RESEARCH ARTICLE

Aqueous extract of black tea (*Camellia sinensis*) prevents chronic ethanol toxicity

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Oxidants have been shown to be involved in alcohol-induced liver injury. High-fat diet may be an additional factor for more acute alcohol-induced liver injury. Moreover, black tea extract (BTE), a phytocompound has been attributed with a plethora of health-promoting actions. This study was designed to test the hypothesis that BTE protects against early ethanol and ethanol + high-fat liquid diet-induced liver injury in rats. Results show that BTE was effective in blunting both ethanol and ethanol + high-fat diet-induced enhanced activities of enzymes like aspartate aminotransferase, alanine aminotransferase and acid phosphatase. BTE was also effective in blunting ethanol and ethanol + high-fat-induced increase in production of malondialdehyde and nitric oxide. Furthermore, BTE could blunt ethanol and ethanol + high-fat diet-induced suppressed activities of superoxide dismutase and catalase. Additionally, BTE was found effective in blunting ethanol + high-fat diet-induced enhanced activity of γ-glutamyl-transpeptidase as well as to recover lower glutathione content. Ethanol with or without high-fat diet-induced histopathological and inflammatory damages could be blunted by BTE. Results of pathology scores of hepatocellular damage caused by ethanol with or without high-fat diet and its recovery by BTE, suggest that BTE also was effective against more intense damaging effects caused by ethanol + high-fat diet. BTE also could blunt body weight loss because of ethanol + high-fat diet. This study demonstrates that BTE prevents chronic ethanol toxicity developed by ethanol with or without high-fat diet, presumably by blunting oxidative stress.

ALCOHOL dependency is a major health and socio-economic problem throughout the world[1,2]. Several animal studies have revealed that high fat-containing diet together with alcohol further aggravates the health situation[3–5]. It has been observed that almost all ingested alcohol is metabolized in the liver and excessive alcohol use can lead to acute and chronic liver disease[6]. It has further been observed that most of the consumed alcohol is eventually broken down by the liver and the products generated and accumulated during alcohol metabolism (e.g. acetaldehyde) are more toxic than alcohol itself[6]. In addition, a group of metabolic products called free radicals can damage liver cells and promote inflammation, impairing vital functions such as energy production. The body's natural defenses against free radicals (e.g. antioxidants) are inhibited by alcohol consumption, leading to increased liver damage[6]. Subsequent studies have confirmed that indeed oxidative stress plays an important role in the initiation and progression of alcohol liver disease (ALD)[7–9].

Despite great progress made in the field in the past two decades, development of suitable medications for the treatment of alcohol dependency or alcohol-induced health injury remains a challenging goal for alcohol research. It is widely accepted that alcoholism is a complex heterogeneous disorder. Medicinal plants have been in use for the treatment of alcohol dependency in China for centuries, but have only recently attracted the attention of Western scientists[2]. Of particular interest is the Kudzu (*Pueraria lobata*) isoflavonoid daidzin which has antioxidant properties, as vitamin E is also effective in reducing alcohol-induced sleeping time[10]. Tea (*Camellia sinensis*) is one of the most commonly consumed beverages in the world. It is rich in polyphenolic compounds, collectively known as the tea flavonoids. These have antioxidant properties *in vitro* and have been proposed as a protective dietary component, reducing the risk of various cancers and other diseases[11]. Black tea, the major form of tea consumed in Western countries, has been found more efficient than green tea and is an excellent chemopreventor against reactive oxygen and nitrogen species[12]. It also causes a significant increase in plasma antioxidant activity[13]. Additionally, aqueous extract of black tea has shown to quench reactive oxygen species (ROS) such as singlet oxygen, superoxide and hydroxyl radicals, prevent the oxidative cross-linking of test proteins and inhibit single-strand breakage of DNA in whole cells[14]. It has also been reported that black tea attenuates carbon tetrachloride (CCl_4)-induced hepatic injury[15]. Recently, it has been reported that melanin derived from tea confers marked protection of the liver against hydrazine-induced oxidative toxicity[16].

Tea as a beverage and alcohol as a social drink along with high-fat consumption, contribute significantly in the day-to-day life of affordable urban and rural population all over the world. Black tea extract (BTE) has been attributed with a plethora of health-promoting actions, while ethanol and high-fat are both known to have health-deteriorating effects. The purpose of this study was to examine the protective effects of black tea (*Camellia sinensis*) extract (BTE) in two animal models of chronic ethanol toxicity with or without

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high-fat diet supplement. The reason for selecting two different animal models of chronic ethanol toxicity was to make an assessment of the protective efficacy of BTE against different degrees of liver injury since ethanol-containing liquid diet has been reported to cause more pronounced damage to the liver than ethanol alone[17]. Additionally, these models of experimental liver damage in rats simulate many of the features of human liver pathology.

Materials and method

Plant material

The black tea extract was prepared from CTC (Curl, Tear and Crush) BOP (Broken Orange Pikoe) grade black clonal tea. Preparation of aqueous extract of black tea was done following the method of Wei *et al*[18]. Briefly, 1.25 g of tea leaves was added to 25 ml of boiling water and was steeped for 15 min. The infusion was cooled to room temperature and then filtered. The tea leaves were extracted a second time with 25 ml boiling water and filtered, and the two filtrates were combined to obtain a 2.5% aqueous-tea extract. The BTE was fed[18] to animals by gavage technique at a dose of 1 ml/100 g body weight at a temperature of 37°C.

Animals

Male Wistar rats weighing 122 ± 4.5 g were used in the experiment. They were maintained in a 12 h light/dark cycle at 25 ± 2°C. All animal experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Culture, Government of India. Ethical care and treatment of animals were undertaken following the guidelines of the Animal Care and Ethics Committee of Presidency College, Kolkata.

Diet and treatment

Two different rat models of experimental liver injury caused by either ethanol or ethanol + high-fat were used in this study. Animals were divided into six groups: Group A (control), Group B (ethanol-treated), Group C (ethanol + BTE-supplemented), Group D (high-fat), Group E (high-fat + ethanol-treated) and Group F (high-fat + ethanol + BTE-supplemented). They were pair-fed with nutritionally adequate, regular and high-fat liquid diet (Table 1)[17] with or without ethanol. Animals of groups B, C, E and F were fed with 15% (v/v) ethanol[19] (volume that contributed isocalorically 36% of total energy) at a single dose per day for 30 days by gavage technique. Simultaneously but at different hours of the day, animals of groups C and F were fed with 2.5% (w/v) BTE by gavage technique for 30 days at a single dose of 1 ml/100 g body wt/day[18]. Animals of groups A and D were administered by gavage technique deionized water, 1 ml/100 g body wt/day, as vehicle. Daily records of body weight of all groups of animals were maintained during the whole experimental period.

