRAP assay result supports the conception that the ellagic acid reduces metal ions. The *in vitro* antioxidant activity was correlates with the results of *in vivo* antioxidant assays in the mitochondrial fractions. The free radical scavenging activity and the reducing ability of ellagic acid, which are evidenced by *in vitro* studies, confirm its mechanism of action. These mechanisms may be responsible for the protective effect of ellagic acid on mitochondria against experimentally-induced cardio toxicity.

Ellagic acid is absorbed rapidly and metabolized in the liver by methyl conjugation by the action of enzyme catechol-O-methyltransferase (Cerda et al. 2005). The $C_{max}$ of ellagic acid was 31.9 ng/ml and was rapidly eliminated by 4 h (Mertens-Talcott et al. 2006). The unbound ellagic acid appeared to best fit the kinetics of a 2-compartment model with first order absorption and lag time with rapid distribution time ($t_{1/2}$ = 0.77 h) in rat blood (Lei et al. 2003). The available information on pharmacokinetics of ellagic acid can be used to develop a suitable formulation for clinical studies to achieve maximum efficacy with minimum dose.

Pretreatment with ellagic acid (7.5 and 15 mg/kg) in normal control rats did not show any significant effect on the biochemical parameters and ultra structure of mitochondria, which confirmed the safety, non toxicity of ellagic acid. Considering the protective effects of ellagic acid on mitochondrial damage and its pharmacokinetics, the results of this study open the way to promote products which are rich in ellagic acid. Ellagic acid can act as a good source of antioxidants with the cardioprotective action. According to our *in vivo* findings, a 70-kg person requires 1050 mg of ellagic acid per day, but *in vitro* studies suggested that 50 μmol of ellagic acid can produce maximum antioxidant effect. A detailed study on the dose-response relationship is necessary to utilize ellagic acid as a cardioprotective drug in human in the future. Pharmacological dose for the treatment after myocardial infarction should be decided based on degree of pathological conditions.

## 5. Conclusion

The protective effect of ellagic acid against lipid peroxidation, mitochondrial damage and the regulative capacity of ellagic acid on lipid profile, antioxidant status and TCA cycle enzymes strongly suggest that ellagic acid is a possible drug moiety for mitochondrial protection and can regulate the pathological conditions caused by stress-induced mitochondrial damage in the myocardium. Collectively, these data are supports the potential important to include ellagic acid in regular diet, especially since current therapies with drugs have many side effects and the myocarial infarction is heterogeneous. In addition, this paper reccomends the inclusion of fruits (eg, pomegranates, strawberries and black berrries) and food sources (eg, walnuts) high in ellagic acid in a healthy diet to prevent myocardial infarction.

## Disclosure

Both the authors (DQ & MK) were designed, organized and carried out the experimental works and wrote the manuscript. Both of the authors declared that the contents are true and approved the final manuscript.

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**Table 1**.
Effect of ellagic acid on heart mitochondrial lipid peroxidation products and endogenous antioxidants

