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(-) Epigallocatechin gallate restores ethanol-induced alterations in hepatic detoxification system and prevents apoptosis

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SUMMARY

The present study was designed to estimate the protective effect of (-) epigallocatechin gallate (EGCG) on ethanol-induced liver injury in rats. Chronic ethanol administration (6 g/kg/day × 60 days) caused liver damage that was manifested by the elevation of markers of liver dysfunction - aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, bilirubin and γ-glutamyl transferase in plasma and reduction in liver glycogen. The activities of alcohol metabolizing enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase were found to be altered in alcohol-treated group. Ethanol administration resulted in the induction of cytochrome p450 and cytochrome-b_5 activities and reduction of cytochrome-c reductase and glutathione-S-transferase, a phase II drug metabolizing enzyme. Further, ethanol reduced the viability of isolated hepatocytes (ex vivo) as assessed by trypan blue exclusion test and induced hepatocyte apoptosis as assessed by propidium iodide staining. Treatment of alcoholic rats with EGCG restored the levels of markers of liver injury and mitigated the alterations in alcohol metabolizing and drug metabolizing enzymes and cyt-c-reductase. Increased hepatocyte viability and reduced apoptotic nuclei were observed in alcohol + EGCG-treated rats. These findings suggest that EGCG acts as a hepatoprotective agent against alcoholic liver injury.

Key words: EGCG; Ethanol; Drug metabolizing enzymes; Hepatocytes; Apoptosis

INTRODUCTION

Alcohol abuse can lead to acute and chronic liver disease due to accumulation of toxic metabolites which are more toxic than alcohol itself (Kurose *et al.*, 1996) and hence alcoholism is considered a major health problem throughout the world. About 90% of the ingested alcohol is metabolized in the liver which offers three metabolic pathways catalysed by the cytosolic enzyme-alcohol dehydrogenase (ADH), the cytosol and mitochondrial aldehyde dehydrogenase (ALDH) and the microsomal ethanol oxidizing system (MEOS). ADH catalyzes the transformation of ethanol into acetaldehyde, which inturn is converted to acetate through ALDH. These reactions generate an excess of reducing equivalents in the liver, primarily in the form of NADH resulting in redox imbalance. The MEOS system requires the participation of the P-450 cytochrome which couples ethanol and nicotinamide adenine dinucleotide phosphate (NADPH) oxidation to the reduction of molecular oxygen to hydrogen peroxide.

Chronic alcohol intoxication is accompanied by a decrease in the activities of ethanol metabolizing

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enzymes-ADH and ALDH and in consequence by an increase in acetaldehyde accumulation (Puntarulo and Cederbaum, 1989). Induction of a cytochrome P-450 enzyme, CYP2E1 by ethanol increases superoxide generation (Lieber, 2005). Ethanol can increase the activity of CYP2E1 and a link between CYP2E1 induction, free radical formation and alcohol-induced cell injury has been proposed (Cederbaum *et al.,* 2001). Increased CYP2E1 causes mitochondrial damage, release of cytochrome c, activation of caspases followed by reduction in cell viability and apoptosis (Higuchi *et al.,* 2001).

There has been a great deal of interest in the role of complementary and alternative medicine in the treatment of various acute and chronic diseases. Of the various herbal and botanical agents available, we focused our interest on the polyphenols found in green tea. The green tea polyphenols include (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin, (-)-epicatechin gallate, and (-)-epicatechin (Varilek *et al.,* 2001) of which, EGCG is the major constituent and is also the component with the highest antioxidant properties (Guo *et al.,* 1996).

Studies indicate that ingested catechins are absorbed and can have biological effects. The potential of tea polyphenols to prevent or ameliorate chronic diseases including cancer, cardiovascular disease and aging (McKay and Blumberg, 2002; Weisburger, 2002) is currently the subject of considerable scientific investigation. However very few animal studies have examined the protective role of EGCG in ethanol-induced tissue damage (Arteel *et al.,* 2002; Ostrowska *et al.,* 2004). The antioxidant properties of EGCG are reported to be responsible for the protective properties. We hypothesize that the benefits of EGCG in alcohol toxicity might be to modulate the activities of alcohol and drug metabolizing enzyme systems. Therefore the current study was initiated to investigate the effect of EGCG *in vivo* on the markers of liver injury, alcohol metabolizing enzymes, cytochrome P-450 components (Phase I) and glutathione-S-transferase (Phase II) enzymes of rats sub-chronically intoxicated with ethanol. In addition, the benefits of EGCG in terms of its ability to prevent apoptosis were also investigated.