Table 1. Composition of high-fat and regular laboratory diet

	High-fat diet	Regular laboratory diet
Protein (% of total calorie)	18	18
Carbohydrate (% of total calorie)	47	77
Fat (% of total calorie)	35	5

Serum preparation

After the treatment period was over, the animals of all groups were anaesthetized and sacrificed by cervical dislocation, which is one of the recommended physical methods of euthanasia by the IAEC. Blood was drawn from heart and serum was separated for the assay of aspartate amino transferase (AST), alanine amino transferase (ALT), acid phosphatase (ACP) and γ-glutamyltranspeptidase (GGT).

Preparation of tissue extracts

The abdomen was opened, liver was quickly removed and part of the right lobe was placed in a beaker containing ice-cold Tris-HCl buffer (pH 7.4). It was minced into small pieces on ice and homogenized immediately in a glass-homogenizing tube equipped with a Teflon pestle. The homogenate was processed according to the method of Koyama *et al.*[20] for the estimation of nitric oxide (NO), malondialdehyde (MDA) and superoxide dismutase (SOD). For catalase (CAT) estimation, the tissue was homogenized in ice-cold isotonic phosphate buffer (pH 7.4) and processed according to the method of Cohen *et al.*[21]. For glutathione (GSH) estimation, liver tissue was homogenized according to the method of Ellman[22] in ice-cold phosphate buffer (pH 8).

Serum analysis

Biochemical assay of enzymes, viz. AST, ALT, ACP and GGT were performed from serum using kit methods. AST and ALT kits were obtained from E. Merck (India) Ltd. ACP and GGT kits were obtained from LABKIT, Spain and HUMAN, Germany respectively.

Estimation of NO production

NO production was estimated by Griess reaction[23], which was expressed in the form of nitrite accumulation. The amounts of nitrite in the sample (μmol unit) were calculated from a sodium nitrite standard curve. The results were expressed as μmol nitrite/mg protein.

Estimation of lipid peroxidation

The quantitative measurement of lipid peroxidation was performed following the thiobarbituric acid (TBA) test. The amount of MDA formed was quantitated by reaction with TBA and used as an index of lipid peroxidation. The results were expressed as nmol MDA/mg protein using molar extinction coefficient of the chromophore (1.56×10^5 cm^2/mmol)[24].

Estimation of antioxidant enzymes: SOD and CAT

SOD was assayed according to the method of Misra and Fridovich[25], comparing the adrenochrome formation in different groups of animals. CAT was assayed by the method of Cohen *et al.*[21]. The enzyme-catalysed decomposition of H_2O_2 was measured and the result was expressed as first-order reaction rate constant/mg protein.

Estimation of GSH

GSH was estimated in the liver samples according to the method of Ellman[22]. Results were expressed as μmol GSH/mg protein. Protein in the homogenate of liver tissue was estimated by the method of Lowry *et al.*[26], using BSA as standard.

Pathological evaluation

After 30 days of ethanol treatment, livers were both cold acetone and formalin-fixed, embedded in paraffin, and stained respectively, with periodic acid-Schiff reagent and hematoxylin-eosin[27] to assess the histopathological changes caused by ethanol with or without high-fat diet + BTE supplementation. Liver pathology was scored as described by Nanji *et al.*[28] as follows: steatosis (the percentage of liver cells containing fat): < 25% = 1 +, < 50% = 2+, < 75% = 3+, 75% > = 4+; inflammation and necrosis: 1 focus per low-power field = 1 +; 2 or more foci = 2 +. Pathology was scored in a blinded manner.

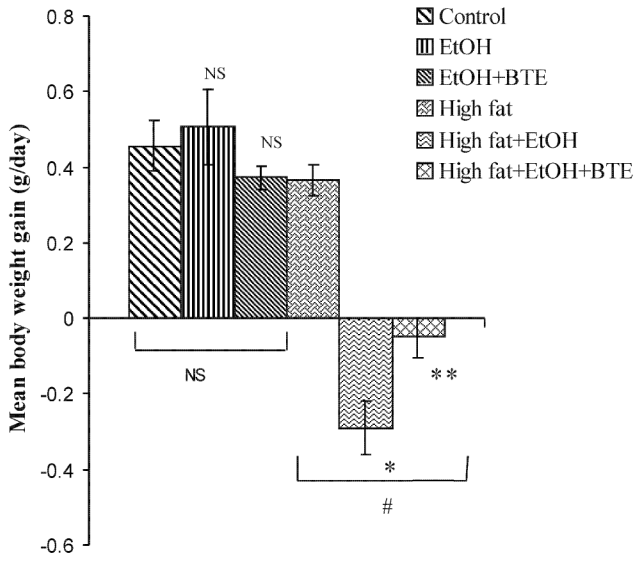

Figure 1. Effect of ethanol (EtOH) [15% (v/v)/100 g body wt/day] and BTE (2.5% at a dose of 1 ml/100 g body wt/day) with or without high-fat diet for 30 days on mean body weight of different groups of rats. Error bars represent mean ± SEM ($n = 6$). #, Significance based on Kruskal–Wallis nonparametric ANOVA test at $P < 0.01$; *, Significance based on Mann–Whitney U multiple comparison test at $P < 0.01$, and ** at $P < 0.05$. NS, Not significant.

Statistical analysis

Data were expressed as mean ± SEM. Kruskal–Wallis nonparametric ANOVA test was performed to find whether or not scores of different groups differ significantly. To test inter-group significant difference, Mann–Whitney U multiple comparison test was performed. SPSS 10.0 software (SPSS Inc, 1999) was used for statistical analysis. Differences were considered significant if $P < 0.05$.

Results

Effect of chronic ethanol administration with or without high-fat diet on mean body weight: Role of BTE

Mean body weight gain (g/day) of the ethanol, ethanol + BTE and control group rats did not vary significantly at any time point during the treatment. Also, compared to high-fat diet group, ethanol + high-fat diet group rats did not gain body weight at all; rather their mean body weight decreased. However, such decrease in body weight was significantly blunted when BTE was simultaneously administered (Figure 1).

Effect of chronic ethanol administration with or without high-fat diet on serum AST, ALT and ACP activities: Role of BTE

The results of the changes in activities of the serum enzymes, AST, ALT and ACP, on chronic ethanol administration (30 days) with or without high-fat diet are represented in Figure 2. The results show that on ethanol administration, there was a significant increase in the activities of AST (24%, $P < 0.01$), ALT (31%, $P < 0.01$) and ACP (43%, $P < 0.01$) over control values; this effect of ethanol was blunted significantly (AST 97%, $P < 0.01$; ALT 113%, $P < 0.01$; ACP 70%, $P < 0.05$) by BTE. On chronic ethanol + high-fat diet administration (30 days), activities of all three enzymes were increased significantly (AST 57%, $P < 0.01$; ALT 20%, $P < 0.01$ and ACP 119%, $P < 0.01$). This effect of ethanol + high-fat diet, however, was blunted significantly (AST 49%, $P < 0.01$; ALT 101%, $P < 0.01$ and ACP 34%, $P < 0.01$) by BTE in case of all the enzymes.