| Groups | Thiobarbituric acid reactive substances (nmol/mg protein) | Lipid hydroperoxides (nmol/100mg of protein) | Super oxide dismutase (#Units/100 mg protein) | Glutathione peroxidase (nmol of GSH oxidized/min/ 100 mg protein) | Gltathione-s-transferase (nmol of *CDNB conjugated/min/100 mg protein) | Glutathione (nmol/100 mg protein) |
|---|---|---|---|---|---|---|
| Normal control | $5.43 \pm 0.42^{a}$ | $7.56 \pm 0.65^{a}$ | $15.58 \pm 1.19^{a}$ | $2.77 \pm 0.24^{a}$ | $72.69 \pm 6.14^{a}$ | $6.49 \pm 0.42^{a}$ |
| Normal+ ellagic acid (7.5 mg/kg) | $5.31 \pm 0.51^{a}$ | $7.32 \pm 0.69^{a}$ | $15.54 \pm 1.03^{a}$ | $2.78 \pm 0.19^{a}$ | $73.32 \pm 6.66^{a}$ | $6.46 \pm 0.46^{a}$ |
| Normal+ ellagic acid (15mg/kg) | $5.22 \pm 0.49^{a}$ | $7.28 \pm 0.62^{a}$ | $16.26 \pm 1.06^{a}$ | $2.87 \pm 0.24^{a}$ | $74.01 \pm 5.66^{a}$ | $6.44 \pm 0.45^{a}$ |
| Isoproterenol (100mg/kg) | $9.19 \pm 0.83^{b}$ | $11.43 \pm 1.05^{b}$ | $6.93 \pm 0.60^{b}$ | $1.37 \pm 0.13^{b}$ | $54.48 \pm 4.93^{b}$ | $3.42 \pm 0.21^{b}$ |
| Ellagic acid (7.5 mg/kg)+ isoproterenol (100mg/kg) | $7.10 \pm 0.62^{c}$ | $9.75 \pm 0.83^{c}$ | $10.91 \pm 0.84^{c}$ | $1.81 \pm 0.18^{c}$ | $62.80 \pm 5.50^{c}$ | $4.63 \pm 0.38^{c}$ |
| Ellagic acid (15 mg/kg) + isoproterenol (100mg/kg) | $6.17 \pm 0.56^{d}$ | $8.34 \pm 0.68^{d}$ | $13.24 \pm 1.19^{d}$ | $2.61 \pm 0.22^{d}$ | $70.83 \pm 5.36^{d}$ | $6.04 \pm 0.50^{d}$ |

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).

# Unit- One unit is defined as the enzyme concentration required to inhibit the OD at 560nm of chromogen production by 50% in 1 min. *CDNB: 1-chloro-2,4-dinitrobenzene.

**Table 2.**

Effect of ellagic acid on the levels of heart mitochondrial lipid profile

| Groups | Cholesterol(nmol/mg protein) | Free fatty acids(nmol/mg protein) | Triglycerides(nmol/mg protein) | Phospholipids(nmol/mg protein) |
|---|---|---|---|---|
| Normal control | 38.88 ± 3.27[a] | 15.49 ± 1.51[a] | 20.71 ± 1.28[a] | 575.70 ± 41.70[a] |
| Normal+ ellagic acid (7.5 mg/kg) | 37.65 ± 3.28[a] | 14.87 ± 1.31[a] | 20.16 ± 1.46[a] | 585.41 ± 41.81[a] |
| Normal+ ellagic acid (15mg/kg) | 36.97 ± 3.59[a] | 15.15 ± 1.47[a] | 19.64 ± 1.21[a] | 582.34 ± 45.08[a] |
| Isoproterenol (100mg/kg) | 57.49 ± 5.00[b] | 25.44 ± 2.22[b] | 33.58 ± 2.77[b] | 413.08 ± 35.90[b] |
| Ellagic acid (7.5 mg/kg)+ isoproterenol (100mg/kg) | 48.18 ± 4.55[c] | 21.77 ± 1.94[c] | 27.34 ± 1.96[c] | 469.70 ± 45.14[c] |
| Ellagic acid (15 mg/kg) + isoproterenol (100mg/kg) | 42.02 ± 4.39[d] | 16.21 ± 1.29[d] | 24.07 ± 1.52[d] | 548.22 ± 48.15[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).

