Effect of ellagic acid, a natural polyphenol, on alcohol-induced prooxidant and antioxidant imbalance: a drug dose dependent study

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ABSTRACT

Introduction: Alcohol abuse, alcohol intolerance and other alcohol-related disabilities are some of the most challenging public health problems. Alcohol, by its property of generating free radicals, causes severe damage to the membrane and affects almost all organs of the human body. Ellagic acid (EA), a natural polyphenolic compound found in fruits and nuts, possess several biological properties. Our aim was to investigate, in vivo, the antioxidant potential of ellagic acid against oxidative stress induced by alcohol intoxication.

Methods: Female albino Wistar rats were used for the study. The toxicity was induced by administering 20 percent alcohol orally (7.9 g/kg body weight) for 45 days. Rats were treated with EA at three different doses (30, 60 and 90 mg/kg body weight) via intragastric intubations. The antioxidant property of EA was studied by assessing the activities of liver marker enzymes (gamma-glutamyl transferase and alkaline phosphatase), superoxide dismutase and catalase and the levels of vitamin E, vitamin C and reduced glutathione, nitric oxide (NO), protein carbonyl content (PCC), thiobarbituric acid reactive substances (TBARS) and hydroperoxides.

Results: Oxidative stress was effectively modulated by EA co-administration. EA significantly improved the status of antioxidants and decreased TBARS, hydroperoxides, NO, PCC and liver marker enzymes at the dose of 60 mg/kg body weight when compared with the alcohol-treated group.

Conclusion: The study provides the anti-oxidant and cytoprotective properties of EA at a dose of 60 mg/kg body weight against oxidative stress induced by alcohol.

Keywords: alcohol-induced prooxidant, antioxidant, ellagic acid, lipid peroxidation, liver marker enzymes, polyphenol

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INTRODUCTION

Alcohol-related disorders are one of the challenging current health problems with far reaching medical, social and economic consequences. Long-term alcohol use potentially results in serious illnesses, including alcoholic fatty liver, hypertriglyceridaemia, cirrhosis, cardiovascular disease and inflammation of the pancreas.[1] One of the factors that play a central role in many pathways of alcohol-induced damage is oxidative stress. Oxidative stress in the cells or tissues refers to the enhanced generation of reactive oxygen species (ROS) and/or depletion in the antioxidant defence system, causing an imbalance between prooxidants and antioxidants.[2] Excessive production of oxygen radicals leads to altered enzyme activity, decreased DNA repair, impaired utilisation of oxygen, lipid peroxidation (LPO) and protein oxidation. Some of these alterations induced by oxidative stress have been recognised to be characteristic features of necrosis and subsequently leads to organ damage.[3]

Recently, there is growing interest in understanding the role and mechanism of the phytochemicals: polyphenolics, flavonoids and phenyl propanoids as inhibitors of oxidative stress. Among all phytochemicals, ellagic acid (EA) (Fig. 1) has been receiving the most attention because of its wide array of biological properties, such as radical scavenging, chemopreventive, antiviral and antibacterial properties.[4] It is mostly abundant in berries, walnuts, pecans, pomegranate, cranberries and other plant foods in the forms of hydrolysable tannins called ellagitannins.[5,6] The main focus of the present communication was to evaluate the antioxidant and antilipoperoxidative properties of EA against alcohol-induced toxicity.

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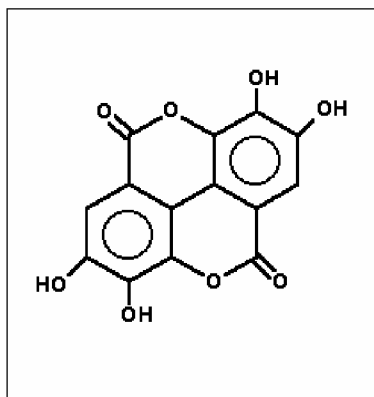Fax: (91) 4144 239 141
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Fig. I Diagram shows the molecular structure of ellagic acid.

