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Original article

Effect of eugenol on oxidative stress, membrane ATPase and glyoxylase system in ethanol fed rats

S. Anbu and J. Padma*
Department of Biochemistry, Sacred Heart College (Autonomous), Tirupattur, Vellore-635 601, Tamil Nadu, India
**Agricultural officer, Government Seed Testing Laboratory, Tiruvannamalai-606 604, Tamil Nadu, India*

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Abstract

Mitochondrial oxidative stress play a crucial role in the development of alcoholic liver disease (ALD). The present study was aimed to evaluate the effect of eugenol on mitochondrial oxidative stress, antioxidants, glyoxylases system and membrane ATPase ethanol fed rats. Adult male albino wistar rats of body weights 150-170 g were divided into four groups and expriment was carried for a period of 45 days. Group I: control rats received isocaloric glucose alone, Group II: control rats received isocaloric glucose plus eugenol (10.0 mg/kg/day), Group III: ethanol alone (6 g/kg/day), Group IV: ethanol administrated rats treated with eugenol (10 mg/kg.b.w/day) for the past 30 days. After the exprimental period, the rats were sacrificed and tissue samples used to obtain mitochondrial fraction. Ethanol fed rats showed marked elevation in lipid peroxidation markers, decreased in antioxidants, membrane ATPase in liver mitochondrial fraction and glyoxylase system in liver. Coadministration of eugenol for last 30 days with ethanol caused significantly alters these changes to normal. These results were also confirmed by histopathological observation, which also showed steastosis and the same was controlled in ethanol fed rats, treated with eugenol. The study reveals the beneficial effects of eugenol in ethanol fed rats possibly *via* inhibition of mitochondrial oxidative stress.

Key words: Eugenol, mitochondrial oxidative stress; alcoholic liver disease, glyoxylase I and II

1. Introduction

Alcohol is one of the most abused drugs world-wide and chronic alcohol consumption produces hepatocellular injury and damage (Diehl, 2002). Hepatotoxicity induced by alcohol is mediated by complex mechanisms that involves the induction of CYP2E1 enzyme system, generates reactive oxygen species (ROS), such as hydroxyradicals, superoxide, 1-hydroxy ethyl radical and others (Albano, 2006). ROS can readily react with cellular macromolecules, causing damage to proteins, lipids and nucleic acids which ultimately leads to loss of structural and functional integrity at the cellular level (De Minicis and Brenner, 2008). One of the earliest effect of ethanol consumption on the liver is change in the structure of mitochondria. The mitochondria are often enlarged and distorted, appearing as swollen with the cristea often disrupted, decreases the capacity of mitochondria to carry out the mitochondrial protein synthesis, which make them less functional. The net results involve the decrease in the rate of ATP synthesis, antioxidants depletion, diminished glutathione transport, oxidative modifications of mitochondrial DNA and finally cellular injury (Fatiha and Jamal, 2014). All these alterations promote both apoptotic and necrotic cell death, thus contributing the progression of alcoholic liver disease (Hoek *et al*., 2002). Oxidative degradation of lipids could generate reactive aldehydes and oxoaldehydes that can alter the metabolic processes and cell signaling (Cotham *et al*., 2004). Aldehydes are shown to play an important role in the induction and progression of oxidative injury. These are removed by the glyoxalases I and II, which catalyze the formation of non-toxic α-hydroxyl acid from these aldehydes (Fu *et al*.,1996).

The hepatoprotective properties of various phytochemicals have been demonstrated in experimental animals (Anbu *et al*., 2016; Anbu and Saravanan, 2013). The chosen phytochemical, eugenol (4-allyl-1- hydroxyl-2- methoxy benzene), is the active principle of plants such as ocimum, clove, cinnamon, basil and nutmeg. It is considered as non-mutagenic, non-carcinogenic and is generally recognized as safe (GRAS) by Food and Drug Administration at a level of 0.3-1.25%, and is used to impart flavor at the level of 0.01% and bakery and dairy industry (Opdyke, 1975). Eugenol has been shown to possess antioxidant and antibacterial properties (Ito *et al*., 2005). The hepatoprotective action of eugenol against experimental CCl_4 intoxication in rats has been demonstrated (Nagababu and Lakshmaiah, 1992). Our previous study has shown the protective effect of eugenol against alcoholic liver disease (Anbu and Anuradha, 2012). However, the possible protective mechanism yet remains unclear. Hence, the present study was undertaken to evaluate the impact of eugenol on mitochondrial oxidative oxidative stress, antioxidans, membrane ATPase and glyoxylase system in ethanol fed rats.