**Table 3.** Effect of ellagic acid on the activities of mitochondrial enzymes

| Groups | Isocitrate dehydrogenase | Succinate dehydrogenase | Malate dehydrogenase | α-ketoglutarate dehydrogenase |
|---|---|---|---|---|
| Normal control | 570.83 ± 50.12[a] | 170.99 ± 15.67[a] | 413.08 ± 30.97[a] | 72.15 ± 4.82[a] |
| Normal+ ellagic acid (7.5 mg/kg) | 574.14 ± 48.83[a] | 173.56 ± 15.77[a] | 417.06 ± 32.76[a] | 73.83 ± 5.43[a] |
| Normal+ ellagic acid (15mg/kg) | 576.10 ± 49.24[a] | 177.74 ± 16.24[a] | 420.65 ± 34.58[a] | 74.65 ± 6.08[a] |
| Isoproterenol (100mg/kg) | 364.74 ± 33.16[b] | 89.80 ± 7.69[b] | 234.65 ± 17.92[b] | 40.98 ± 3.97[b] |
| Ellagic acid (7.5 mg/kg)+ isoproterenol (100mg/kg) | 500.20 ± 48.16[c] | 122.61 ± 11.01[c] | 315.92 ± 21.59[c] | 55.62 ± 5.13[c] |
| Ellagic acid (15 mg/kg) + isoproterenol (100mg/kg) | 587.33 ± 45.24[a] | 153.06 ± 11.15[d] | 358.82 ± 27.26[d] | 69.83 ± 5.67[d] |

units: isocitrate dehydrogenase: nmol of α-ketoglutarate formed/h/mg protein; succinate dehydrogenase: nmol of succinate oxidized/min/mg protein; malate dehydrogenase: nmol of nicotinamide adenine dinucleotide hydrogenase oxidized/min/mg protein; α-ketoglutarate dehydrogenase: nmol of ferrocyanide formed/h/mg protein. 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 captions**

Fig. 1. Effect of ellagic acid on the levels of calcium in the heart mitochondria. 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. Columns not sharing a common letter (w, x, y, z) differ significantly with each other ($p<0.05$, DMRT).

Fig. 2. Transmission electron microscopic analysis of heart mitochondria.

Fig. 2 a: Normal control group showing normal architecture of the heart mitochondria.

Fig. 2 b: Normal + ellagic acid (7.5 mg/kg)-treated rats showing normal architecture of the heart mitochondria.

Fig. 2 c: Normal + ellagic acid (15 mg/kg)-treated rats showing normal architecture of the heart mitochondria.

Fig. 2 d: Isoproterenol-induced (100 mg/kg) heart mitochondria showing swelling of mitochondria and disruption of cristae with vacuolation marked with ↓.

Fig. 2 e: Ellagic acid (7.5 mg/kg) + isoproterenol-induced heart mitochondria (×25,000) showing reduced separation of cristae with mild swelling and vacuolation.

Fig. 2 f: Ellagic acid (15mg/kg) + isoproterenol-induced heart mitochondria showing mild separation of cristae without swelling and vacuolation.

Fig. 3. The $DMPD^{\bullet+}$ scavenging effect of ellagic acid.

Fig. 4. Ferric reducing antioxidant power (FRAP) assay of ellagic acid.

