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EVALUATION OF SYNERGISTIC EFFECT OF FERULIC ACID WITH ASCORBIC ACID FOR THE MANAGEMENT OF HYPERLIPIDEMIA

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ABSTRACT
Ferulic acid is the most abundant phenolic compound found in vegetables and cereal grains. In vitro and animal studies have shown ferulic acid has anti-hyperlipidemic. The present study was designed to evaluate the effect of the combination of ferulic acid and ascorbic acid on antioxidant defense system and lipid peroxidation against isoproterenol (ISO)-induced myocardial infarction in rats. Induction of rats with isoproterenol (150 mg/kg body weight daily, i.p.) for 2 days resulted in a marked elevation in lipid peroxidation, serum marker enzymes (LDH, CPK, GOT, and GPT), and a significant decrease in activities of endogenous antioxidants (SOD, GPx, GST, CAT, and GSH). Pre-co-treatment with the combination of ferulic acid (20 mg/kg body weight/day) and ascorbic acid (80 mg/kg body weight/day) orally for 6 days, significantly attenuated these changes when compared to the individual treatment groups. Histopathological observations were also in correlation with the biochemical parameters. Thus, ferulic acid and ascorbic acid significantly counteracted the pronounced oxidative stress effect of ISO by the inhibition of lipid peroxidation, restoration of antioxidant status, and myocardial marker enzymes levels. In conclusion, these findings indicate the synergistic protective effect of ferulic acid and ascorbic acid on lipid peroxidation and antioxidant defense system during ISO-induced myocardial infarction and associated oxidative stress in rats.

KEYWORDS: Ascorbic acid, Ferulic acid, Antioxidant, Hyperlipidemia, Isoproterenol.

1. INTRODUCTION

Hyperlipidemia (HL) is a heterogenous group of disorder that is characterized by increased level of one or more plasma lipids, especially cholesterol, triglyceride (TG) and/or plasma lipoprotein like very low density lipoprotein (VLDL), low density lipoprotein (LDL), and reduced high density lipoprotein (HDL).[1-3] Among various metabolites, lipids constitute the most abundant class of cellular metabolites having varied structural and functional diversity.[4] The interaction with lipids or their metabolites determines the localization, structure and function of many proteins. Therefore, the cooperation between lipid-protein and protein-protein interactions is responsible for many cellular, metabolic and signaling processes.[5] Nowadays, the prevalence of HL has increased due to sedentary lifestyle, unhealthy diet and physical inactivity.[6] Consequently, the imbalance in blood lipid level may alter normal functioning of a lipid-protein signaling network and may indicate presence of some underlying ailment. Hence, the lipid profile assessment has become a preliminary test for diagnosis of an underlying medical condition.[4]

The major dietary form of vitamin C, is an important water-soluble antioxidant that may affect the risk of atherosclerosis and coronary heart disease.[7,8] Unlike most mammals, humans do not have the ability to make their own vitamin C. Therefore, we must obtain vitamin C through our diet. In a recent prospective study, a low plasma ascorbic acid (AA) was associated with an increased risk of myocardial infarction.[9] AA is known to act as an efficient antioxidant. It acts both directly by reaction with aqueous peroxyl radicals and indirectly by restoring the antioxidant properties of vitamin E.[10]

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is an antioxidant in vitro and may therefore contribute to cardioprotective effects of whole grain consumption. Ferulic acid (FA) is found especially in wheat, corn, rice, tomatoes, spinach and cabbage, and is clinically used to treat angina pectoris and hypertensive diseases in China. Previous studies show that it had significant effects on improving blood fluidity and inhibits platelet aggregation. Hence the present study was aimed at investigating the effect of a combination of ferulic acid and ascorbic acid in reducing isoproterenol induced changes with respect to lipid metabolism.[11]