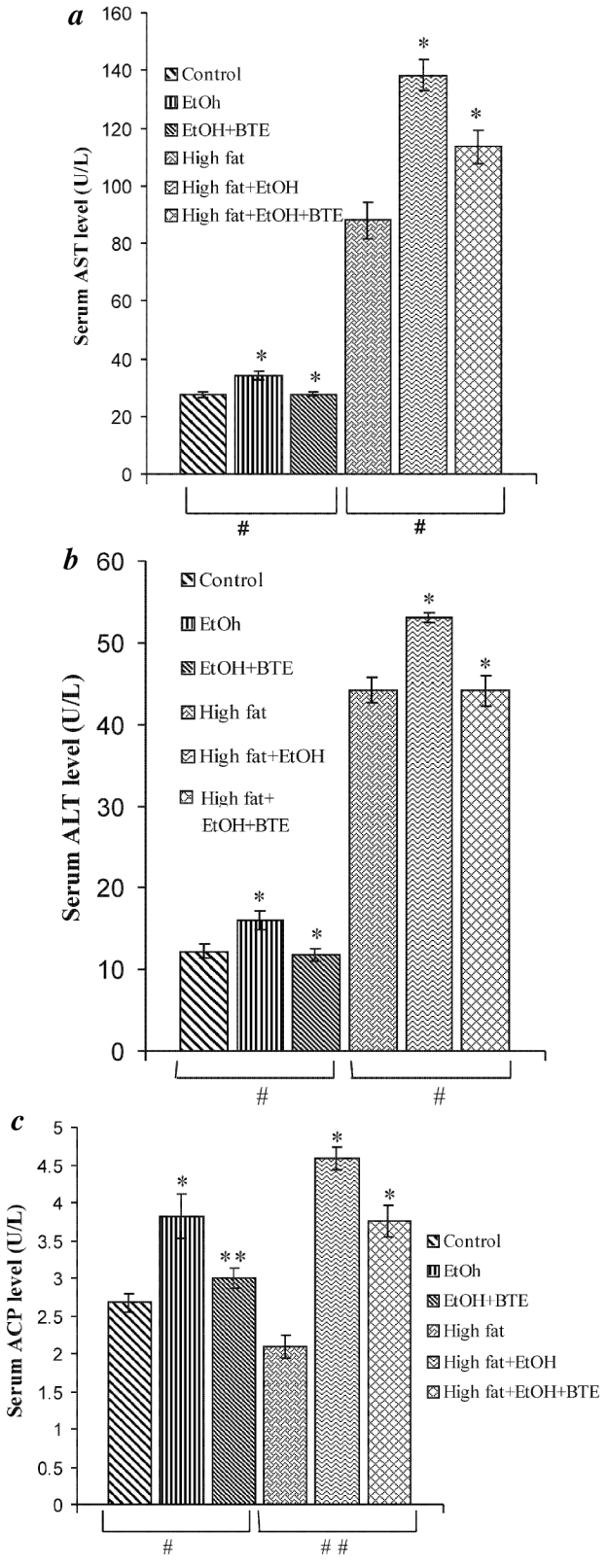

Figure 2. Effect of EtOH [15% (v/v)/100 g body wt/day] and BTE (2.5% at a dose of 1 ml/100 g body wt/day) with or without high-fat diet for 30 days on serum AST, ALT and ACP activities in rat. Error bars represent mean ± SEM ($n = 6$). #, Significance based on Kruskal–Wallis nonparametric ANOVA test at $P < 0.01$ and ##, at $P < 0.001$. *, Significance based on Mann–Whitney U multiple comparison test at $P < 0.01$ and **, at $P < 0.05$.

Effect of chronic ethanol + high-fat diet administration on GGT activity: Role of BTE

On chronic administration (30 days) of ethanol + high-fat diet, the activity of GGT was increased significantly (65%, $P < 0.01$). This effect of ethanol + high-fat diet, however, was blunted significantly (101%, $P < 0.01$) by BTE (Table 2).

Effect of chronic ethanol administration with or without high-fat diet on NO production in liver: Role of BTE

The results of the changes of oxidative enzyme activity profiles (in terms of product formation) in different groups of rats on chronic ethanol administration (30 days) with or without high-fat diet are presented in Figure 3. Nitrite accumulation, an indicator of NO synthesis, was expressed in terms of μmol/mg protein. Nitrite accumulations in ethanol-treated group of rats were found to increase significantly (44%, $P < 0.01$) over control group; this effect of ethanol was diminished significantly (139%, $P < 0.01$) by BTE. After administration of ethanol with high-fat diet for 30 days, NO production was increased significantly (25%, $P < 0.01$) compared to high-fat diet-supplied group of rats. This effect of ethanol + high-fat diet could be significantly blunted by about 147% ($P < 0.01$) by BTE.

Effect of chronic ethanol administration with or without high-fat diet on MDA production in liver: Role of BTE

MDA formation, an indicator of lipid peroxidation, was expressed in terms of nmol/mg protein. The level of MDA, similar to NO production, was significantly increased (52%,

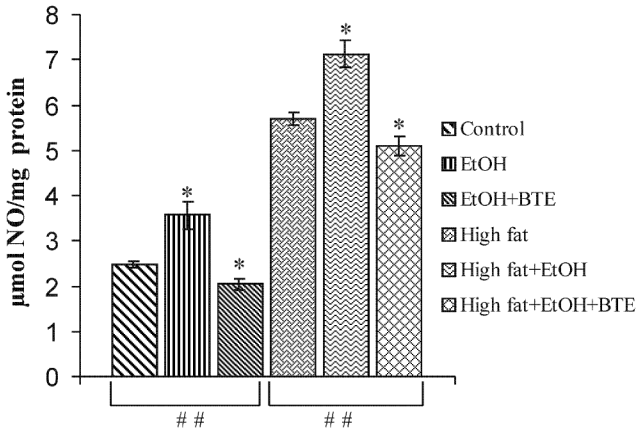

Figure 3. Effect of EtOH [15% (v/v)/100 g body wt/day] and BTE (2.5% at a dose of 1 ml/100 g body wt/day) with or without high-fat diet for 30 days on NO production in rat liver tissue. Error bars represent mean ± SEM ($n = 6$). ##, Significance based on Kruskal–Wallis nonparametric ANOVA test at $P < 0.001$. *, Significance based on Mann–Whitney U multiple comparison test at $P < 0.01$.