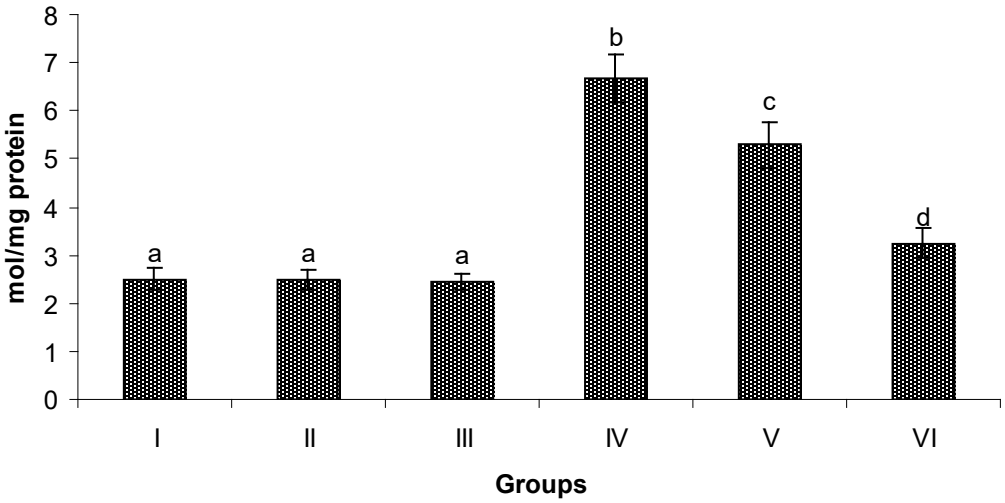


**Figure 1**

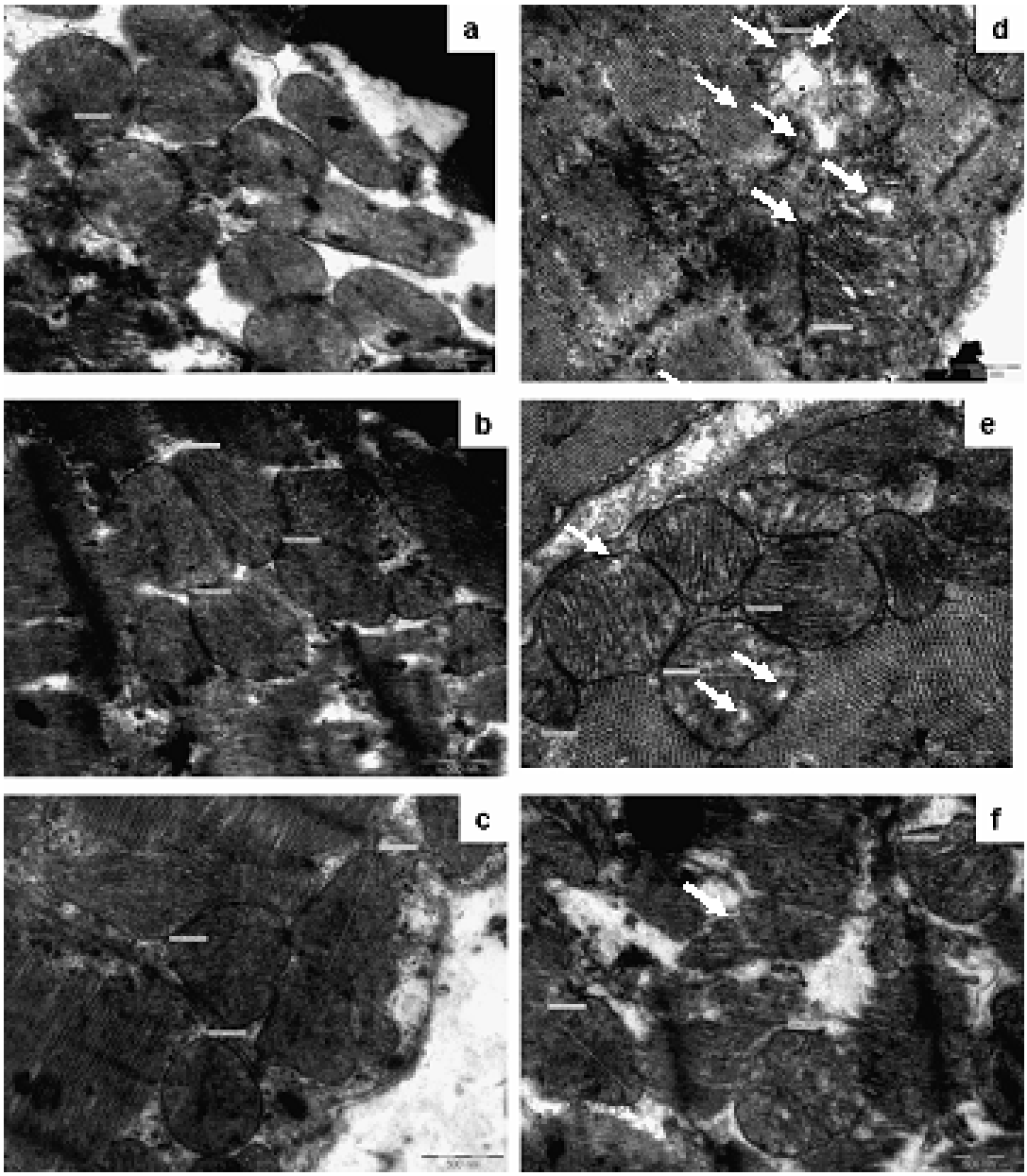


**Figure 2**

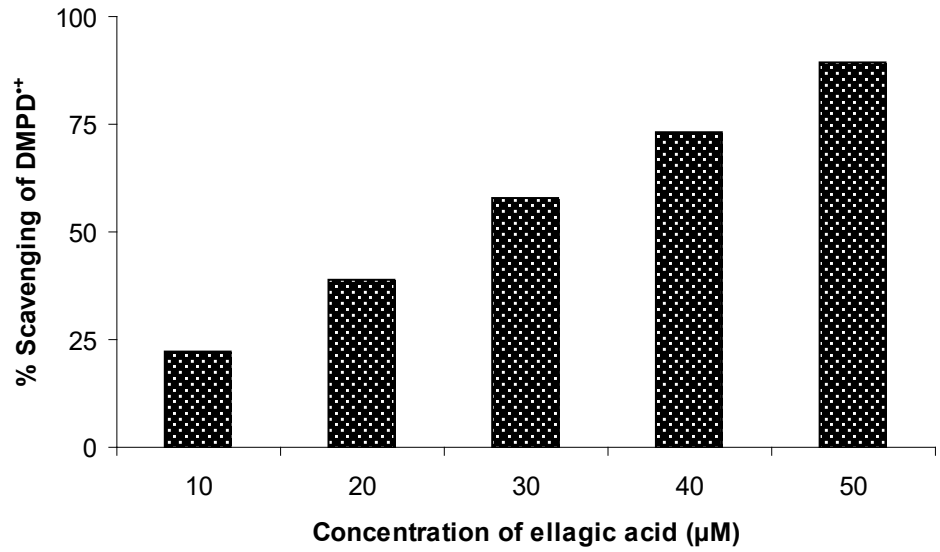
100
75
50
25
0
% Scavenging of DMPD•+
10
20
30