Myocardial infarction induced by isoproterenol (L-b-(3,4-dihydroxyphenyl)-a-iso propyla mino ethanol

hydrochloride), shows many metabolic and morphologic aberrations in theheart tissue of the experimental animals similar to those observed in human myocardial infarction. Isoproterenol, asynthetic catecholamine causes severe oxidative stress in the myocardium resulting in infarct like necrosis of heartmuscle with an increase in the levels of lipid in the myocardium, It also increases the low density lipoprotein-cholesterol levels in the blood which in turn leads to the build-up of a harmful depositin the arteries, thus favouring coronary heart disease.[11]

Lipid peroxidation is one of the main manifestations of oxidative damage initiated by reactive oxygen species and has been linked to the altered membrane structure and enzyme inactivation. The peroxidative damages induced in the cell are encountered by elaborate defense mechanism including enzymic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S-transferase) and nonenzymic antioxidant (reduced glutathione). Biological compounds with antioxidant properties contribute to the protection of cells and tissues against deleterious effects of reactive oxygen species and other free radicals. As a previous report showed a synergistic antioxidant interaction of FA with AA in isolated rat liver microsomal membranes exposed to free radicals, but any *in vivo* studies on whether (or) not this synergistic interaction of FA with AA could offer protection to the myocardium during myocardial oxidative stress induced injury has not been previously evaluated. Hence, the present study aims to evaluate the effect of FA and AA in combination on tissue defense system and on lipid peroxidation status during ISO-induced myocardial infarction in rats.

2. MATERIALS AND METHODS

2.1. Chemicals

Ferulic acid, L-ascorbic acid, and isoproterenol hydrochloride were procured from Sigma Chemical Co., St. 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl(DPPH) All other chemicals used were of analytical grade.

2.2. Free radical (DPPH·) scavenging activity

Principle: The scavenging reaction between (DPPH˙) and an antioxidant (H-A) can be written as:

$$(DPPH) + (H\text{-}A) \longrightarrow DPPH\text{-}H + (A)$$

(Purple) (Yellow)

Antioxidants react with DPPH˙, which is a stable free radical and is reduced to DPPH-H and as a consequence the absorbance decreased from the DPPH˙ radical to the DPPH-H form. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Procedure: The free radical scavenging activity of the compounds was measured by DPPH using the method described by Oktay et al (2003).[12] About 1ml 0.2mM of ethanolic DPPH solution was added to 3 ml each of compounds. After 30 min. absorbance was measured at 517nm. Radical scavenging activity was expressed as inhibition percentage and was calculated using the formula:

% Radical scavenging activity = [(control O.D – sample O.D)/control O.D] × 100

2.3. Animal treatment & diet

The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Bhopal, India. Animal room was maintained at a temperature of 25 ± 1ºC, a humidity of 50 ± 10% and a 12-h light/dark cycle and the mice were allowed to access food and water ad libitum. Adult male albino rats of Wistar strain, weighing approximately 140–160 g take for experiment.

Experimental design

The rats were divided into eight groups (n = 6 in each group). Group 1, control; Group 2, ISO induced; Group 3, FA only; Group 4, AA only; Group 5, FA+AA; Group 6, FA+ISO; Group 7, AA+ISO; and Group 8, FA+AA+ISO. FA (20 mg/kg body weight) and AA (80 mg/kg body weight) was administered orally for 6 days both alone and in combination. This particular dosage was fixed after trying out different doses, 10, 20, 40, and 60 mg/kg body weight for FA and 20, 40, 60, 80, and 100 mg/kg body weight for AA for 2, 4, 6, 8, and 10 days prior to the induction of ISO. An amount of 150 mg/kg body weight of ISO was administered intraperitoneally for 2 days.[13] Both FA and AA offered protection in a dose-dependent manner. As the dosage of 20 mg/kg body weight for FA and 80 mg/kg body weight for AA was found to exhibit maximum cardioprotective effect against ISO-induced damage, this particular dosage was fixed as the optimum dosage for the study.