Author for correspondence: Dr. S. Anbu
Assistant Professor, Department of Biochemistry, Sacred Heart College (Autonomous), Tirupattur, Vellore-635 601, Tamil Nadu, India
E-mail: anbu@shctpt.edu
Tel.: +91-9787727941

2. Materials and Methods

2.1 Animals

Male albino wistar rats (150-180 g) were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital, Chidambaram, Tamil Nadu, India. The animals were housed in plastic cages in a well ventilated room and maintained in a 12 h light/12 h dark cycle. The animals had free access to standard pellet diet (Pranav Agro Industries Ltd, Bangalore, India) and water *ad libitum*. The study was approved by Institutional Animal Ethics Committee, Rajah Muthiah Medical College and the study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Vide letter No: 160/1999/CPCSEA-385).

2.2 Experimental design

The animals were divided into 4 groups of 6 rats each.

Group I: Control rats received glucose from a 40% stock glucose solution twice daily, which was isocaloric to ethanol.

Group II: Control rats received eugenol (10 mg/kg body weight in olive oil) daily for 45 days along with 40% stock glucose solution twice daily, which was isocaloric to ethanol.

Group III: Rats received ethanol (6 g/kg body weight) from a 30% stock solution twice daily for 45 days.

Group IV: Rats received eugenol (10 mg/kg body weight in olive oil) from the15th day along with ethanol (6 g/kg body weight).

After the experimental period of 45 days, the animals were fasted over night, anesthetized with ketamine hydrochloride (30 mg/kg, i.p) and then sacrificed by cervical decapitation. Liver was dissected out, washed in ice-cold saline, weighed and homogenate was prepared in 0.1M Tris-buffer pH 7.4.

2.3 Biochemical assays

2.3.1 Preparation of mitochondrial fraction

Mitochondra was isolated by differential centrifugation following the method of Johnson and Lardy (1967). The liver tissues were quickly removed and washed in ice-cold saline. A 10% liver homogenate was prepared in 10 mM Tris-HCl, pH 7.4 containing 0.25M sucrose. The homogenate was centrifuged at 600 x g for 10 min at below 4°C and the supernatant was centrifuged at 10,000 x g for 20 min at 4°C. The pellets were washed with ice-cold 0.15 M KCl and after washing, they were suspended in the buffer solution. The protein content was measured by the method of Lowry *et al.* (1951) and succinate dehydrogenase (SDH) activity by Slater and Bonner (1952).

2.3.2 Oxidative stress markers

The extent of lipid peroxidation was determined by analyzing the levels of thiobarbituric acid reactive substances (TBARS) by Niehaus and Samuelsson (1968) and hydroperoxides (HP) by Jiang *et al.* (1992). For TBARS assay, TBA-TCA-HCl containing (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl in 1:1:1 ratio) was mixed with 0.1 ml of mitochondrial fraction and placed in a water bath for 15 min, cooled and centrifuged at 1000 x g for 10 min. The absorbance of clear supernatant was measured against a reagent blank at 535 nm. For hydroperoxides, 0.2 ml of the mitochondrial fraction was treated with FOX reagent containing 88 mg butylated hydroxytoluene, 7.6 mg of xylenol orange and 9.8 mg of ammonium iron (II) sulphate in 90 ml of methanol and 10 ml of 250 mM sulphuric acid. The mixtures were incubated at 37ºC for 30 min and the colour developed was read at 560 nm. The S-nitrosothiol and protein carbonyl contents were estimated by the method of Cook *et al.* (1996) and Lavine *et al.* (2000).

2.3.3 Estimation of enzymic and nonenzymic antixidants system

The enzymic (superoxide dismutase, gluathione-s-tranferase and glutathione peroxidase) and non-enzymic antioxidants (glutathione reductase, vitamin C and E) were measured by the methods described in detail elsewhere (Anbu and Saravanan, 2013).