METHODS

30 female albino rats, Wistar strain, of body weight ranging from 150 g to 170 g, were bred in the central Animal House, Rajah Muthiah Medical College, Tamil Nadu, India. The animals were housed in polypropylene cages (47 cm × 34 cm × 18 cm) in an air-conditioned room with controlled temperature (25 ± 2°C) and automatic lighting (alternating 12-hour periods of light and dark). The animals were fed on the standard pellet diet (Hindustan Lever Limited, Mumbai, India), and water was given *ad libitum*. The standard pellet diet comprised 21% protein, 5% lipids, 4% crude fibre, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamins and 55% nitrogen-free extract (carbohydrates). It produces a metabolisable energy of 3,600 kcal. The animals used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council for Medical Research, Hyderabad, India and approved by the Animal Ethical Committee, Annamalai University (Reg. No.160/1999 CPSEA) (Approval number: 248, dt. 16.12.2004). Absolute ethanol used in our study was obtained from Hayman Private Limited, England and ellagic acid was purchased from Sigma Chemical Company, St Louis, USA. All other chemicals and solvents used were of analytical grade.

The animals were categorised into five groups (n = 6). Group I: control; Group II: 20% ethanol (7.9 g/kg body weight)[7] with 0.2% dimethyl sulphoxide (DMSO); Group III: 20% ethanol + EA (30 mg/kg body weight); Group IV: 20% ethanol + EA (60 mg/kg body weight) and Group V: 20% ethanol + EA (90 mg/kg body weight). The rats in the control group received glucose solution which is equal to the isocalorific value to ethanol (14.025 g/kg body weight) along with 0.2% of DMSO via intragastric intubations. EA was dissolved in 0.2% DMSO and co-administered with 20% ethanol to all the three EA groups. At the end of the experimental period of 45 days, the rats were kept overnight fasting and sacrificed

by cervical dislocation after anaesthetising the animals with intramuscular injections of ketamine hydrochloride (30 mg/kg body weight). The liver was excised, cleared of blood and immediately transferred to ice-cold containers containing 0.9% NaCl for assessing the biochemical parameters.

Blood was collected in a heparinised tube and plasma was separated by centrifugation at 1,000 g for 15 minutes for the estimation of liver marker enzymes. Liver samples were homogenised in 0.1 M Tris-HCl buffer (2 ml/100 mg tissue), pH 7.4. The homogenate was centrifuged at 3,000 rpm for five minutes and the supernatant was used for the estimation of biochemical parameters. The total protein in the tissue extract was determined after trichloroacetic acid precipitation by the method of Lowry et al.[8] To assess the membrane damage, the activities of liver marker enzymes: γ-glutamyl transferase (GGT) by the method of Fiala et al,[9] and alkaline phosphatase (ALP) by the method of King and Armstrong,[10] were assayed. ALP activity was assayed using disodium phenyl phosphate as substrate. After preincubation of the buffer (0.1 M bicarbonate buffer pH 10) with substrate for ten minutes, 0.2 ml of serum was added and incubated for 15 minutes at 37°C. The liberated phenols from the substrate was reacted with Folin-Phenol reagent (1 ml). The suspensions were centrifuged and the supernatant was collected. 2 ml of 10% sodium bicarbonate was added to the supernatant and the colour developed was read at 680 nm after ten minutes.

GGT was analysed by adding 2 ml buffer (Tris-HCl 120 mm, MgCl₂ 12 mM, glucyl glycine 90 mM, pH 7.8) to 0.2 ml substrate (L-γ-glutamyl-p-nitroanilide 48 mm in 150 mM HCl), warmed to 37°C. 0.1 ml serum was added, mixed and incubated at 37°C. The reaction was then stopped by adding 2 ml of glacial acetic acid and the absorbance was read at 450 nm. The extent of LPO was determined by analysing the levels of thiobarbituric acid reactive substances (TBARS) by Niehaus and Samuelsson[11] and hydroperoxides (HP) by Jiang et al.[12] To the tissue homogenate, TBA TCA-HCl in the ratio of 1:1:1 (0.37% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl) was mixed and placed in a water bath for 15 minutes, cooled and centrifuged at room temperature for ten minutes at 1,000 rpm. The absorbance of clear supernatant was measured against a reference blank at 535 nm.