After the experimental period the rats were sacrificed, blood was collected, and the serum separated was used for the estimation of protein[14], assaying the activity of lactate dehydrogenase (LDH)[15], creatine phosphokinase (CPK)[16], oxaloacetate transaminase (GOT), and pyruvate transaminase (GPT).[17] Immediately after the sacrifice, the heart was excised, washed in ice-cold isotonic saline and homogenized. The homogenate was used for assaying the activity of antioxidant enzymes superoxide dismutase (SOD)[18], catalase (CAT)[19], glutathione-peroxidase (GPx)[20], glutathione-S-transferase (GST)[21], reduced glutathione (GSH)[22], and levels of lipid peroxides.[23]

A portion of the heart tissue was fixed in 10% buffered neutral formalin solution for histological studies. After fixation, tissues were embedded in paraffin wax, solid sections were cut at 5µmand stained with hematoxylin and eosin and viewed under light microscope for histological changes.

Statistical analysis All the grouped data were evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as Mean ± S.D for six animals in each group.

3. RESULTS AND DISCUSSION

3.1. Cardiac marker enzymes

Table 1 shows the activities of marker enzymes i.e., LDH, CPK, GOT, and GPT in the serum of control and experimental group of rats. Marked elevation ($p < 0.05$) in the activities of these enzymes were observed in group 2, ISO intoxicated rats. Rats pre-co-treated with the combination of FA and AA (group 8) showed significant decrease in the activities of serum marker enzymes when compared to group 6 (ISO+FA), group 7 (ISO+AA), and group 2 (ISO intoxicated) rats.

Table 1: Effect of ferulic acid and ascorbic acid on the activities of marker enzymes in serum of control and experimental group of rats.

Group	Glutamate oxaloacetate transaminase	Glutamate pyruvate transaminase	Creatine phosphokinase	Lactate dehydrogenase
Control	76.06 ± 7.43	24.49 ± 2.27	83.13 ± 8.27	976.41 ± 95.68
Isoproterenol (ISO)	137.24 ± 10.89a	47.49 ± 4.18a	220.67 ± 22.37a	1537.97 ± 150.19a
Ferulic acid (FA)	75.93 ± 5.15	24.44 ± 1.78	76.93 ±7.31	961.16 ± 95.34
Ascorbic acid (AA)	75.89 ± 2.19	24.12 ± 2.57	77.17 ± 7.70	955.10 ± 93.13
FA+AA	75.73 ± 5.54	24.11 ± 2.36	76.24 ± 12.24	940.67 ± 90.16
FA+ISO	115.61 ± 10.65ab	31.88 ± 3.11ab	121.66 ± 29.43ab	1118.93 ± 108.00ab
AA+ISO	109.50 ±10.85ab	32.66 ± 3.22ab	126.72 ± 12.36ab	1110.22 ± 10.76ab
FA+AA+ISO	84.37 ± 8.41bcd	24.96 ± 2.47bcd	79.37 ± 11.55bcd	981.33 ± 97.29bcd

Results are expressed as mean ± S.D. ($n = 6$) $p < 0.05$ compared with a Group 1 (Control), b Group 2 (Isoproterenol), c Group 6 (FA+ISO), and d Group 7 (AA+ISO). Activity is expressed as μmol of pyruvate liberated/mg of protein/h for GOT, GPT, and LDH, μmol of phosphorus liberated/mg of protein/h for CPK.

3.2. Myocardial antioxidants

Table 2 presents the activities of antioxidant enzymes (GST, GPx, SOD, and CAT) and level of GSH in the heart of control and experimental group of rats. Significant decrease in the levels of antioxidants was observed in group 2, ISO intoxicated rats when compared to group 1, control rats. Pre-cotreatment with the combination of FA and AA (group 8) significantly prevented these alterations and restored the altered levels to near normal when compared with group 6 (ISO+FA), group 7 (ISO+AA), and group 2 (ISO intoxicated) rats.

Table 2: Activities of antioxidant enzymes in the heart of control and experimental group of rats.