40
50
Concentration of ellagic acid (µM)

**Figure 3**

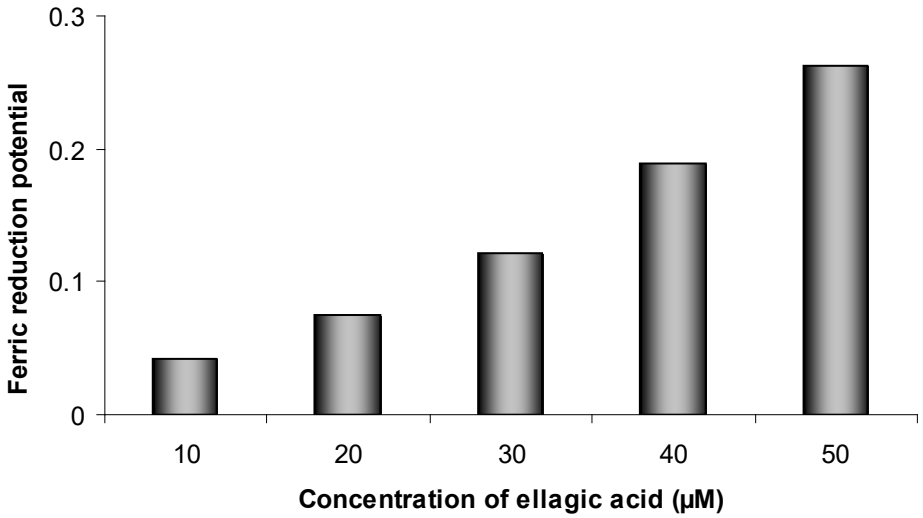
0.3
0.2
0.1
0
Ferric reduction potential
10
20
30
40
50
Concentration of ellagic acid (µM)

**Figure 4**