2.3.4 Membrane ATPase

The activities of total ATPase, Na^+, K^+-ATPase, Ca^{2+} and Mg^{2+} ATPases were assayed by Evans (1969), Bonting (1970), Hjertan and Pan, (1983) and Ohinishi *et al.* (1982), respectively.

2.3.5 GSH and the glyoxalase system

Reduced glutathione (GSH) was determined by the method of Ellman (1959). Glyoxalase I was assayed in the liver homogenate by measuring the rate of formation of S-D-lactoylglutathione (Thoranelly, 1993). The assay mixture contained 7.9 mM methyl glyoxal (MG), 1mM GSH and 14.6 mM magnesium sulfate and 182 mM imidazole HCl, pH 7.0. After 5 min. 0.1 ml of sample (50 µg protein) was added and increase in absorption at 240 nm was measured and the activity was calculated using the coefficient 2.86 /mM/cm. The enzyme activity is calculated as µ mole/g/min of the product formed. One unit of the enzyme is defined as the amount of enzyme catalyzing the formation of 1µ mol of S-D-lactoylglutathione/min/mg protein under the assay conditions. Glyoxalase II was assayed by measuring formation of GSH from S-D-lactoylglutathione (Martins *et al.*,1999).The reaction was started by the addition 0.5 mL of 1.5 mM S-D-lactoylglutathione to 0.1 mL of sample and GSH formation was measured after 15 min by reaction with 0.75 mM DTNB. The activity was expressed as m mol GSH formed/min/mg protein.

2.3.6 Histopathological investigation

For histopathology study, liver tissue was excised from two animals in each group, washed and placed in 10% formalin. They were later sectioned with a microtome, dehydrated in alcohol, embedded in paraffin and stained with hematoxylin and eosin (H and E).

2.4 Statistical analysis

Results are presented as Mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a statistical software package (SPSS for Windows, V. 13.0, Chicago, IL, USA). Probability values at $p < 0.05$ were considered to be statistically significant.

3. Results

3.1 Oxidative stress

The concentration of TBARS, hydroperoxides, nitrosothiol and iron in mitochondrial fractions of control and experimental rats were given in Table 1. TBARS, hydroperoxides, nitrosothiol and iron levels were significantly higher in ethanol treated rats (Groups III)

as compared to those of the experimental control rats (Group I) ($p < 0.05$). Whereas, ethanol fed rats treated with eugenol (Group IV) significantly lowered these levels when compared to the ethanol alone treated rats (Groups III). Treatment with eugenol to control rats (Group I) did not shown any alterations.

Table 1: Effect of eugenol on lipid peroxidative markers, nitrosothiol and iron in mitochondrial fraction

Parameters	CON	CON + EUG	EtOH	EtOH +EUG
TBARS (n moles/ mg proteins	0.93 ± 0.08^a	0.94 ± 0.06^a	3.13 ± 0.20^b	1.46 ± 0.84^c
LPH (n moles/ mg proteins)	1.96 ± 0.86^a	1.93 ± 0.07^a	3.13 ± 0.29^b	2.32 ± 0.85^c
Ntrosothiol (µ moles/mg protein)	30.60 ± 2.7^a	31.51 ± 2 .83^a	50.16 ± 4.51^b	36.92 ± 1.6^c
Protein carbonyl (n mole/mg protein)	1.49 ± 0.14^a	1.47 ± 0.26^a	2.99 ± 0.22^b	1.73 ± 1.13^c

Values are Mean ± SD of 6 rats from each group. CON-control: CON + EUG - control + eugenol; EtOH - ethanol: EtOH +EUG - ethanol + eugenol. Values not sharing common alphabets as superscripts significantly differ from each other at the level of $p<0.05$ (ANOVA followed by DMRT).

3.2 Effect of eugenol on antioxidants system

Effects of eugenol on enzymic and non-enzymic antioxidants system were given in Tables 2 and 3. Ethanol fed rats showed significantly lowered the antioxidant system when compared the normal control rats. On the other hand, ethanol fed rats treated with eugenol significantly increased the antioxidants system, when compared to the ethanol alone treated rats.