For HP, 0.1 ml of tissue homogenate was treated with fox reagent (butylated hydroxytoluene), xylenol orange and ammonium ion sulphate were added to 90 ml of methanol and 10 ml of 250 mM sulphuric acid, and incubated at 37°C for 30 minutes. The colour developed was read at 560 nm colorimetrically. The level of nitric oxide (NO) was estimated by the method of Lepoivre et al.[13] To 0.5 ml of tissue homogenate, 0.1 ml
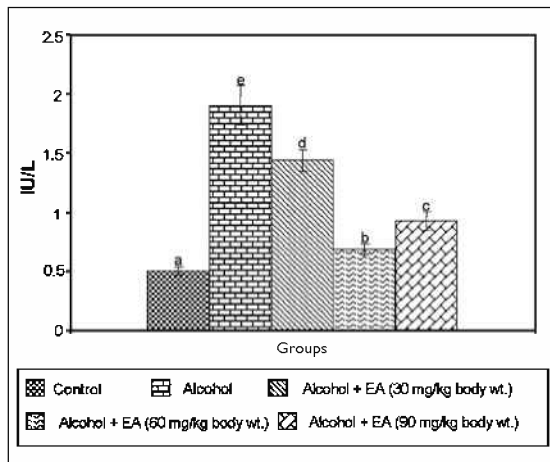
Fig. 2 Bar chart shows the activities of GGT in plasma (values are mean ± S.D from 6 rats in each group). ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at $p \leq 0.05$.
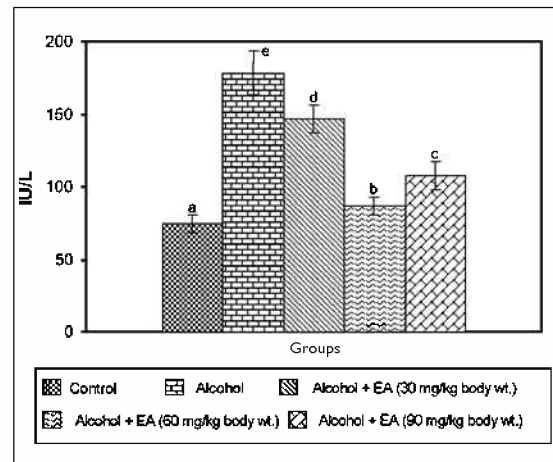

Fig. 3 Bar chart shows the activities of ALP in plasma (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at $p \leq 0.05$.

Fig. 4 Bar chart shows the levels of TBARS in tissues (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at $p \leq 0.05$.
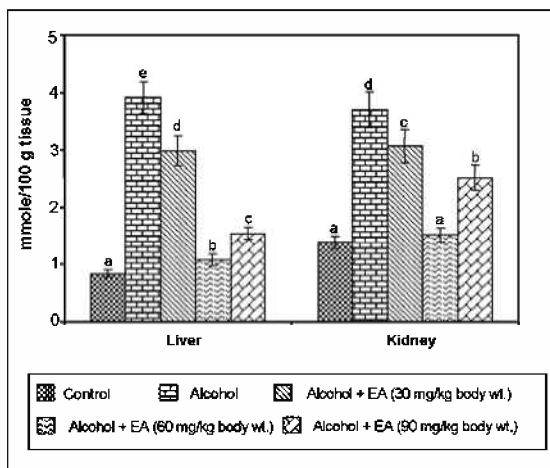

Fig. 5 Bar chart shows the levels of HP in tissues (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at $p \leq 0.05$.
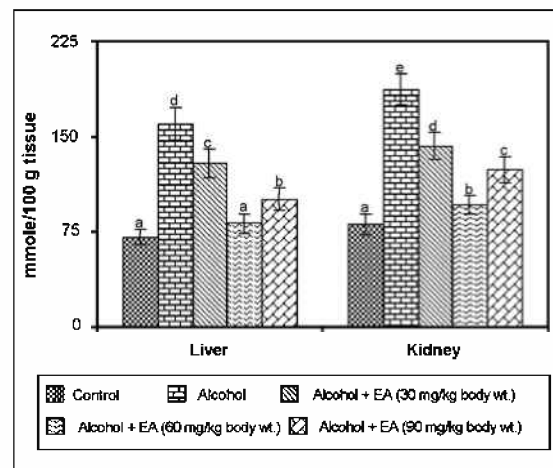
of sulphosalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. The protein-free supernatant was used for the estimation of nitrite levels. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris-HCl buffer and mixed well. To this, 530 µL of Griess reagent was added and incubated in the dark for 10–15 minutes and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