MATERIALS AND METHODS

Animals and Chemicals

Healthy male albino Wistar rats (150 – 170 g) purchased from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical Collage, Annamalai University were housed in polypropylene rat cages in a room with controlled temperature (24 ± 2°C) and light (lights on 0,600 to 1,800). They were fed with standard pellet diet (Agro Corporation Private Limited, Bangalore, India) and water *ad libitum*. The experimental and animal-handling procedures were approved by the Institutional Animal Ethics Committee (IAEC). EGCG was purchased from Hunan Kinglong Bio-Resource Co., Ltd (China). Trypan blue was obtained from Himedia Pvt Ltd (India). Alcohol dehydrogenase, cytochrome c, propidium iodide and type IV collagenase were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade.

Experimental design

The animals were randomly divided into four groups of six each. Alcoholic rats (toxicity control) received ethanol (6 g/kg/day) as an aqueous solution for 60 days by intragastric intubation. Normal control rats received glucose solution equivalent to the calorific value of ethanol (from 40% glucose solution). After the induction of toxicity (i.e. initial 30 days), treatment groups received EGCG (100 mg/kg/day) for next 30 days along with ethanol. This experimental design is clearly presented in Table 1. The total experimental duration was 60 days. Rats in all the groups were fasted overnight and anesthetized with ketamine hydrochloride (30 mg/kg b.w.) and sacrificed by

Table 1. Experimental design

Group	Treatment and Duration	
	1 - 30 days	31 - 60 days
I	Isocaloric Glucose (40% stock) twice a day (09:00 a.m. and 05:00 p.m)	
II	Ethanol (3 g/kg twice a day i.e 6 g/kg/day)	
III	Ethanol (6 g/kg/day)	Ethanol + EGCG (50 mg/kg twice a day) i.e 100 mg/kg/day
IV	Glucose (40% stock)	Glucose (40% stock) + EGCG (50 mg/kg twice a day) i.e 100 mg/kg/day

cervical dislocation. Blood was collected with heparin as anticoagulant. Plasma was separated by centrifugation at 2,000 rpm. The liver tissue was sliced into pieces and homogenized in cold 50 mM phosphate buffer (pH 7.4) to give 10% homogenate (w/v). The homogenate was centrifuged at 1,000 rpm for 10 min at 0°C in cold centrifuge. The supernatant was separated and used for various estimations.

Biochemical estimations

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by the method of Reitman and Frankel (1957) and that of alkaline phosphatase (ALP) by the method of Kind and King (1954). Serum bilirubin was estimated by the method of Malloy and Evelyn (1937) while gammaglutamyl transferase (GGT) and lactate dehydrogenase (LDH) activities were assayed by the methods of Oralowsky and Meister (1963) and King (1965) respectively. Estimation of liver glycogen was done by the method of Montgomery (1957). ADH and ALDH activities were assayed by the method of Agarwal and Goedde (1990). Glutathione-S-transferase (GST) was assayed by the method of Habig *et al.* (1974). Protein content was determined by the method of Lowry *et al.* (1951).

Analysis of cytochromes and electron transport component content

Cytochrome P-450 was assayed by the method of Omura and Sato (1964) using carbon monoxide difference spectra of dithionite-reduced microsomes. The extinction difference of the hemoprotein was taken as 91 $mM^{-1}.cm^{-1}$ (450 minus 490 nm). Cytochrome b_5 was determined by Omura and Sato (1964) by measuring the reduced minus oxidized difference spectrum by the addition of NADH, taking the extinction coefficient of the cytochrome between 424 and 409 nm as 185 $mM^{-1}.cm^{-1}$. Cytochrome c reductase activity was determined by the method of Umeki *et al.* (1984).