Table 2: Effect of eugenol on non-enzymic antioxidants in mitochondrial fraction

Parameters	CON	CON + EUG	EtOH	EtOH +EUG
Vitamin C (µg/mg protein)	9.80 ± 0 .95^a	9 .50 ± 0.66^a	6.09 ± 0.43^b	8.74 ± 0.52^c
Vitamin E (µg/mg protein)	4.72 ± 0.34^a	4.67 ± 0.34^a	3.07 ± 0.29^b	3.84 ± 0.35^c
GSH (µg/mg protein)	14.18 ± 1.02^a	13.94 ± 1 .02^a	8.58 ± 0.65^b	12.40 ± 0.90^c

Values are Mean ± SD of 6 rats from each group. CON-control: CON + EUG - control + eugenol; EtOH - ethanol: EtOH +EUG - ethanol + eugenol. Values not sharing common alphabets as superscripts significantly differ from each other at the level of $p<0.05$ (ANOVA followed by DMRT.

Table 3: Effect of eugenol on enzymic antioxidants in mitochondrial fraction

Parameters	CON	CON + EUG	EtOH	EtOH +EUG
SOD (Units/mg protein)	2.51 ± 0 .21^a	2.63 ± 0.26^a	1.43 ± 0.62^b	2.18 ± 0.18^c
GST (µ mole/mg protein/min)	93.21 ± 5.79^a	92.56 ± 4.55^a	78.11 ± 6.42^b	84.86 ± 6.09^c
GPx (µ mole GSH reduced/min)	7.90 ± 0.43^a	7.69 ± 0 .86^a	4.73 ± 0.41^b	6.23 ± 0.58^c

Values are Mean ± SD of 6 rats from each group. CON-control: CON + EUG - control + eugenol; EtOH - ethanol: EtOH +EUG - ethanol + eugenol. Values not sharing common alphabets as superscripts significantly differ from each other at the level of $p<0.05$ (ANOVA followed by DMRT.

3.3 Effect of eugenol on membrane ATPase

Table 4 revealed the effect of eugenol on membrane ATPase. Ethanol fed rats showed significantly lowered the membrane ATPase when compared to control rats. On the other hand, ethanol fed rats treated with eugenol (Group IV) significantly increased the membrane ATPase when compared to the ethanol alone treated rats. Control rats treated with eugenol alone did not shown any significant alterations.

Table 4: Effect of eugenol on ATPase (µmoles of phosphorous liberated/mg of protein/hr) in mitochondrial fraction

Parameters	CON	CON + EUG	EtOH	EtOH +EUG
Total ATPase	1.41 ± 0 .11^a	1 .40 ± 0.11^a	0.89 ± 0.07^b	1.25 ± 0.10^c
Ca^{2+} ATPase	0.22 ± 0.02^a	0.22 ± 0.02^a	0.15 ± 0.01^b	0.19 ± 0.01^c
Na^{+}/K^{+} ATPase	0.40 ± 0.03^a	0.38 ± 0 .03^a	0.28 ± 0.02^b	0.32 ± 0.02^c
Mg^{2+} ATPase	0.52 ± 0.04^a	0.51 ± 0 .04^a	0.30 ± 0.02^b	0.42 ± 0.03^c

Values are Mean ± SD of 6 rats from each group. CON-control: CON + EUG - control + eugenol; EtOH - ethanol: EtOH +EUG - ethanol + eugenol. Values not sharing common alphabets as superscripts significantly differ from each other at the level of $p<0.05$ (ANOVA followed by DMRT).

3.4 Glutathione and glyoxylases I and II

Table 5 showed the concentration of GSH and the activities of glyoxalases I and II of control and experimental rats. The level of GSH and the activities of glyoxalase I and II were significantly reduced in the ethanol fed rats (Group III). Cotreatment with eugenol (Group IV) significantly restored the glyoxylases activity as compared to untreated ethanol fed rats (Group III). Control rats received eugenol alone (Group II) did not show any significant alteration when compared to the normal control rats (Group I).