Reduced glutathione (GSH) was determined by the method of Ellman.[14] To the homogenate, 10% TCA

was added and centrifuged. 1.0 ml of the supernatant was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5,5'-dithiobisnitrobenzoic acid in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm. Vitamin E was estimated by the method of Baker et al.[15] To 0.5 ml of tissue homogenate, 1.5 ml of ethanol and 2.0 ml of petroleum ether were added and centrifuged. The supernatant was evaporated to dryness at 80°C, and then 0.2 ml of 2,2'-dipyridyl solution and 0.2 ml of ferric chloride solution were added. This was mixed well, kept in the dark for five minutes, and 4 ml of butanol was added. The intense red colour developed was read at 520 nm. Vitamin C was estimated by the
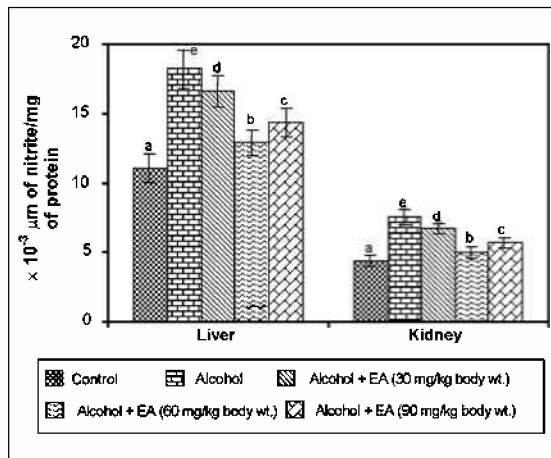
Fig. 6 Bar chart shows the levels of NO in tissues (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at p ≤ 0.05.
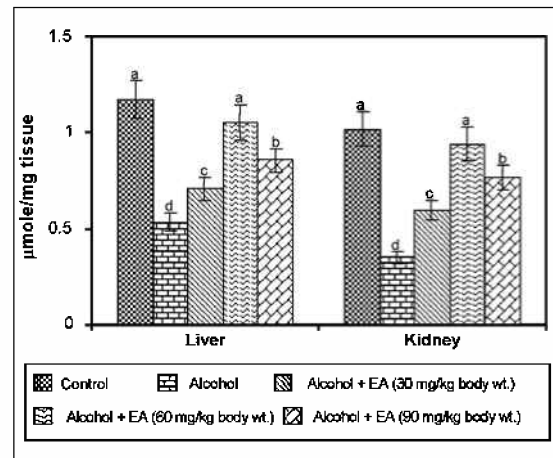

Fig. 7 Bar chart shows the levels of vitamin C in tissues (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at p ≤ 0.05.

method of Roe and Kuether.[16] To 0.5 ml of plasma, 1.5 ml of 6% TCA was added and allowed to stand for five minutes and centrifuged. The supernatant was collected and 0.3 g of acid-washed norit was added, shaken vigorously and filtered. 2 ml of the filtrate was taken and 0.5 ml of dinitrophenyl hydrazine (DNPH) was added, stoppered and placed in a water bath at 37°C for exactly three hours. After the incubation, the tubes were placed in an ice bath and 2.5 ml of 85% H_2SO_4 was added drop by drop. The contents of tubes were mixed well and allowed to stand at room temperature for 30 minutes. The colour developed was read at 540 nm.

Superoxide dismutase (SOD) was assayed utilising the technique of Kakkar et al.[17] 0.5 ml of tissue homogenate was mixed with ethanol and chloroform mixture and centrifuged. To the supernatant, assay mixture (sodium pyrophosphate buffer [0.025 M, pH 8.3], phenazine methosulphate, nitroblue tetrazolium and reduced nicotinamide adenine dinucleotide [NADH]) was added and incubated at 30°C for 90 s. The reaction was stopped by the addition of glacial acetic acid and mixed with n-butanol. The intensity of the chromogen in the butanol was measured at 560 nm. Catalase (CAT) was assayed colorimetrically at 620 nm and expressed as μmole of H_2O_2 consumed/min/mg protein as described by Sinha.[18] The reaction mixture contained phosphate buffer (0.01 M, pH 7.0), tissue homogenate and 2 M H_2O_2. The reaction was stopped by the addition of dichromate-acetic acid reagents (5% potassium dichromate and glacial acetic acid were mixed in a ratio of 1:3).