Isolation of rat hepatocytes

Hepatocytes were isolated according to the method of Berry and Friend (1969), as modified by Seglen (1976). Rats were fasted for 24 h prior to the hepatocyte isolation. The rats were anesthetized with sodium pentobarbital (62.5 mg/kg) subcutaneous and hepatocytes were isolated by collagenase perfusion technique. Cell viability of isolated hepatocytes, from both control and experimental rats, was measured by the trypan blue exclusion test.

Trypan blue exclusion test

One drop of hepatocyte stock suspension (1 - 1.2 × 10^6 cells/ml) was mixed with three drops of Trypan blue solution (0.2%). The unstained viable cells were distinguished visually from the blue stained dead cells and protection was expressed in terms of percent viable cells (Moldeus *et al.*, 1978).

Propidium Iodide staining

Cells isolated from rat liver were washed thrice in phosphate buffered saline (PBS), incubated for another 10 min with 50 μl of propidium iodide (5 mg/ml) and mounted in slides and then

examined by fluorescence microscopy (Olympus BX51). Quantification of data was obtained by examination of a defined area of each slide.

Statistical analysis

Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Values are expressed as means ± SD of six rats in each group. A probability of $P < 0.05$ was considered as significant.

RESULTS

Effect of chronic ethanol administration with or without EGCG on plasma AST, ALT, ALP, GGT, LDH activities and levels of plasma bilirubin and liver glycogen

The results of the changes in activities of the marker enzymes, AST, ALT, ALP, GGT, LDH and levels of bilirubin and glycogen on chronic ethanol administration with or without EGCG are represented in Table 2. The results show that upon administration of ethanol there was a significant increase in the activities of AST (81%, $P < 0.05$), ALT (54%, $P < 0.05$), ALP (129%, $P < 0.05$), GGT (327%, $P < 0.05$), LDH (99%, $P < 0.05$) and levels of plasma bilirubin (125%, $P < 0.05$) as compared to control rats. The liver marker glycogen was significantly decreased in ethanol treated rats (45%, $P < 0.05$) and on treatment with EGCG, the activities of marker enzymes, bilirubin and liver glycogen levels were restored to near-normal values.

Effect of EGCG on hepatocyte viability and apoptosis

Table 3 represents the percentage viability of isolated rat heptocytes from control and experimental animals and it can be seen that there was a significant decrease in the cell viability of about 43% in ethanol-treated rat as compared to control. Upon treatment with EGCG the viability was reduced to only 9% as compared to control rats. Normal rats treated with EGCG showed no significant difference as compared to control.

Ethanol is known to induce apoptosis in

Table 2. Effect of EGCG on biochemical serum and liver parameters against alcohol induced liver injury

Parameters	CON	ETOH	ETOH + EGCG	CON + EGCG
Plasma (IU/L)				
AST	84.65 ± 3.09	152.77 ± 11.86[a]	85.64 ± 8.31[b]	84.73 ± 8.47
ALT	92.89 ± 8.40	143.10 ± 8.68[a]	98.20 ± 7.16[b]	94.60 ± 6.55
ALP	85.66 ± 5.74	197.01 ± 13.04[a]	86.77 ± 5.23[b]	82.06 ± 7.25
GGT	11.23 ± 1.10	48.05 ± 2.66[a]	13.26 ± 1.12[b]	11.39 ± 0.60
LDH	326.28 ± 30.46	649.21 ± 43.90[a]	347.03 ± 20.48[b]	325.94 ± 29.23
Bilirubin (mg/dl)	0.93 ± 0.09	2.10 ± 0.13[a]	1.04 ± 0.03[b]	0.94 ± 0.05
Liver (mg/g tissue)				
Glycogen	43.29 ± 4.09	23.66± 1.58[a]	40.15 ± 2.34[b]	41.69 ± 2.61

Values are means ± SD of six animals. [a]significant as compared to CON ($P < 0.05$, DMRT). [b]significant as compared to ETOH ($P < 0.05$, DMRT).

Table 3. Protective effect of EGCG on viability of isolated rat hepatocytes against ethanol-induced toxicity

Parameters	CON	ETOH	ETOH + EGCG	CON + EGCG
Percentage Viability (Trypan blue exclusion test)	89.12 ± 3.03	50.84 ± 2.45[a]	81.08 ± 2.87 [b]	88.65 ± 4.70

Values are means ± SD of six animals. [a]significant as compared to CON ($P < 0.05$, DMRT). [b]significant as compared to ETOH ($P < 0.05$, DMRT).