Table 5: Effect of eugenol on glyoxalase system in liver of control and experimental animals

Parameters	CON	CON + EUG	EtOH	EtOH +EUG
Glyoxalase I (mol/g/min)	21.53±1.64^a	21.52±1.64^a	12.85±0.98^b	17.93±1.37^c
Glyoxalase II (m mol GSH /mg protein/min)	0.285±0.09^a	0.264±0.04^a	0.152±0.01^b	0.239±0.08^c
GSH (m mol/mg protein)	152.27±11.60^a	151.77±11.56^a	98.26±7.48^b	125.02±9.52^c

Values are Mean ± SD of 6 rats from each group. CON-control: CON + EUG - control + eugenol; EtOH - ethanol: EtOH +EUG - ethanol + eugenol. Values not sharing common alphabets as superscripts significantly differ from each other at the level of $p< 0.05$ (ANOVA followed by DMRT).

3.5 Histology

Figures 1-4 represented the histological sections of liver from experimental animals. Figure 1 showed the liver section of a control animal in which the portal triad and hepatocytes appear normal. Figure 2a represented the liver histology of alcohol-treated rat in which the portal triad was eroded and was infiltrated with inflammatory cells. Most of the hepatocytes showed microvesicular type of fatty changes and little macrovesicular type of fatty changes (Figure 2b). In control, rat treated with eugenol, hepatocytes were within normal limits (Figure 3) with Kuppfer cell hyperplasia. Figure 4 represented the liver section of alcohol and eugenol treated rat, showing Kuppfer cell hyperplasia. Most of the hepatocytes within normal limits, only occasionally hepatocytes exhibit microvesicular type of fatty change.

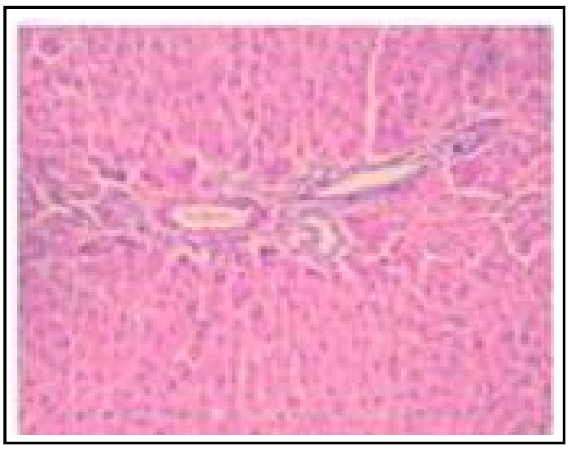

Figure 1: Liver of control rat H and E **20x**.The hepatocytes are normal and are arranged in trabecular pattern.

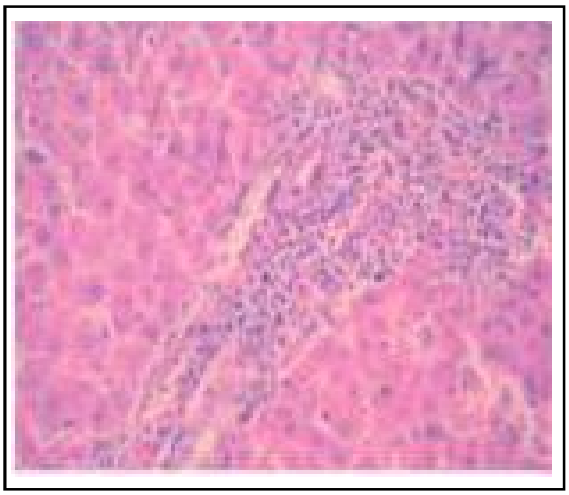

Figure 2a: Liver of alcoholic rat, H and E **20x**.The portal triad is damaged and surrounded by inflammatory infiltration of lymphocytes.

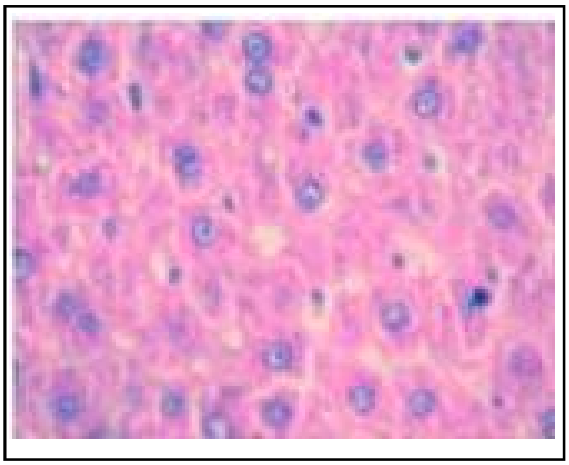

Figure 2b: Liver of alcoholic rat, H and E **20x** Most of the hepatocytes show microvesicular type of steatosis. Few hepatocytes exhibit macrovesicular type of steatosis.