The protein carbonyl content (PCC) was determined by the method of Levine et al.[19] The sample was divided into two portions containing 1–2 mg protein each. To one portion, an equal volume of 2 M HCl was added

and incubated at room temperature for one hour, and shaken intermittently. The other portion was treated with an equal volume of 10 mM DNPH in 2 M HCl and incubated for one hour at room temperature. After incubation, the mixture was precipitated with 10% TCA and centrifuged. The precipitate was washed with ethanol:ethyl acetate (1:1), twice dissolved in 1 ml of 6 M guanidine HCl, centrifuged at low speed and the supernatant was taken. The difference in absorbance between the DNPH-treated and HCl-treated samples was determined at 366 nm.

Statistical analysis was performed by one-way analysis of variance (ANOVA) and the groups were compared by Duncan's multiple range test (DMRT) using the Statistical Package for Social Sciences version 11.0 (SPSS Inc, Chicago, IL, USA). Results were expressed as mean ± standard deviation for six rats in each group. A value of p ≤ 0.05 was considered to be statistically significant.

RESULTS

The activities of hepatic marker enzymes in plasma are shown in Figs. 2 and 3. The activities of GGT and ALP were significantly increased in the alcohol-administered group, which were decreased significantly on treatment with the three doses of EA. The decrease was more significant in the 60 mg/kg body weight-treated group, compared to the 90 mg/kg and 30 mg/kg body weight groups. The levels of lipid peroxidative indices are shown in Figs. 4 and 5. The levels of TBARS and HP were significantly increased in the alcohol-administered rats. Co-administration of EA resulted in a significant decrease in the levels of TBARS and HP in both liver and kidney. The levels of NO in the liver and kidney are shown in
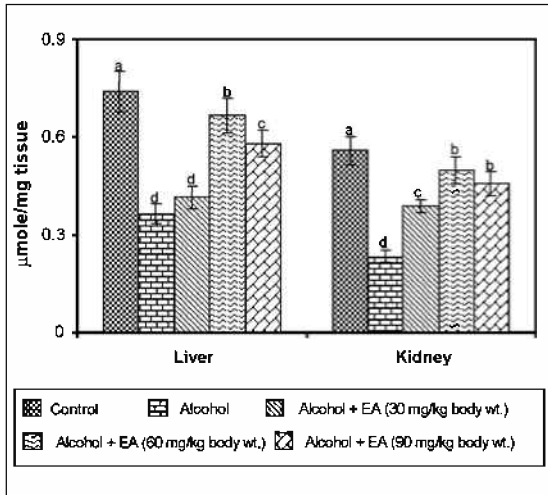
Fig. 8 Bar chart shows the levels of vitamin E in tissues (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at $p \leq 0.05$.
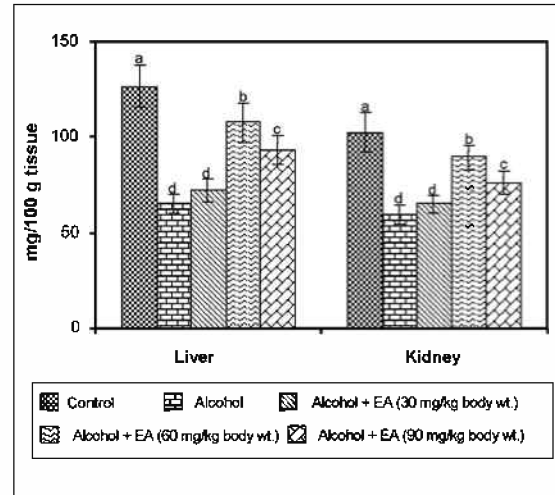
Fig. 9 Bar chart shows the levels of GSH in tissues (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at $p \leq 0.05$.