Group	GSH	GST	GPx	SOD	CAT
Control	2.55 ± 0.21	0.48 ± 0.10	1.99 ± 0.15	9.63 ± 0.82	14.66 ± 1.11
Isoproterenol (ISO)	0.89 ± 0.14a	0.20 ± 0.02a	1.03 ± 0.19a	4.34 ± 0.34a	8.28 ± 0.56a
Ferulic acid (FA)	2.56 ± 0.21	0.46 ± 0.12	2.05 ± 0.24	9.62 ± 0.31	14.48 ± 1.12
Ascorbic acid (AA)	2.59 ± 0.15	0.47 ± 0.14	2.00 ± 0.24	9.63 ± 0.84	14.50 ± 1.44
FA+AA	2.62 ± 0.20	0.54 ± 0.10	2.08 ± 0.20	9.65 ± 0.39	14.67 ± 1.25
FA+ISO	1.45 ± 0.18ab	0.34 ± 0.02ab	1.51 ± 0.52ab	8.03 ± 0.53ab	12.31 ± 1.24ab
AA+ISO	1.47 ± 0.20ab	0.35 ± 0.03ab	1.52 ± 0.47ab	8.09 ± 0.55ab	12.53 ± 1.15ab
FA+AA+ISO	2.51 ± 0.57bcd	0.49 ± 0.04bcd	1.95 ± 0.19bcd	9.60 ± 0.38bcd	14.40 ± 1.45bcd

Results are expressed as mean ± S.D. ($n = 6$) $p < 0.05$ compared with aGroup 1 (Control), bGroup 2 (Isoproterenol), cGroup 6 (FA+ISO), and dGroup 7 (AA+ISO). Activity is expressed as nmol/g heart tissue for GSH, μmol of GSH oxidized/min/mg of protein for GPx; units/min/mg of protein for GST; 50% inhibition of epinephrine auto-oxidation for SOD; μmol of hydrogen peroxide decomposed/min/mg of protein for CAT.

3.3. Myocardial lipid peroxides

Figure 1 indicates the level of lipid peroxides (LPO) in the heart of control and experimental group of rats. Maximum induction of LPO was observed in group 2, ISO intoxicated rats. The distorted metabolic change was significantly decreased in group 8 rats pre-co-treated with FA and AA when compared to group 6 (ISO+FA), group 7 (ISO+AA), and group 2 (ISO intoxicated) rats.

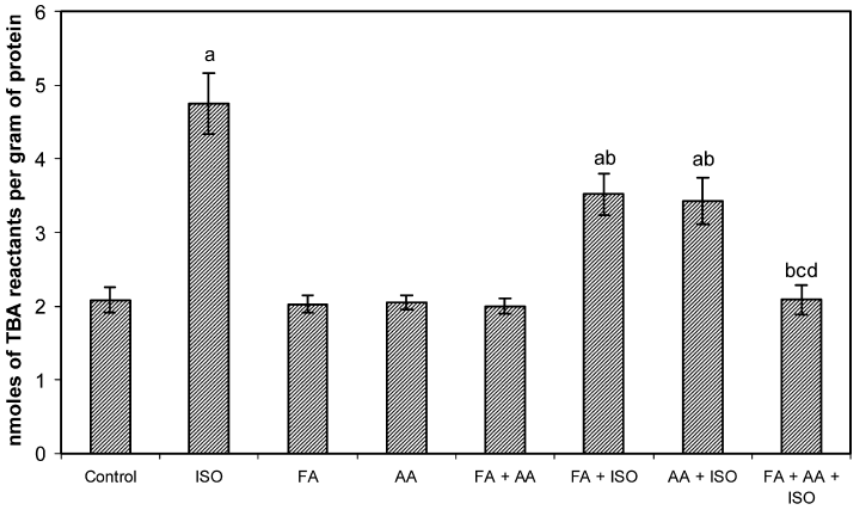
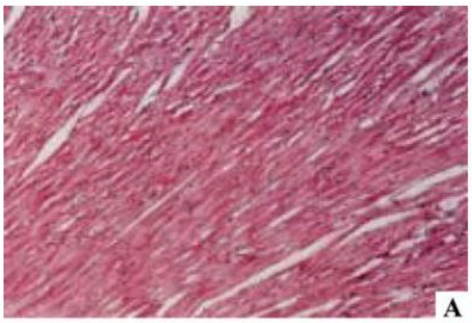