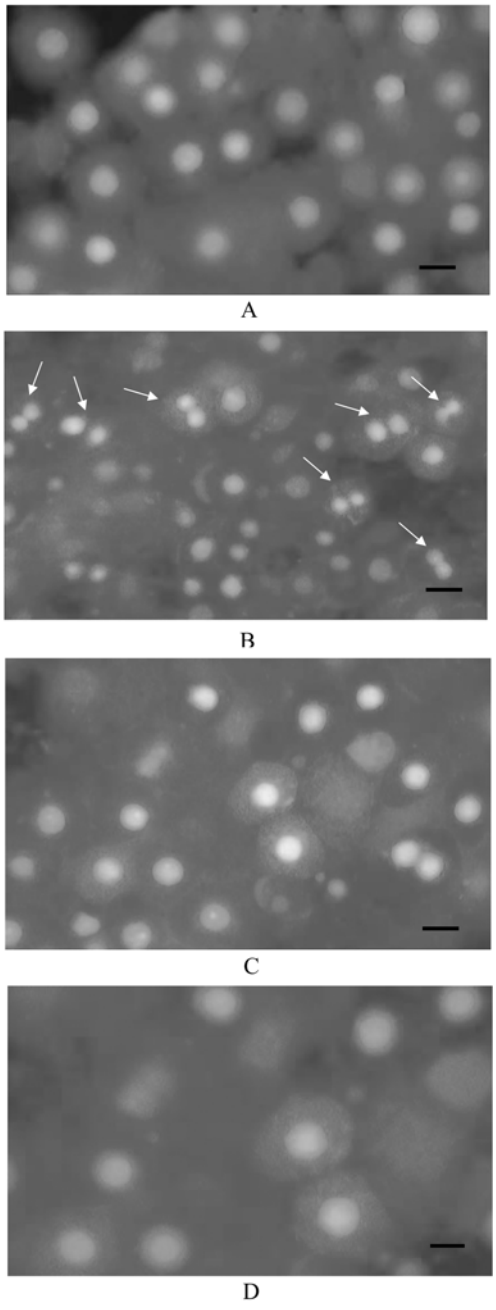

Fig. 1. Propidium iodide staining of isolated rat hepatocytes. Apoptotic cells with fragmented nuclei (arrows) are seen in EtOH treated rat hepatocytes (Fig. 1B). Hepatocytes from rats treated with ethanol and EGCG (Fig. 1C) show less apoptotic bodies and exhibit similar hepatocyte morphology as that of control (Fig. 1A) Hepatocytes from normal rats treated with EGCG (Fig. 1D) appear normal as that of control (Scale bar = 30 mm).

hepatocytes. Ethanol-treated rats showed more apoptotic nuclei as indicated by arrows (Fig. 1B) when compared to control (Fig. 1A). Treatment with EGCG reduced hepatocyte apoptosis (Fig. 1C) and the cells appear to be similar to that of control.

Effect of EGCG on alcohol metabolizing, phase I and phase II enzymes

Table 4 shows the activities of ADH, ALDH, cytochrome P-450, cytochrome b5, cytochrome C reductase and the phase II enzyme GST in liver of control and experimental animals. The activities of ADH and ALDH decreased significantly ($P < 0.05$) in liver after chronic ethanol administration whereas in EGCG treated rats the activities were restored to near-normal.

The activities of cytochrome P-450 and cytochrome b_5 were significantly increased whereas the activities of cytochrome c reductase and glutathione-S-transferase were decreased in alcohol treated animals when compared with control. Administration of EGCG significantly reduced activity of these phase I enzymes with significant increase in the activities of cytochrome c reductase and GST (Table 4).