Fig. 10 Bar chart shows the activities of SOD in tissues. Unit* - enzyme reaction which gives 50% inhibition of NBT reduction/minute (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at $p \leq 0.05$.
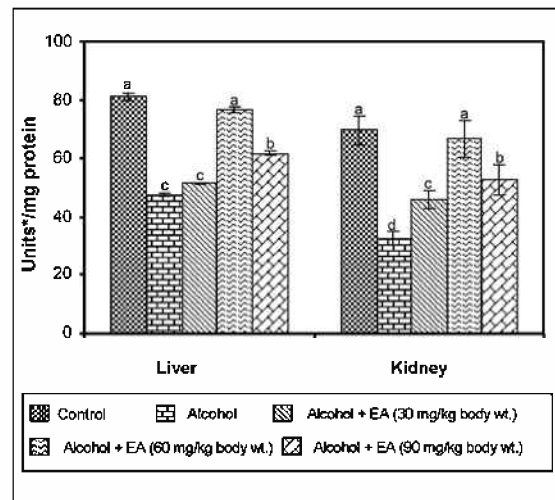
Fig. 11 Bar chart shows the activities of CAT in tissues. Unit* - μmoles of H_2O_2 liberated/minute (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at $p \leq 0.05$.
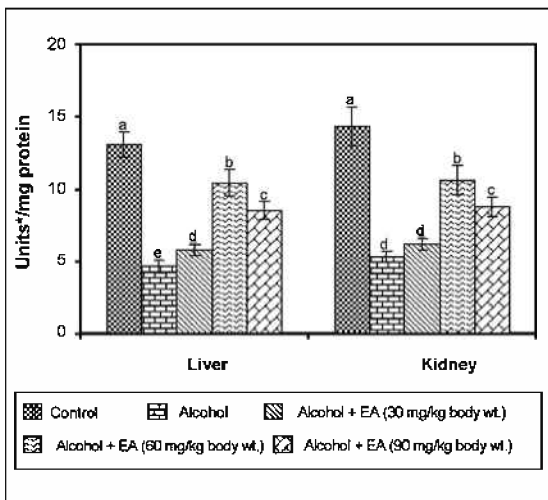
Fig. 6. The levels of NO were significantly increased in the alcohol-fed rats when compared to the rats in the control group. Treatment with EA restored the levels of NO to near normal.

The levels of non-enzymatic antioxidants: vitamin C (Fig. 7) and vitamin E (Fig. 8), GSH (Fig. 9) and activities of enzymatic antioxidants, SOD and CAT (Figs. 10 & 11), were significantly depleted in the alcohol groups, which were positively modulated by EA treatment. Better positive modulation was observed in the group treated with 60 mg/kg body weight of EA, when compared to the other two doses (30 and 90

mg/kg body weight). The levels of PCC in liver and kidney are shown in Fig. 12. The alcohol-administered group showed a significant increase in the levels of PPC in the liver and kidney. Treatment with EA (60 mg/kg body weight) significantly decreased the levels of protein carbonyl groups, when compared to the other two doses (30 and 90 mg/kg body weight).

DISCUSSION

Oxidative stress has been related to several alcohol-related illnesses including cancer, liver pathology, myopathy, cerebellar atrophy, testicular injury and