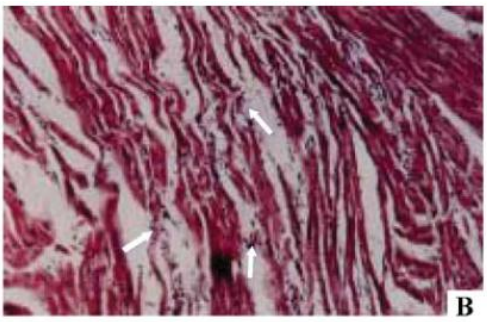
Fig. 1: Levels of lipid peroxides in the heart of control and experimental group of rats. Results are expressed as mean ± S.D. (n = 6) p < 0.05 compared with a Group 1 (Control), b Group 2 (Isoproterenol), cGroup 6 (FA+ISO), and d Group 7 (AA+ISO).

3.4. Histological examination of cardiac tissue

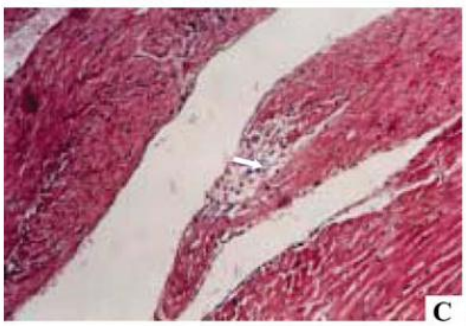
The following observations were made in the heart of control and experimental groups of rats. Figure 2A shows the architecture of normal cardiac tissue. The rats intoxicated with ISO (Fig. 2B) elicited severe cardiac damage as evidenced by pathological changes in the architecture of the heart viz. necrotic changes in the cardiac tissue with intense infiltration of leucocytes. FA pre-co-treated rats with ISO administration (Fig. 2C) showed mild subendocardial degenerative changes. AA pre-co-treated rats with ISO administration (Fig. 2D) showed separation of cardiac muscle bundles and focal areas of hyalinization with inflammatory collections. Combined pre-co-treatment with FA and AA (Fig. 2E) showed better cardioprotection as observed by the absence of adverse pathological changes in the heart of ISO-induced myocardia infarcted rats. Rats administered with the combination of FA and AA (Fig. 2F) shows normal architecture of cardiac tissue.



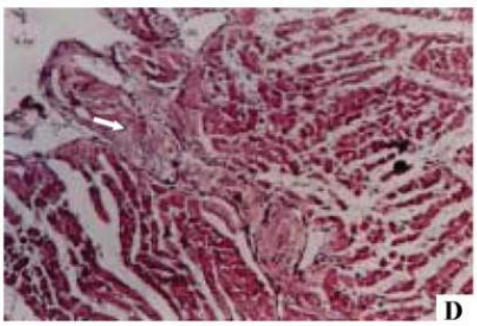
Section of heart tissue from control rat showing normal architecture



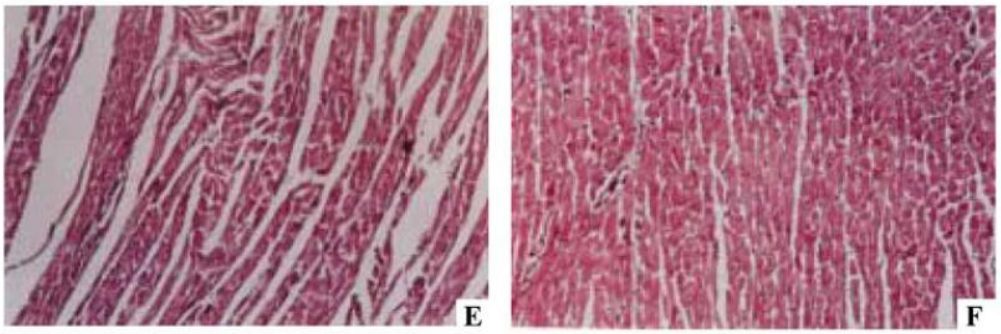
The rats given isoproterenol showed necrotic changes in the cardiac tissue with intense infiltration of leucocytes (→)