Table 2. Effect of ethanol (EtOH) [15% (v/v)/100 g body wt/day] with high-fat diet and EtOH + high-fat + BTE (2.5% at a dose of 1 ml/100 g body wt/day) on serum GGT activity in rat

Parameter	High-fat (Group D)	High-fat + EtOH (Group E)	High-fat + EtOH + BTE (Group F)	Significance level#	Significance level*	
					D vs E	E vs F
GGT (U/l)	4.13 ± 0.22	6.8 ± 0.21	4.11 ± 0.15	$P < 0.01$	$P < 0.01$	$P < 0.01$

Data are mean ± SEM ($n = 6$); #Significance based on Kruskal–Wallis nonparametric ANOVA test; *Significance based on Mann–Whitney U multiple comparison test.

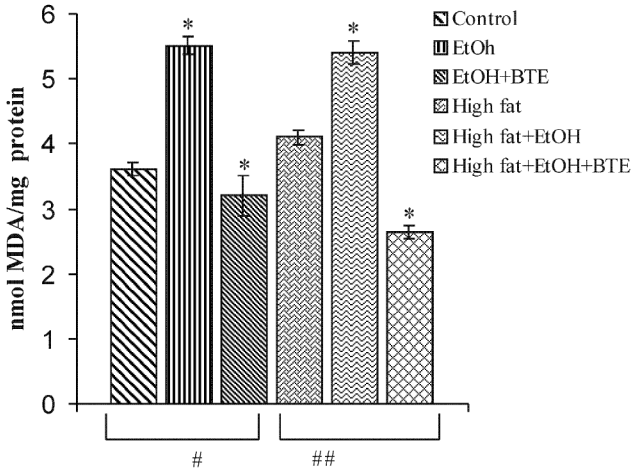

Figure 4. Effect of EtOH [15% (v/v)/100 g body wt/day] and BTE (2.5% at a dose of 1 ml/100 g body wt/day) with or without high-fat diet for 30 days on MDA production in rat liver tissue. Error bars represent mean ± SEM ($n = 6$). #, Significance based on Kruskal–Wallis non-parametric ANOVA test at $P < 0.01$ and ##, at $P < 0.001$. *, Significance based on Mann–Whitney U multiple comparison test at $P < 0.01$.

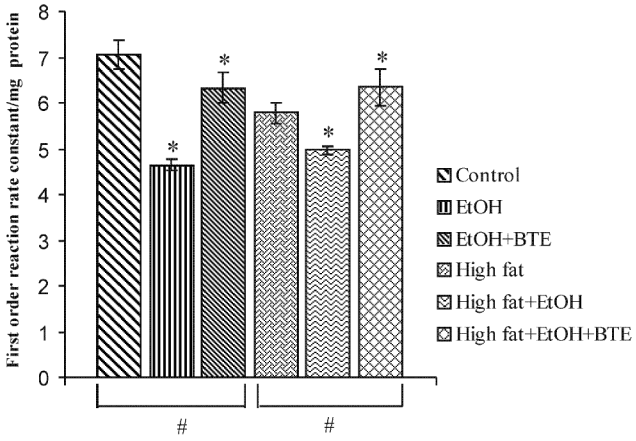

Figure 5. Effect of EtOH [15% (v/v)/100 g body wt/day] and BTE (2.5% at a dose of 1 ml/100 g body wt/day) with or without high-fat diet for 30 days on CAT activity in rat liver tissue. Error bars represent mean ± SEM ($n = 6$). #, Significance based on Kruskal–Wallis non-parametric ANOVA test at $P < 0.01$. *, Significance based on Mann–Whitney U multiple comparison test at $P < 0.01$.

$P < 0.01$) compared to control group. BTE significantly (122%, $P < 0.01$) blunted the enhanced, MDA production caused by ethanol only (Figure 4). On chronic (30 days) ethanol + high-fat diet administration, MDA production was increased significantly ($P < 0.01$) by about 32% compared to high-fat diet-treated group of rats. BTE significantly ($P < 0.01$) blunted this increase in MDA production (211%; Figure 4).

Effect of chronic ethanol administration with or without high-fat diet on CAT activity: Role of BTE

The results of the changes in CAT in different groups of rats on chronic ethanol administration (30 days) with or without high-fat diet are presented in Figure 5. The results show that on ethanol administration, there was a significant decrease in CAT activity (34%, $P < 0.01$), compared to control values; this response could be significantly blunted by BTE (17%, $P < 0.01$). On chronic (30 days) ethanol + high-fat diet administration, compared to high-fat diet-treated rats, the activity of CAT was found to be decreased by about 14% ($P < 0.01$), which was increased significantly (169%, $P < 0.01$) by BTE treatment.

Effect of chronic ethanol administration with or without high-fat diet on SOD activity: Role of BTE

The results of the changes of SOD activity in different groups of rats on chronic ethanol administration (30 days) with or without high-fat diet are presented in Figure 6. SOD was expressed in terms of μg/mg protein. It was observed that on ethanol administration, compared to control, there was a significant decrease in SOD activity (23%, $P < 0.01$), which was significantly blunted by BTE supplementation (171%, $P < 0.01$). After administration of ethanol with high-fat diet for 30 days, SOD activity was decreased significantly ($P < 0.01$) by about 17% compared to high-fat diet-treated rats. This decreased effect of ethanol + high-fat diet could be significantly increased by about 104% ($P < 0.01$) by BTE.

Effect of chronic ethanol administration with high-fat diet on GSH content in liver: Role of BTE

Table 3 depicts the total GSH content of the liver of rats treated with ethanol with or without high-fat diet. The results show that GSH content was significantly decreased (48%,

Table 3. Effect of EtOH [15% (v/v)/100 g body wt/day] + high-fat diet and EtOH + high-fat + BTE (2.5% at a dose of 1 ml/100 g body wt/day) on total GSH level in rat liver tissue

Parameter	High-fat (Group D)	High-fat + EtOH (Group E)	High-fat + EtOH + BTE (Group F)	Significance level#	Significance level*	
					D vs E	E vs F
GSH (μmol/mg protein)	1.45 ± 0.23	0.752 ± 0.01	0.851 ± 0.26	$P < 0.001$	$P < 0.01$	$P < 0.01$

Data are mean ± SEM ($n = 6$); #Significance based on Kruskal–Wallis nonparametric ANOVA test; *Significance based on Mann–Whitney U multiple comparison test.

Table 4. Effect of EtOH [15% (v/v)/100 g body wt/day] and EtOH + BTE (2.5% at a dose of 1 ml/100 g body wt/day) on pathology scores in rats

	Control (Group A)	EtOH (Group B)	EtOH + BTE (Group C)	Significance level#	Significance level*	
					A vs B	B vs C
Inflammation	0	0.995 ± 0.23	0.22 ± 0.07	$P < 0.01$	$P < 0.01$	$P < 0.01$

Pathological changes were scored as described in the text. Data are mean ± SEM ($n = 6$). #Significance based on Kruskal–Wallis nonparametric ANOVA test. *Significance based on Mann–Whitney U multiple comparison test.