Fig. 12 Bar chart shows the levels of PCC in tissues (values are mean ± S.D from 6 rats in each group) ANOVA followed DMRT. Bars not sharing the common superscript differ significantly at $p \leq 0.05$.
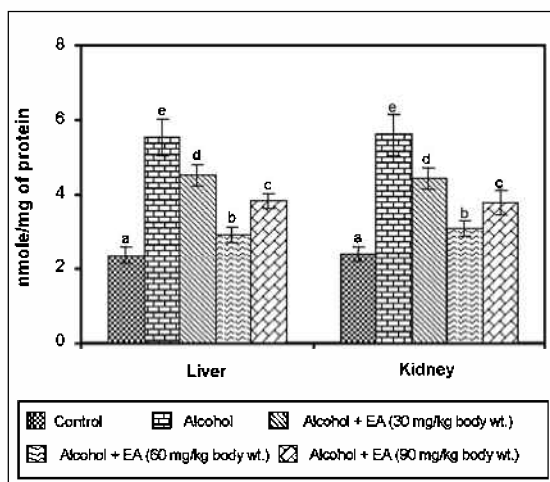
immunosuppression.[20] In our study, we observed a significant increase in the activities of liver marker enzymes (indices of hepatic dysfunction), i.e. ALP and GGT, during chronic alcohol-administration. Ethanol metabolism via alcohol dehydrogenase and CYP2E1, results in the formation of cytotoxic aldehyde, which in turn is oxidised to acetate by aldehyde oxidase or xanthine oxidase, giving rise to ROS, which can damage the biomembrane, resulting in leakage of liver marker enzymes into the circulation.[21] Moreover, various pathways play a role in ethanol-induced tissue injury, including changes in cellular nicotinamide adenine dinucleotide (NAD^+)/NADH,[22] production of acetaldehyde, induction of CYP2E1, formation of 1-hydroxyethyl radicals, ethanol-mediated mitochondrial damage,[23] endotoxin-derived activation of Kupffer cells, and subsequent production of tumour necrosis factor-α.[24] From the above suggestive mechanisms, we could expect that the activities of GGT and ALP to be significantly elevated in the alcohol group.

Treatment with EA (30, 60 and 90 mg/kg body weight) effectively decreased the activities of GGT and ALP in the plasma. This can be attributed to the antioxidant property of EA. It is reported that phenolic compounds can act by scavenging free radicals.[25] EA, being a phenolic compound and an antioxidant,[26] quenches free radicals and inhibits LPO. Moreover, EA has been shown to inhibit the biotransformation of ethanol to acetaldehyde[27] and consequently decreases ROS formation through aldehyde oxidase or xanthine oxidase. Furthermore, it has been reported that EA decreases the liver marker enzymes during CCl_4-

induced toxicity.[28] Hence, it could be suggested that the leakage of enzymes from the hepatocellular membrane is decreased by the liver cell membrane-stabilising action of EA.

Oxidative injury induced by alcohol can be monitored in experimental animals by detecting lipid peroxidative products such as lipid TBARS and HP.[29] In our study, we observed increased levels of TBARS and HP in the liver and kidney of the alcohol-administered group. Ethanol administration results in excessive generation of free radicals such as hydroxy ethyl radical, superoxide radical ($O2^{-}$), hydroxyl radical ($OH^{\cdot}$), peroxyl radical and hydrogen peroxide.[2] All these radicals formed from the ethanol-mediated process have a great potential to react rapidly with lipids which in turn leads to LPO.[21] It is generally accepted that the enhanced LPO is one of the toxic manifestations of ethanol ingestion. Furthermore, extensive damage to tissue via free radicals-mediated LPO can result in membrane disorganisation and subsequently decreases the membrane fluidity.[30] Thus, the increased activities of GGT and ALP in our study are suggestive of severe hepatic injury during alcohol ingestion.

Treatment with EA resulted in significant decreased levels of TBARS and HP. It has been shown that two lactone groups of EA (Fig. 1) can act as a hydrogen bond donor and acceptor,[31] which might be involved in the free radical scavenging action and decreased free radicals-mediated LPO. Further studies have shown that EA suppresses LPO in necrosis of skin flaps,[32] possibly by its antioxidant action. Thus, EA effectively quenches free radicals, inhibits LPO and protects tissues from damage. NO plays an important role in various kinds of tissue injury either directly or by interacting with reactive oxygen intermediates to form a more toxic species.[33] NO is produced in Kupffer and endothelial cells during endotoxaemia and inflammation. Excessive NO can inactivate antioxidants and antioxidases by reacting with some active groups in their molecular structure, and also harm the function of the mesangial cells in the kidney.[34] It has been reported that alcohol intoxication stimulates the immune system, which in turn leads to enhanced NO production.[35] Thus, consistent with the above report, we observed increased levels of NO in the liver and kidney during chronic administration of alcohol.