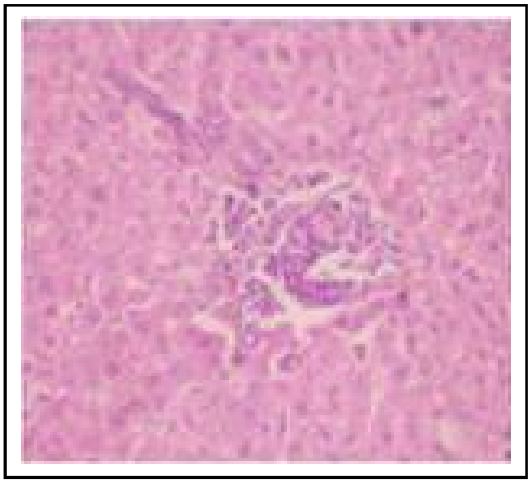

Figure 3: Liver of control rat treated rat with eugenol H and E 20x. The hepatocytes are within normal limits.

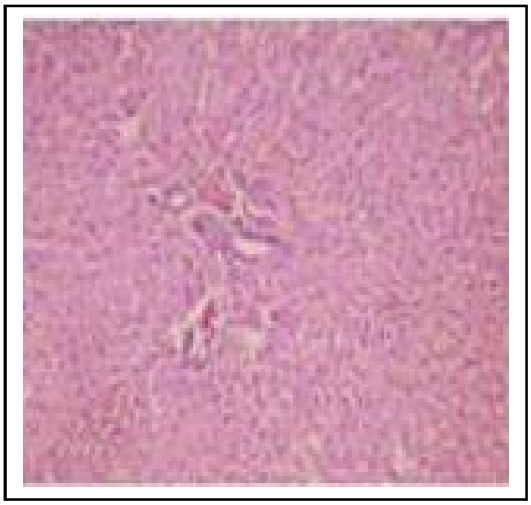

Figure 4: Liver of alcoholic rat treated with eugenol H and E **20x**. Most of the hepatocytes are within normal limits. Few hepatocytes exhibit microvesicular type steatosis. Kuppfer cell hyperplasia is also seen.

4. Discussion

The extensive liver damage by ethanol was is related to its ability to induce ROS production to deplete redox ratio and to cause mitochondrial membrane damage (Bailey *et al*., 1999). Induction of CYP2E1 by ethanol predisposes to the membrane permeability transition (MPT) in the mitochondria through an increase in the production of ROS. MPT is subsequently followed by events such as cytochorome 'c' release into the cytosol and caspase activation culminating in apoptosis (Higuchi *et al*., 2001). In addition to this, ROS formation within the mitochondria can lead to extensive mitochondrial protein carbonylation and suppression of vital mitochondrial functions, such as respiration and oxidative phosphorylation and inner membrane barrier function. This explains the increased lipid peroxidation products observed in the present study. Further, aldehydes are highly reactive and are generated in high concentrations particularly under disease condition (Conklin *et al*., 2007). Ethanol toxicity has been shown to be associated increased accumulation of protein-aldehyde adducts (Chen *et*

al.,2000). The glyoxalase system is one mechanism by which the α-oxoaldehydes could be metabolized and reduced activity, represent a defect in the detoxification of oxoaldehydes in ethanol-fed animals. Elevated glyoxylase system by eugenol treated contributes to the detoxification of aldehydes generated as a product of lipid peroxidation from the ethanol metabolism.

The incereased response of glyoxalaseI because of treatment with eugenol was reported to help in the removal of toxic species and reduces the oxidative stress (Tiku *et al.*, 2004). Eugenol was found to be an inducer of phase II enzyme rather than phase I enzyme of drug detoxification (Thompson *et al*., 1990). Since eugenol is metabolized and cleared rapidly from the body (Nagababu *et al*., 1995), it was given concurrently with alcohol.