Table 5. Effect of EtOH [15% (v/v)/100 g body wt/day] with high-fat diet and EtOH + high-fat + BTE (2.5% at a dose of 1 ml/100 g body wt/day) on pathology scores in rats

	High-fat (Group D)	High-fat + EtOH (Group E)	High-fat + EtOH + BTE (Group F)	Significance level#	Significance level*	
					D vs E	E vs F
Steatosis	0.05 ± 0.05	2.22 ± 0.51	0.275 ± 0.13	$P < 0.05$	$P < 0.05$	$P < 0.05$
Inflammation	0.165 ± 0.07	1.73 ± 0.08	0 .22 ± 0.11	$P < 0.01$	$P < 0.01$	$P < 0.01$
Necrosis	0	1.2 ± 0.1	0 .05 ± 0.05	$P < 0.001$	$P < 0.01$	$P < 0.01$
Total	0.215 ± 0.12	5.17 ± 0.69	0.545 ± 0.29			

Pathological changes were scored as described in the text. Data are mean ± SEM ($n = 6$). #Significance based on Kruskal–Wallis nonparametric ANOVA test. *Significance based on Mann–Whitney U multiple comparison test.

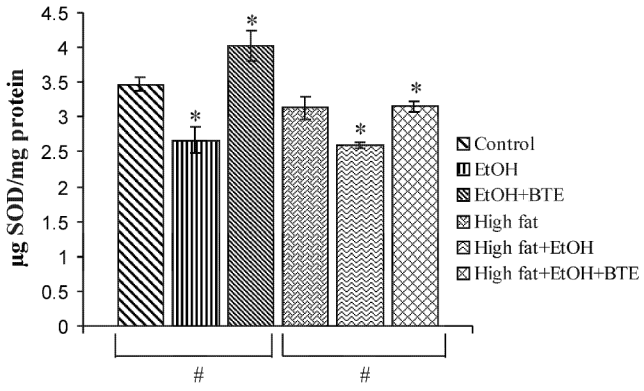

Figure 6. Effect of EtOH [15% (v/v)/100 g body wt/day] and BTE (2.5% at a dose of 1 ml/100 g body wt/day) with or without high-fat diet for 30 days on SOD activity in rat liver tissue. Error bars represent mean ± SEM ($n = 6$). #, Significance based on Kruskal–Wallis nonparametric ANOVA test at $P < 0.01$. *, Significance based on Mann–Whitney U multiple comparison test at $P < 0.01$.

$P < 0.01$) in ethanol + high-fat diet-treated group of rats compared to high-fat diet group. BTE treatment was found to increase significantly the formation of GSH (14%, $P < 0.01$).

Pathological changes

Effect of chronic ethanol administration on liver histology – Role of BTE: Figure 7 shows photomicrographs of rat liver following ethanol treatment with or without high-fat diet. With ethanol only, low magnification (100X) shows some pathological changes of liver injury (Figure 7 *b*), compared to control (Figure 7 *a*). However with higher magnification (400X), these changes could be seen as (i) presence of inflammatory cells within and around central vein, and (ii) absence of normal radiating pattern of cell plates (Figure 7 *c*). Pathology score of such sections of livers was only on the basis of inflammatory changes and is represented in Table 4. BTE blunted these pathological changes of ethanol treatment and decreased the number of inflammatory foci (Figure 7 *d*). Administration of ethanol + high-fat diet for 30 days caused severe glycogen depletion (absence of PAS stain which is specific for glycogen; Figure 7 *e*). BTE supplementation also could blunt this glycogen depletion (Figure 7 *f*). On further examination of fatty infiltration (Figure 7 *g* and *h*), inflammation and focal necro-