DISCUSSION

Ethanol can solubilize the lipid components and alter ion-channels of biomembranes leading to altered membrane permeability and fluidity. The increase in membrane permeability and in the terminal situation disruption of membranes can be linked to the translocation of the liver enzymes into the blood. It has been well documented that both AST and ALT are the most sensitive markers of hepatocellular injury while increased GGT could be another index, along with elevated ALP and bilirubin (Coodley, 1969). Reduced glycogen content could be associated with reduced synthetic capacity of the liver due to ethanol toxicity. EGCG administration could blunt ethanol-induced increases

Table 4. Effect of EGCG on alterations in alcohol metabolizing enzymes and detoxification system in liver of chronic ethanol administered rats

Parameters	CON	ETOH	ETOH + EGCG	CON + EGCG
ADH[a]	0.75 ± 0.07	0.47 ± 0.03[a]	0.71 ± 0.07[b]	0.77 ± 0.07
ALDH[a]	1.05 ± 0.05	0.66 ± 0.07[a]	0.99 ± 0.06[b]	1.05 ± 0.04
Cytochrome P-450[b]	0.91 ± 0.07	1.30 ± 0.10[a]	0.98 ± 0.06[b]	0.96 ± 0.04
Cytochrome b_5[b]	0.49 ± 0.03	0.62 ± 0.02[a]	0.51 ± 0.01[b]	0.50 ± 0.02
Cytochrome c reductase[c]	1.11 ± 0.10	0.81± 0.07[a]	1.06 ± 0.08[b]	1.10 ± 0.03
Glutahione-S-transferase[d]	5.47 ± 0.33	2.81 ± 0.26[a]	5.22 ± 0.22[b]	5.39 ± 0.32

Values are means ± SD of six animals. [a]significant as compared to CON ($P < 0.05$, DMRT). [b]significant as compared to ETOH ($P < 0.05$, DMRT). [c]mol NAD/min/mg protein; nmol/mg protein; mol/min/mg protein; [d]moles of CDNB-GSH/min/mg protein.

in activities of various marker enzymes of heptocellular injury, viz. AST, ALT, ALP and GGT and could enhance glycogen levels suggesting that EGCG possibly has a protective influence against ethanol -induced hepatocellular injury and degenerative changes.

Chronic alcohol feeding decreased the activity of alcohol metabolizing enzymes ADH and ALDH (Hasumura *et al.*, 1975). Acetaldehyde the metabolic product of ethanol, might damage the mitochondria thereby reducing the ALDH enzyme which inturn impairs further metabolism. Changes in the redox potential due to ADH and ALDH could cause alterations in the metabolism of lipid, glycogen and protein (Matsuzaki and Leiber, 1977). Treatment with EGCG showed restoration of these two enzymes that could result in the maintenance of redox ratio, and normal metabolic activities in the liver.

During oxidative metabolism in the microsomal environment involving the cytochrome P-450 system, electrons flow from NADPH or NADH through cytochrome P450 reductase to cytochrome-b_5 or cytochrome P-450. Cytochrome P-450 is the terminal component of the electron transport system responsible for many drug reactions.

The enhancement of cytochrome b_5 content in ethanol-fed rats may be due to the increase in electron transport to cytochrome P-450, the content of which is increased in relation to ethanol oxidation (Sinclair *et al.*, 1981). CYP2E1 induction results in superoxide production, oxidative stress and cell injury in the liver. Induction of CYP2E1 may predispose to the membrane permeability transition (MPT) in the mitochondria through an increase in the production of ROS, 1-hydroxy ethyl radical and products such as acetaldehyde (Cederbaum, 2001). The MPT is the regulatable opening of a large, nonspecific pore across the outer and inner mitochondrial membrane. MPT development is followed by cytochrome c release into the cytosol, caspase activation and apoptosis (Higuchi *et al.*, 2001).

GST is a critical drug metabolizing enzyme that primarily functions in conjugating toxic metabolites with endogenous ligands (reduced glutathione) favouring their elimination. The action of this phase II enzyme on the substrates generated by the action of phase I enzymes leads to their solubilization and excretion. Treatment with EGCG increased the activity of the enzyme.

Ethanol treatment resulted in apoptosis and decreased cell viability. Increased levels of ethanol has been reported to promote apoptosis by the following events: formation of toxic metabolites (Neuman *et al.*, 1998), inducibility of cytochrome P-450 system (Ingelman-Sundberg *et al.*, 1993), depletion of GSH, mitochondrial depolarization and damage (Cameron *et al.*, 1998), formation of proinflammatory cytokines and iron overload.