Ferulic acid and isoproterenol treated rat showing mild subendocardial degenerative changes (→)



Ascorbic acid and isoproterenol treated rat showing separation of cardiac muscle bundles and focal areas of hyalinization with inflammatory collections (→)



Ferulic acid + Ascorbic acid + Isoproterenol treated rat showing normal architecture

Ferulic acid + Ascorbic acid administered rat showing normal architecture

Fig. 2: (A–F). Histopathological studies of the heart tissue of control and experimental group of rats.

The formation of free radicals as well as accumulation of lipid peroxides has been recognized as one of the possible biochemical mechanism for the myocardial damage caused by ISO.[99] It has been reported that ISO-induced myocardial necrosis show membrane permeability alterations which bring about the loss of function and integrity of myocardial membranes.[24] Of all the macromolecules to leak from damaged tissues, enzymes, because of their tissue specificity and catalytic activity, are the best markers of tissue damage.[101]

Serum levels of CPK, LDH, and transaminases are the diagnostic indicators of myocardial infarction.[25] Increased activities of these marker enzymes in the serum are indicative of cellular damage and loss of functional integrity of cell membrane.[26] A significant increase observed in the activities of CPK, LDH, and transaminases in ISO intoxicated rats is due to the leakage of enzymes from the heart as a result of necrosis induced by ISO.[27] That is, the myocardial membrane becomes permeable or may rupture, due to deficient oxygen supply or glucose, thereby resulting in the leakage of enzymes. These molecules find their way into the blood stream, thus increasing their concentration in the serum.[28]

The combination of FA and AA seems to preserve the structural and functional integrity of the myocardial membrane as evident from the significant reduction in the elevated levels of these serum marker enzymes in the rats pre-co-treated with the combination of FA and AA when compared to the individual treatment groups, thereby establishing the cardioprotective effect of the combination of FA and AA.

Antioxidants constitute the foremost defense system that limit the toxicity associated with free radicals. Hence, these antioxidants are expected to be consumed by enhanced radical reactions.[29] Significant reduction in the levels of GSH, GPx, and GST was observed in ISO intoxicated rats. Decreased GSH level may be due to its increased utilization during the burst of reactive oxygen species production, in protecting 'SH' group containing proteins from LPO.[30] Further, the decrease in the activities of GPx and GST may be due to the reduced availability of GSH. Pre-co-treatment with the combination of FA and AA significantly prevented the alterations in the levels of GSH, GPx, and GST and restored the levels to near normalcy when compared to individual treatment groups. This effect may be due to the free radical scavenging properties of FA and AA.[31] Further AA has also been reported to preserve the intracellular concentrations of GSH that likely helps maintain nitric oxide levels and potentiates its vasoactive effects.[32] That is, AA scavenges the free radicals that inactivate nitric oxide (NO) and therefore increases the availability of NO.[33] NO and donors of NO have been shown to improve coronary and cardiac performance during coronary ischaemia and to protect against cellular damage and cytotoxicity from reactive oxygen species.[34] Nevertheless, GSH may react with NO to form S-nitroglutathione[35] which is far more potent than NO itself. Thus, AA may have a direct protecting effect both through NO pathway and as a free radical scavenger.

Activated LPO is an important pathogenic event in myocardial infarction.[36] SOD and CAT are anti-peroxidative enzymes that protect the cellular constituents against oxidative damage. A significant reduction in the activities of SOD and CAT with a concomitant increase in LPO observed ingroup 2, ISO intoxicated rats may be due to excessive generation of free radicals by ISO. Pre-co-treatment with the combination of FA and AA significantly restored the levels of SOD and CAT to near normal and concomitantly decreased LPO levels when compared to individual treatment groups. This shows the antioxidant potential of the combination of FA and AA against injury caused by free radicals.