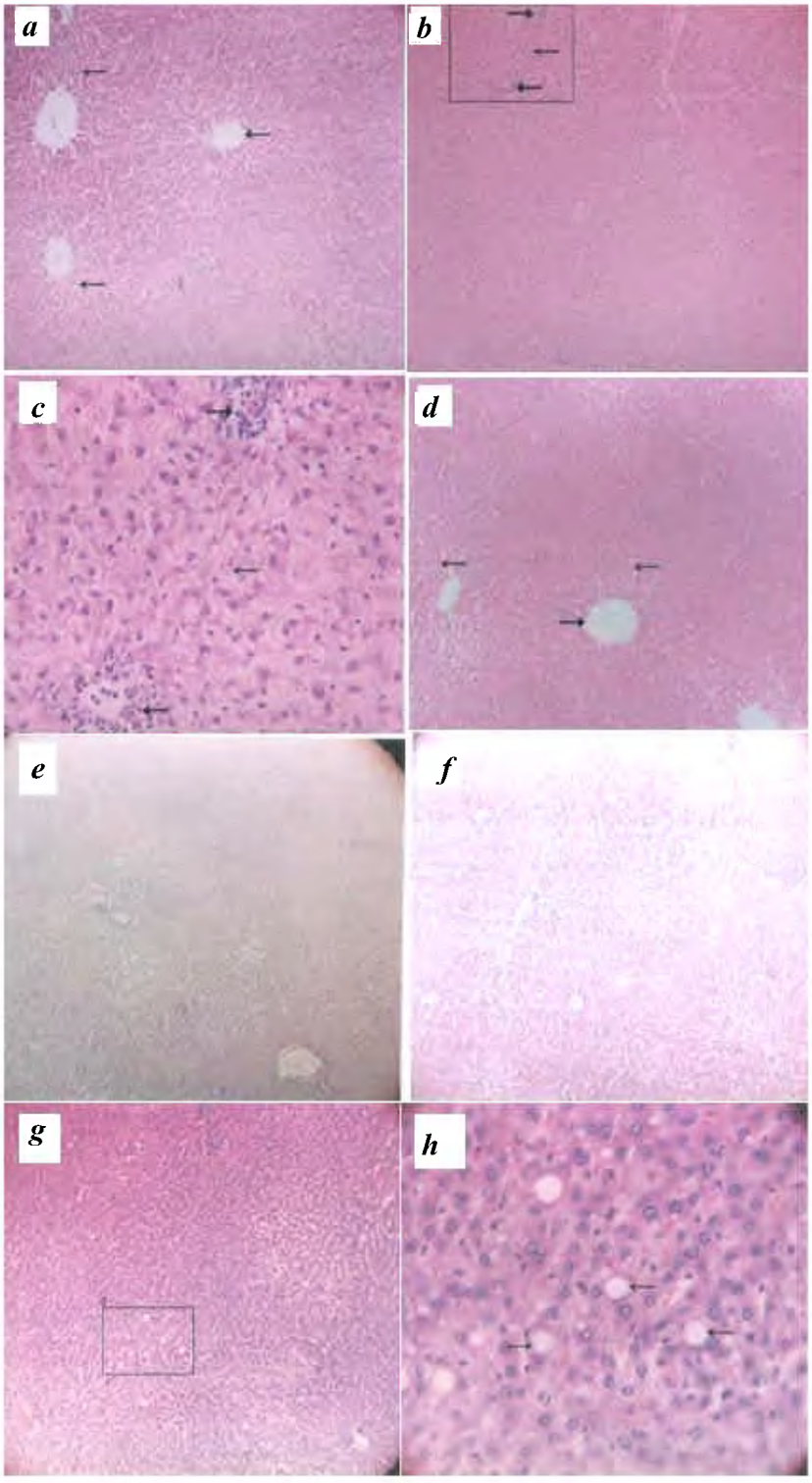

Figure 7. Representative photomicrographs of liver sections from rats treated with ethanol (15% v/v per 100 g body wt/day) with or without high-fat diet and BTE supplementation (2.5% at a dose of 1 ml/100 g body wt/day) for 30 days. Tissues were stained with haematoxylin–eosin and PAS. ***a***, Representative section (100X) from control rat, no pathologic changes are present. Normal architecture of the liver was vivid, i.e. normal pattern of the central vein (◄—) with radiating pattern of hepatocytes (◄—), which are normal in shape and size. ***b***, Representative section (100X) from rat treated with ethanol. ***c***, With higher magnification (400X) typical characteristics of injury are seen: (i) presence of inflammatory cells within and around the central vein (◄—), and (ii) disorganization of normal radiating pattern of cell plates around central vein (◄—). ***d***, Representative section (100X) from rat treated with ethanol + BTE; a complete protection is observed: (i) normal pattern of the central vein, presence of no inflammatory cells (◄—), (ii) normal radiating pattern of cell plates (◄—). ***e***, Representative section (100X) from rat treated with high-fat diet + ethanol, whose intensity of staining revealed massive depletion of glycogen. ***f***, Representative section (100X) from rat treated with high-fat diet + ethanol + BTE, whose intensity of staining revealed a near-normal level of glycogen. ***g***, Representative section (100X) from rat treated with high-fat diet + ethanol, which reveals prominent signs of moderate fatty infiltration. ***h***, Higher magnification (400X) of inset in (***g***) shows signs of fatty infiltration.

sis, a total pathology score of 5.17 ± 0.69 was obtained (Table 5). BTE blunted these liver injury effects of ethanol + high-fat diet administration and the total pathology score of livers from rats was 0.545 ± 0.29 (Table 5).

Discussion

The most encouraging findings of this study are: (i) BTE effectively could prevent ethanol-induced biochemical changes of liver toxicity, and (ii) this action of BTE also could be seen against more pronounced liver toxicity caused by ethanol + high-fat diet.

Earlier it has been well documented that both AST and ALT are considered among the most sensitive markers of hepatocellular injury[29]. ACP, which is secreted from the lysosomes, is also a marker enzyme for assessing liver damage. When the integrity of the lysosomal membrane changes and/or the membrane of the lysosome is ruptured by deleterious influences, these acid hydrolases enter the blood stream, producing transient increase in the activity of lysosomal enzymes in the serum[29]. Excess alcohol consumption also has been linked with altered liver metabolism and liver damage, with leakage of cytoplasmic liver enzyme GGT into blood[30]. GGT index has been reported high in alcoholic liver disease[29] and measurement of GGT has been claimed to be an extremely sensitive test and marker of ethanol-induced hepatic damage[1]. Results indicate that BTE administration could blunt ethanol-induced increase in activities of different marker enzymes of heptocellular injury, viz. AST, ALT, ACP and GGT with or without high-fat diet (Figures 2 and 7; Table 2) suggesting that BTE possibly has a protective influence against ethanol with or without high-fat diet-induced hepatocellular injury and degenerative changes.

With respect to food intake and gain in mean body weight (g/day), animals in the ethanol with or without high-fat diet groups did not alter significantly (Figure 1). Rather, there was a significant decrease in mean body weight of the animals in ethanol + high-fat diet group, which could be blunted significantly by BTE. This decrease in body weight by ethanol + high-fat diet and its recovery by BTE, despite similar food consumption, suggests that BTE possibly has a positive anabolic effect. This observation has an indirect support from an earlier raised question whether or not polyphenol esters and complex polyphenols of BTE, similar to that of soybean protein isolate, stimulate the synthesis of growth hormone[31].

This has been confirmed in our histological studies of hepatic tissue (Figure 7; Tables 4 and 5), where BTE supplementation was found to recover ethanol with or without high-fat diet-induced histopathological changes. Results of pathology score (Tables 4 and 5) further established that BTE is protective against ethanol-induced liver injury. It is well established that acute hypoglycaemia may result from drug–alcohol interactions and alcohol ingestion, and reflect an inhibition of gluconeogenesis due to depletion of glycogen in the liver[30,32]. Light microscopic examination of PAS-stained liver sections in this study have revealed that BTE supplementation could restore the depletion of glycogen (Figure 7 *e* and *f*). Such hepatoprotective effect of BTE in our studies corroborates well with earlier observations that tea polyphenols have a variety of physiological functions such as antioxidative, anti-bacterial, anti-diabetic, anti-neoplastic, hypolipidemic, hypotensive and anti-inflammatory[33,34].

Formation of ROS, oxidative stress and hepatocellular injury have been implicated to alcoholic liver disease. It has been documented that Kupffer cells are the major sources of ROS during chronic ethanol consumption, and these are primed and activated for enhanced formation of pro-inflammatory factors[8]. Additionally, alcohol-induced liver injury has been associated with increased amount of lipid peroxidation[35]. It may thus be plausible that in our study, loss of membrane structure and integrity because of lipid peroxidation was accompanied with an elevated level of activities of marker enzymes, AST, ALT, ACP and GGT. Indeed, BTE-supplementation in our study was potentially effective in blunting lipid peroxidation, suggesting that BTE possibly has antioxidant property to reduce ethanol with or without high-fat diet-induced membrane lipid peroxidation and thereby to preserve membrane structure.

Alcoholic liver disease is characterized by steatosis, inflammation, necrosis and ultimately fibrosis and cirrhosis[36]. NO, a reactive free radical generated from L-arginine by NO synthase, is well recognized as a physiological messenger molecule. Excessive amounts of NO, however, are potentially toxic and have been implicated in numerous pathological situations and chronic inflammation[37]. Also, NO is a cytotoxic agent involved as a mediator in inflammatory disorders[38] and because of its cytotoxicity, overproduction is deleterious to cells[39]. In our studies, administration of ethanol with or without high-fat diet was found to cause a significant increase in NO production that, we speculate, might be responsible for hepatocellular injury and inflammatory changes. BTE-supplementation could blunt this enhanced NO level significantly, almost to control or even less than control level. Earlier, it was reported that black tea scavenges NO and peroxynitrite, and also inhibits excessive production of NO by the inducible form of NO synthase (iNOS)[40]. Therefore, it seems logical to infer that BTE, because of its antioxidant property, might be capable of protecting the hepatic tissue from ethanol-induced injury and inflammatory changes.