Many studies carried out over the past few

years have shown that the polyphenolic fractions isolated from green tea inhibit oxidant stress and possess antiinflammatory activity (Sarafini *et al.,* 1996; Leung *et al.,* 2001; Yang *et al.,* 2001). Ostrowska *et al.* (2004) reported that green tea enhanced antioxidative abilities in the liver, brain and serum of rats and protected liver cells and their organelles (morphological examinations) against ethanol action.

Prevention of ethanol toxicity by EGCG was associated with reduced liver damage, as reflected by a decrease in the leakage of enzymes from liver and increase in the synthetic capacity of the liver (glycogen). EGCG has an inhibitory effect on ethanol-inducible cytochrome P-450 IIE1 activity. It may be inferred that EGCG may block this key enzyme which otherwise could activate the xenobiotics including chemical carcinogens. EGCG protected hepatocyte viability and also prevented the formation of apoptotic nuclei.

In one study EGCG, at the concentrations (10 – 50 μM) used, did not inhibit CYP2E1 activity but was shown to prevent alcohol-induced liver injury by possible protection against oxidative stress and by protection of mitochondria against depletion in membrane potential (Jimenez-Lopez and Cederbaum, 2004). It has been suggested that EGCG can prevent damage due to CYP2E1-dependent oxidative stress possibly by its antioxidant activity downstream CYP2E1 (Jimenez-Lopez and Cederbaum, 2004). However several derivatives of epicatechin including EGCG were shown to inhibit cytochrome P-450 including CYP2E1 activity in membrane fraction of genetically engineered *Salmonella typhimurium* strains (Muto *et al.,* 2001). Catechin incorporation into cell membranes was shown to prevent or reduce the morphological and biochemical alterations of hepatocytes induced by hepatotoxic agents (Varga and Buris, 1989; 1990). EGCG modulates signaling pathways that trigger cell cycle arrest and apoptosis in different human cell lines (Hofmann *et al.,* 2003). EGCG prevents the depletion of mitochondrial membrane potential and inhibits by blocking CYP2E1-generated oxidant stress and the generation of hydroxyl radicals, degradation of lipid hydroperoxides and aldehyde formation (Jimenez-Lopez and Cederbaum, 2004; Ostrowska *et al.,* 2004).

A number of factors including antioxidant property, a reduction in metabolic activation of toxicant and chelation of metal ions could be involved in the benefits of EGCG. For instance, flavonoids can inhibit microsomal phenol hydroxylase in a dose-dependent manner (Hendrickson *et al.,* 1994). Catechins, silymarin, quercetin, morin and a number of these substances have been reported to be effective in protection against hepatotoxicity of CCl_4, -amanitin, galactosamine and ethanol (Hahn *et al.,* 1968; Perrissoud and Weibel, 1980; Perrissoud and Testa, 1986; Wu *et al.,* 1993).The protective effect of three flavonoids, catechin, quercetin and diosmetin were tested on iron-loaded rat hepatocyte cultures and the catechins were found to be the most effective in preventing iron-mediated toxicity (Morel *et al.,* 1993). These studies reveal that hepatoprotective effects of these flavonoids could be related to the iron chelation and modulation of alcohol metabolism in addition to antioxidant potential.

Clinical and animal studies have repeatedly documented the protective effects of EGCG against oxidative stress-related diseases (Hertog *et al.,* 1993; Sueoka *et al.,* 2001; Arteel *et al.,* 2002; Levites *et al.,* 2002). It is possible that EGCG may modify specific factors that underly the toxicity of ethanol cited above such as, MPT development, activation of caspases, release of cytochrome-c and iron overload. More mechanistic studies to ascertain the role of EGCG in the inhibition of caspases or iron chelation are essential. It would also be important to compare the protection by EGCG with other antioxidants such as vitamins E and C since the catechins are reported to have intermediate reactivity between the water soluble and lipid soluble antioxidants (Lotito and Fraga, 2000).

In summary the present paper reveals that in

rats intoxicated with ethanol, EGCG restores the liver function parameters, modulates the metabolism of alcohol and drug metabolizing system and diminishes apoptotic process. The results point out a need for further studies on the intracellular mechanisms of protection by EGCG against ethanol.

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