Eugenol was a methoxy phenol and it owes its antioxidant activity to phenolic group. The ability to trap the peroxy radical ($ROO^{\bullet}$) by donation of phenolic hydrogen atom which takes place at a faster rate than the attack on organic substrates such as the phospholipids (Fujisawa *et al*., 2002). Ito *et al.* (2005) have studied the structure activity relationship which revealed the important role of phenolic ring and chain structure. Eugenol has been shown to have radical scavenging activity against species such as $OH^{\bullet}$, O_2, NO, $DPPH^{\bullet}$ (Ogata *et al*., 2005) and metal chelating activity (Ito *et al*., 2005). The antioxidant nature of eugenol has been shown in a number of *in vivo* and *in vitro* studies (Ito *et al*., 2005). For instance, eugenol has been shown to inhibit Cu^{2+} H_2O_2 catalysed lipid peroxidation in human RBC (Nagashima *et al*., 1989) and lipooxygenase - dependent lipid peroxidation (Naidu, 1995). Eugenol at a concentration of 5 m mol in plasma can prevent LDL oxidation indicating that it can act as a physiological antioxidant *in vivo* (Fischer and Dengler, 1990). It has been suggested that eugenol protects the membrane from free radicals attack by incorporating into membrane and also mitochondrial functions, resulting in dearrangement, particularly at proton transferring sites (Usha *et al*., 2007)

Mitochondria possesses multiple antioxidant defense system, including superoxide dismutase, glutathione peroxidase, thioredoxin and its reeducates, as well as water and lipid soluble antioxidant (Vitamin C and E). Vitamin C and E synergistically mops up free radicals generated in ethanol metabolism (Oyinbo *et al.*, 2006). The reduced levels of β-tocopherol and ascorbic acid may be due to their increased utilization for scavenging ethanol derived radicals. We have observed significant increased levels of these antioxidants in ethanol fed rats treated with eugenol. Mitochondrial GSH (mGSH) plays an essential role in the mitochondrial defense against constant ROS generation, particularly H_2O_2 because as a cofactor of GPx. Previous studies have shown that chronic alcohol feeding in various models results in depletion of mGSH (Ruiz *et al*., 1994). Decreased SOD catalyzes the dismutation of superoxide radicals anion to H_2O_2. The decreased SOD activity could be due to oxidative inactivation of enzymes proteins due to excessive ROS. Previous report has shown eugenol protects rat liver mitochondria from iron induced lipid peroxidation (Nagababu *et al*., 1995) and eugenol enhances the endogenous antioxidants (Kabuto *et al*., 2007). Eugenol being natural antioxidant, could scavenge the mitochondrial ROS, preventing the oxidation of proteins. This may contribute to decreased protein carbonylation. Our results correlated with this report and treatment eugenol significantly elevated the mGSH and SOD that contribute to the detoxification of peroxides and replenish the antioxidants status.

The membrane bound enzymes such as Na^+/K^+ ATPase, Mg^{2+} ATPase and calcium ATPase are intrinsic proteins responsible for the transport of Na^{2+}, K^+, Mg^{2+} and Ca^{2+} ions across the cell membrane against concentration (Bonilla *et al*., 1991). Membrane lipid peroxidation results in altered membrane fluidity, altered permeability to ions (Marshansky *et al.*, 1983) and changes in the activities of membrane bound enzymes (Viani *et al*., 1991). It has been shown that eugenol incorporated into the membrane and decrease membrane fluidity, which results in normalization of ATPase activities in liver mitochondrial fractions (Parasakthy *et al*., 1996). Our results also confirmed that ethanol fed rats treated with eugenol significantly maintains the ATPase activities.

Live histology confirmed the protective efficacy of eugenol. Many plant derived antioxidants showed protection against alcohol induced liver toxicity (Pradhan and Girish, 2006). In this study, eugenol, an antioxidant component of several herbs was found to be protective against alcoholic liver disease. It was reported that eugenol reversed fatty changes and portal inflammation in the liver and lowers plasma and tissue lipids in diabetic rats (Rai *et al.*, 1997).

5. Conclusion

In conclusion, eugenol protects against ethanol- induced liver damage *via* the modulation of mitochondrial oxidative stress, antioxidant system, membrane ATPase in mitochondrial fraction and also enhances the detoxification system. However, further molecular mechanism studies are warranted.

Conflict of interest

We declare that we have no conflict of interest.

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