Our study further revealed that chronic exposure to ethanol with or without high-fat-diet decreased the activities of the ROS scavenging enzymes, viz. SOD and CAT. This is in line with assumption suggested earlier by Sandhir and Gill[1], that decrease in the activity of antioxidant enzymes SOD, glutathione peroxidase and GSH following ethanol exposure may be due to the damaging effects of free radicals, or alternatively could be due to a direct effect of acetaldehyde, formed from oxidation of ethanol, on these enzymes. BTE-

supplementation, in our studies, could restore the activity of both these antioxidant enzymes and possibly could reduce generation of free radicals and hepatocellular damage. This was found to be similar to the earlier observation that tea flavonoids have antioxidant properties[11,41] and are a powerful chemopreventor of oxidative damage caused by free radicals[12,42]. Thus, results of these studies together with those of earlier ones, suggest that BTE has an ability to protect the liver from ethanol-induced damage through its direct antioxidative effect.

GSH is a naturally occurring antioxidant important in the antioxidant defence of the body. It has been reported that determination of total GSH, as well as its reduced and oxidated fractions, can serve as a key to know the amount of antioxidant reserve in the blood and probably in the organism and also, contribute in evaluating the possibilities available for the recuperation of alcoholic patients[43,44]. Therefore, the levels of glutathione are of critical importance in liver injury caused by toxic substances such as ethanol. It has been claimed that binding of acetaldehyde, a metabolite of ethanol, with GSH may contribute to reduction in the levels of GSH[1]. Recently, it has been reported that tea polyphenols function as antioxidants through the induction of antioxidant enzymes, such as glutathione S-transferases and superoxide dismutases[45]. Our results are in line with this earlier report because we found that after BTE-supplementation, elevated GSH level in rats with high-fat diet + ethanol could be blunted to normal level. This ability of black tea to protect the liver from high-fat diet + ethanol-induced damage might be attributed to its ability to restore the activity of antioxidative enzymes.

Light microscopic evidence of histopathological changes in the hepatic tissue of rat induced by ethanol with or without high-fat diet and its reversal by BTE-supplementation further emphasizes the hepatoprotective effect of aqueous extract of black tea, which has not been reported previously.

In summary, we demonstrate that BTE prevents ethanol with or without high-fat diet-induced oxidative stress, hepatic injury and inflammatory changes. Since these models of hepatic damage in the rat simulate many of the features of human liver pathology, we suggest that natural antioxidants and scavenging agents in BTE might be effective as plant hepatoprotectors and thus may have some obvious therapeutic implications.

1. Sandhir, R. and Gill, K. D., Hepatoprotective effects of Liv-52 on ethanol induced liver damage in rats. *Indian J. Exp. Biol.*, 1999, **37**, 762–766.
2. Rezvani, A. H., Overstreet, D. H., Perfumi, M. and Massi, M., Plant derivatives in the treatment of alcohol dependency. *Pharmacol. Biochem. Behav.,* 2003, **75**, 593–606.
3. French, S. W., Miyamoto, K. and Tsukamoto, H., Ethanol-induced hepatic fibrosis in the rat: Role of the amount of dietary fat. *Alcohol. Clin. Exp. Res.* (*Suppl.*), 1986, **10**, 13S–19S.
4. Nomura, F. *et al.*, Fatty liver in rats induced by excessive intake of a nutritionally adequate liquid diet. *Int. J. Obes.,* 1987, **11**, 603–608.
5. Arteel, G. E., Uesugi, T, Bevan, L. N., Gabele, E., Wheeler, M. D., McKim, S. E. and Thurman, R. G., Green tea extract protects against early alcohol-induced liver injury in rats. *Biol. Chem.*, 2002, **383**, 663–670.
6. Kurose, I., Higuchi, H., Kato, S., Miura, S. and Ishii, H., Ethanol-induced oxidative stress in the liver. *Alcohol. Clin. Exp. Res.*, 1996, **20**, 77A–85A.
7. Davlos, M. and Rolnaldo, N., Alcoholic hepatitis. *Rev. Gastroenterol. Peru*, 1998, **18**, 151–164.
8. Bautista, A. P., Free radicals, chemokines and cell injury in HIV-I and SIV infections and alcoholic hepatitis. *Free Radic. Biol. Med.*, 2001, **31**, 1527–1532.
9. Arteel, G. E., Oxidants and antioxidants in alcohol-induced liver disease. *Gastroenterology*, 2003, **124**, 778–790.
10. Xie, C-I. *et al.*, Diadzin, an antioxidant isoflavonoid, decreases blood alcohol levels and shortens sleep time induced by alcohol intoxication. *Alcohol. Clin. Exp. Res.*, 1994, **18**, 1443–1448.
11. Langley-Evans, S. C., Antioxidant potential of green and black tea determined using the ferric reducing power (FRAP) assay. *Int. J. Food Sci. Nutr.*, 2000, **51**, 181–188.
12. Sarkar, A. and Bhaduri, A., Black tea is a powerful chemopreventor of reactive oxygen and nitrogen species: Comparison with its individual catechin constituents and green tea. *Biochem. Biophys. Res. Commun.*, 2001, **284**, 173–178.
13. Leenan, R., Roodenburg, A. J., Tijburg, L. B. and Wiseman, S. A., A single dose of tea with or without milk increases plasma antioxidant activity in humans. *Eur. J. Clin. Nutr.*, 2000, **54**, 87–92.
14. Thiagarajan, G., Chandani, S., Sundari, C. S., Rao, S. H., Kulkarni, A. V. and Balasubramanium, D., Antioxidant properties of green and black tea, and their potential ability to retard the progression of eye lens cataract. *Exp. Eye Res.*, 2001, **73**, 393–401.
15. Sur-Altiner, D. and Yenice, B., Effect of black tea on lipid peroxidation in carbon tetrachloride treated male rats. *Drug Metab. Drug Interact.*, 2000, **16**, 123–128.
16. Hung, Y. C., Sava, V. M., Blagodarsky, V. A., Hong, M. Y. and Huang, G. S., Protection of tea melanin on hydrazine-induced liver injury. *Life Sci.*, 2003, **72**, 1061–1071.
17. Lieber, C. S. and Decarli, L. M., Animals models of chronic ethanol toxicity. *Methods Enzymol.*, 1994, **233**, 585–594.
18. Wei, H., Zhang, X., Zhao, J. F., Wang, Z. Y., Bickers, D. and Lebwohl, M., Scavenging of hydrogen peroxide and inhibition of ultraviolet light-induced oxidative DNA damage by aqueous extracts from green and black teas. *Free Radic. Biol. Med.*, 1999, **26**, 1427–1435.
19. Luma, P. and Vorne, M., Changes in the hepatic microsomal enzyme activity during long-term ethanol feeding in rat. *Acta Pharmacol. Toxicol.*, 1976, **38**, 260–266.
20. Koyama, I., Tsugikazu, K., Yoshikatsu, S. and Munetsugu, K., A possible mechanism for the changes in hepatic and intestinal alkaline phosphatase activities in bile duct ligated rats and guinea pigs. *Biochim. Biophys. Acta*, 1983, **760**, 169–174.
21. Cohen, G., Dembiec, D. and Marcus, J., Measurement of catalase activity in tissue extract. *Anal. Biochem.,* 1970, **34** , 30–37.
22. Ellman, G. L., Tissue sulfhydryl groups. *Arch. Biochem.*, 1959, **82**, 70–77.
23. Giuseppina, M. R., Rosario, M., Oreste, G., Maria, P. and Raffaele, D. C., Prolactin induction of nitric oxide synthase in rat C6 glioma cells. *J. Neurochem.*, 1999, **73**, 2272–2277.
24. Wills, E. D., Evaluation of lipid peroxidation in lipids and biological membranes. In *Biochemical Toxicology – A Practical Approach* (eds Snell, K. and Mullock, B.), IRL Press, Oxford, 1987, p. 138.
25. Misra, H. P. and Fridovich, I., The role of superoxide anion in the autooxidation of epinephrine and simple assay for superoxide dismutase. *J. Biol. Chem.*, 1972, **247**, 3170–3175.
26. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J., Protein measurement with folin phenol reaction. *J. Biol. Chem.*, 1951, **193**, 265–271.