AA has been demonstrated to be an efficient antioxidant that acts both directly by reaction with aqueous peroxyl radicals and indirectly by restoring the antioxidant properties of fat soluble vitamin E.[37] That is, AA traps peroxyl radicals in the aqueous phase and inhibits LPO. AA reacts with to copheroxyl radicals to yield tocopherol and an ascorbic radical at the surface of the cell membrane, thus regenerating reduced tocopherol and transferring the oxidative challenge to aqueous phase.[38]

Previous studies reveal that AA improves the variety of established cardiovascular risk factors, such as increased arterial stiffness, surrogate markers such as those of oxidative stress, flow-mediated dilation (or) decreased platelet aggregation may be due to the antioxidative and free-radical-scavenging effect[39] or increased NO bioavailability[40] resulting from increased NO production via upregulation of endothelial NO synthase gene expression and/or increased tetra hydrobiopterin stabilization.[41]

Earlier performed studies support the strong efficiency of FA as a scavenger of different free radicals, including anion superoxide (O2−), hydroxyl radicals (OH•), NO−, and hydroxyl and peroxyl radicals observed in several biological models. Normally, phenolic compounds act by scavenging free radicals[42] and quenching the lipid peroxidative chain. The hydroxy and phenoxy groups of phenolic compounds donate their electron to the free radicals and quench them. The phenolic radical in turn forms a quinone methide intermediate, which is excreted via the bile.[43] Thus, FA being a phenolic compound might have inhibited LPO in our study.

FA possesses three distinctive structural motifs that can possibly contribute to the free radical scavenging capability of this compound. The presence of electron donating groups on the benzene ring [3-methoxy and more importantly 4- hydroxyl] of FA gives additional resonance structures of the FA phenoxyl radical, contributing to the stability of this intermediate or even terminating free radical chain reactions.[44] The next functionality – the carboxylic acid group in

FA with adjacent unsaturated C C double bond can provide additional attack sites for free radicals and thus prevent them from attacking the membrane. In addition, the carboxylic acid group also acts as an anchor of FA by which it binds to the lipid bilayer providing some protection against LPO. Clearly, the presence of electron donating substituents enhances the antioxidant properties of FA.[45]

In a previous study, the synergistic interaction of FA with AA, in rat liver microsomal membrane model was shown, suggesting that they can cooperate in preserving the physiological integrity of cells exposed to free radicals. The combined addition of FA and AA strongly inhibited MDA production, and such an inhibition was greater than the sum of the inhibitions obtained individually by the two antioxidants. Such an effect may be explained by the fact that AA, which was less efficient than FA in inhibiting 2,2- azobis(2-amidinopropane)-induced lipid peroxidation in rat liver microsomal membranes, protected FA. This hypothesis is supported by a recent finding that the addition of AA provokeda delay of FA consumption induced by wheat peroxidase (POD).[46] This effect could occur because AA rapidly reduced the phenoxyl radicals formed by wheat peroxidase back to the initial phenol, avoiding the formation of ferulate dimers until it was completely oxidized to dehydroascorbic acid.

In our study, we have also observed, that the combination of FA and AA offered better protection to the myocardium when compared to individual treatment groups. This effect could be due to the addition of AA, whose antioxidant efficiency though less pronounced than FA[47], might have protected FA from its rapid consumption by free radicals, thereby exerting the observed synergistic protection to the myocardium. Further, the pre-co-treatment with combination of FA and AA significantly attenuated the pathological features induced by ISO as evident from the histopathological studies, thus indicating their cardioprotective effect.

In conclusion, our study reveals that the combination of FA and AA proved to be more effective in reducing the extent of myocardial damage and significantly counteracted the oxidative stress during isoproterenol induced myocardial infarction in rats.

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