Treatment with EA effectively decreased the NO levels in liver and kidney. Generally, phenolic compounds effectively scavenge free radicals and inhibit NO production,[36] independent of their antioxidant properties. EA, being a phenolic compound, may have inhibited NO production. Furthermore, EA has been reported to inhibit the elevation of NO production induced by endrin.[37] Thus, the above reports show

that EA with its free radical-scavenging property may have inhibited NO production. ROS production is a naturally-occurring process; a variety of enzymatic and non-enzymatic antioxidants are involved to protect cells against ROS. Vitamin E, a major antioxidant, found in the lipid phase of membrane, acts as a powerful terminator of LPO. Vitamin C plays a vital role in the defence against oxidative stress.[38] In our study, we observed decreased levels of vitamins C and E in both liver and kidney of alcohol-treated rats. Studies have shown that chronic alcoholics are deficient in vitamin E and vitamin C.[39,40]

GSH is a tripeptide (L-γ-glutamylcysteinylglycine), an antioxidant and a powerful nucleophile, critical for cellular protection such as detoxification of ROS, conjugation and excretion of toxic molecules and control of inflammatory cytokine cascade.[41] Depletion of GSH in tissues leads to impairment of the cellular defence against ROS, and may result in peroxidative injury. The levels of GSH were significantly decreased in alcohol-treated rats. Our findings are consistent with the other published reports, which show that GSH concentration is decreased during ethanol ingestion.[42,43] SOD is considered as the first line of defence against the deleterious effects of oxygen radicals in the cells and it scavenges ROS by catalysing the dismutation of superoxide to H_2O_2.[44] There is evidence to indicate that ethanol significantly depresses SOD activities.[2] The inhibition of SOD activity may result in an increased flux of superoxide in cellular compartments which may be the reason for the increased lipid peroxidative indices in our study.

Catalase acts as a preventive antioxidant and plays an important role in the protection against the deleterious effects of LPO. Reports have shown that there is a significant decrease in the activities of catalase.[2,45] Our results are also in correlation with the above observations. Thus, the analysis of antioxidant status in our study indicates that both non-enzymatic and enzymatic antioxidants were decreased due to alcohol-induced toxicity. Co-administration of EA with alcohol significantly modulates the antioxidant status in tissues, suggesting the enhancing effect of EA on cellular antioxidant defences. The antioxidant mechanism of EA may include the following interventions: scavenging of $O2^{\cdot-}$, $OH^{\cdot}$, peroxy radical and peroxynitrite,[46] and decreasing activities of cytochrome P450, particularly CYP2E1.[47] In vitro studies have shown that EA effectively scavenges free radicals and the effect was more potent than α-tocopherol.[48] Further, reports suggest that EA also enhances the GSH-dependent protection.[49] Thus, EA might have quenched free radicals and inhibited LPO, and ultimately decreased the burden to

antioxidants. In addition to cellular lipids, studies have shown that cellular proteins may also be affected by free radical accumulation. The formation of carbonyl derivatives of proteins is suggested to be a useful measure of oxidative damage to proteins.[50] The carbonyl derivatives of proteins may result from oxidative modification of amino acid side chains and reactive oxygen-mediated peptide cleavage. Further, Remmer et al have reported that the primary target of the oxygen-radical attack, promoted by ethanol, is represented on cellular proteins.[51] Our results also support the above report by showing increased PCC in the liver and kidney of the alcohol group.

Treatment with EA significantly decreased the levels of PCC, which may be due to the antioxidant property of EA. EA, by its free radical scavenging action, would prevent the attack of free radicals on amino acids and thus diminish the production of the carbonyl group in EA-treated rats. In our study, all the three doses of EA did not produce the same effect. The low dose of 30 mg/kg body weight was not effective, because its concentration might have been too low and in sufficient to quench all free radicals generated by alcohol. The high dose (90 mg/kg body weight) was also not as effective as the medium dose (60 mg/kg body weight), because at higher concentration, EA may react with some ligands in the system and thus might not be completely available for the quenching of free radicals.

In conclusion, our study has demonstrated that oral administration of EA protects the system from alcohol toxicity by decreasing the liver marker enzymes, lipid peroxidative markers, NO, PCC, and increasing the antioxidant cascade in a dose dependent manner. Among the three doses of EA, 60 mg/kg body weight was found to be the most effective in modulating the biochemical changes during alcohol-mediated tissue injury. Hence, EA can be developed as a drug against alcohol-related disabilities in the near future.

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