27. Bancroft, J. D., Stevens, A. and Turner, D. R., *Theory and Practice of Histological Techniques*, Churchill Livingstone, New York, 1996, p. 104.
28. Nanji, A. A., Mendenhall, C. L. and French, S. W., Beef fat prevents alcoholic liver disease in the rat. *Alcohol. Clin. Exp. Res.*, 1989, **13**, 15–19.
29. Nemesanszky, E., Enzyme test in hepatobiliary disease. In *Enzyme Test in Diagnosis* (eds Moss, D. W. and Rosalki, S. B.), Arnold, London, pp. 23–59.
30. James, W. P. T., Alcohol: Its metabolism and effects. In *Human Nutrition and Dietetics* (eds Garrow, J. S. and James, W. P. T.), Churchill Livingstone, London, 1993, pp. 103–118.
31. Arjmandi, B. H., Alekel, L., Hollis, B. W., Amin, D., Stacewicz-sapuntzakis, M., Guo, P. and Kukreja, S. C., Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. *J. Nutr.*, 1996, **126**, 161–167.
32. Varley, H., Gowenlock, A. H. and Bell, M., In *Practical Clinical Biochemistry*, William Heinemann Medical Books Ltd, London, 1980, 5th edn, p. 403.
33. Matsumoto, N., Tono-Oka, F., Ishigaki, A., Okushio, K. and Hara, Y., The fate of (–)-epigallocatechin gallate (EGCg) in the digestive tract of rats. Proceedings of the International Symposium on Tea Science Japan, 1991, pp. 253–257.
34. Krishnamoorthy, K. K., The nutritional and therapeutic value of tea. Proceedings of the International Symposium on Tea Science Japan, 1991, pp. 6–11.
35. Nanji, A. A., Jokelainen, K., Tipoe, G. L., Rahemtulla, A., Thomas, P., Dannenberg, A. J. and Fisher, A. E., Curcumin prevents alcohol-induced liver disease in rats by inhibiting the expression of NF-KB-dependent genes. *Am. J. Physiol. – Gastrointest. Liver Physiol.*, 2003, **284**, G321–G327.
36. Kono, H., Arteel, G. E., Rusyn, I., Sies, H. and Thurman, R. G., Ebselen prevents early alcohol-induced liver injury in rats. *Free Radic. Biol. Med.*, 2001, **30**, 403–411.
37. Yabuki, M., Kariya, S., Ishisaka, R., Yasuda, T., Yoshioka, T., Horton, A. A. and Utsumi, K., Resistance to nitric oxide-mediated apoptosis in HL-60 variant cells is associated with increased activities of Cu, Zn-superoxide dismutase and catalase. *Free Radic. Biol. Med.*, 1999, **26**, 325–332.
38. Kalf, J. C., Schraut, W. H., Billiar, J. R., Simmons, R. L. and Bauer, A. J., Role of inducible nitric oxide synthase in postoperative intestinal smooth muscle dysfunction in rodents. *Gastroenterology*, 2000, **118**, 316–327.
39. Krippeit-Drews, P. *et al.*, The effects of nitric oxide on the membrane potential and ionic currents of mouse pancreatic B cells. *Endocrinology*, 1995, **136**, 5363–5369.
40. Paquay, J. B., Haenen, G. R., Stender, G., Wiseman, S. A., Tijburg, L. B. and Bast, A., Protection against nitric oxide toxicity by tea. *J. Agric. Food Chem.*, 2000, **48**, 5768–5772.
41. Matsuzaki, T. and Hara, Y., Antioxidative activity of tea leaf catechins. *Nippon Nogei Kagaku Kaishi*, 1985, **59**, 129–134.
42. Sano, M. *et al.*, Antioxidative activities of green tea and black tea in rat organ and blood plasma. Proceedings of the International Symposium on Tea Science, Japan, 1991, pp. 304–313.
43. Lu, S. C., Huang, Z. Z., Yang, J. M. and Tsukamoto, H., Effect of ethanol and high-fat feeding on hepatic gamma-glutamylcysteine synthetase subunit expression in the rat. *Hepatology*, 1999, **30**, 209–214.
44. Zentella, P. M. *et al.*, Blood glutathione in the alcoholic patient with hepatopathy. *Rev. Med. Hosp. Gen. Mex.*, 1995, **58**, 52–58.
45. Frei, B. and Higdon, J. V., Antioxidant activity of tea polyphenols *in vivo*: Evidence from animal studies. *J. Nutr.*, 2003, **133**, 3275S–3284S.

ACKNOWLEDGEMENTS. This work was financially sponsored by the National Tea Research Foundation, Kolkata. We thank Dr J. R. Vedasiromoni, Indian Institute of Chemical Biology, Kolkata for the generous gift of black tea samples. We also thank Dr Santosh Mitra, Ex-Pathologist, Chittaranjan Cancer Hospital & Seva Sadan, Kolkata and Ms Aloka Biswas for technical help.

Received 27 August 2004; accepted 22 November 2004