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Antioxidant effect of 2-hydroxy-4-methoxy benzoic acid on ethanol-induced hepatotoxicity in rats

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Abstract

Alcoholic liver disease (ALD) is one of the most common diseases in society. A large number of studies are in progress to identify natural substances that are effective in reducing the severity of ALD. 2-Hydroxy-4-methoxy benzoic acid (HMBA), the active principle of *Hemidesmus indicus*, an indigenous Ayurvedic medicinal plant in India, is expected to significantly inhibit the development of liver injury in ethanol administration. It is expected to reduce the severity of liver damage in terms of body weight, hepatic marker enzymes, oxidative stress, antioxidant status and histological changes in ethanol-induced hepatotoxic rats. Hepatotoxicity was induced by administering 20% ethanol (5 g kg^{-1} daily) for 60 days to male Wistar rats, which resulted in significantly decreased body weight and an increase in liver–body weight ratio. The liver marker enzymes aspartate transaminase, alanine transaminase, alkaline phosphatase, γ-glutamyl transpeptidase and lactate dehydrogenase were elevated. In addition, the levels of plasma, erythrocyte and hepatic thiobarbituric acid reactive substances, hydroperoxides and conjugated dienes were also elevated in ethanol-fed rats as compared with those of the experimental control rats. Decreased activity of superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, vitamin C and α-tocopherol was also observed on alcohol administration as compared with experimental control rats. HMBA was co-administered at a dose of 200 μg kg^{-1} daily for the last 30 days of the experiment to rats with alcohol-induced liver injury, which significantly increased body weight, significantly decreased the liver–body weight ratio, transaminases, alkaline phosphatase, γ-glutamyl transpeptidase and lactate dehydrogenase, significantly decreased the levels of lipid peroxidative markers, significantly elevated the activity of enzymic and non-enzymic antioxidants in plasma, erythrocytes and liver and also increased levels of plasma and liver vitamin C and α-tocopherol at the end of the experimental period as compared with untreated ethanol-administered rats. The histological changes were also in correlation with the biochemical findings. The results suggest that HMBA administration may afford protection against ethanol-induced liver injury in rats.

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Introduction

Alcoholism is a social and economic problem that is global in scope. Alcohol is the most frequently abused drug throughout the world and has a long history of use. Alcoholism can also be considered one of the costliest diseases of the modern era (Murray & Lopez 1996). In the pathogenesis of ethanol-induced liver injury oxidative stress plays an important role (Lin et al 1988; Zima et al 2001). Peroxidative damage in alcoholic patients and enhanced lipid peroxide production in animals can be correlated with high ethanol consumption (Schlorff et al 1999). Ethanol administration disturbs the balance between the pro- and antioxidant system of the organism, leading to oxidative stress. Generation of oxygen metabolites, such as superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), is believed to be important in the pathogenesis of alcoholic liver injury (Thurman & Handler 1989). Increased generation of oxygen free radicals and ethanol-derived free radicals has been observed at the microsomal level (particularly at the ethanol-inducible cytochrome P450 isoform), the cytosolic xanthine or aldehyde oxidase, as well as through the mitochondrial respiratory chain (Nordmann et al 1992). Polyunsaturated fatty acids are probably the most susceptible target to free radical attack. The reaction of free radicals with the membrane lipid components leads to lipid peroxidation. This process can eventually cause

increased membrane permeability and cell death (Rakonczay et al 2003). To counteract these oxidants, cells have several enzymic antioxidants, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and non-enzymic antioxidants, including reduced glutathione (GSH), ascorbic acid and α-tocopherol. There is evidence that their levels are altered in alcoholics.

The importance of the traditional system of medicine has gained recognition all over the world and several indigenous drugs form an indispensable part of health care (Chopra et al 1958; Nadkarni 1989). A phytotherapeutic approach to modern drug development can provide many invaluable drugs from traditional medicinal plants.

2-Hydroxy-4-methoxy benzoic acid (HMBA) exists in *Hemidesmus indicus* (Asclepiadaceae), which is a widely distributed medicinal plant in India. The root bark of this plant has been used as a traditional medicine in the treatment of biliousness, blood diseases, diarrhoea, respiratory disorders, skin diseases, syphilis, fever, bronchitis, asthma, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite, burning sensation and rheumatism (Nadkarni 1989). Jain & Singh (1994) reported that *H. indicus* is employed in traditional medicine for gastric ailments. It mainly consists of essential oils and phytosterols such as hemidesmol, hemidesterol and saponins. The pure compound 2-hydroxy-4-methoxy benzoic acid (HMBA), $C_8H_8O_4$ was isolated and purified from the methanolic root extract of *H. indicus* root bark.

HMBA (Figure 1) is a white needle-shaped crystal which is soluble in water, methanol and chloroform and has a melting point of 155–158°C and lambda max 260 nm. By spectral analysis, the presence of a benzene ring, methoxy group and hydroxyl group was confirmed. The molecular weight of the compound is 168 (Alam et al 1994). The concentration of HMBA is in the range of 0.03–0.54% in the root bark of *H. indicus* (Nagarajan & Rao 2003). HMBA is known to possess potent anti-inflammatory, antipyretic and antioxidant properties (Alam & Gomes 1998a). The compound effectively neutralizes viper-venom-induced changes in serum phosphatase and transaminase activity in male albino rats and is also known to reduce free radical formation as estimated by thiobarbituric acid reactive substances (TBARS) and SOD activity (Alam & Gomes 1998a). The compound also has an adjuvant effect and antiserum potentiation activity against viper venom (Alam & Gomes 1998b). ACGIH, IARC, NIOSH, NTP and OSHA do not list it for carcinogenicity. The protective effect of *H. indicus* against rifampicin- and isoniazid-induced hepatotoxicity in rats (Prabakan et al 2000), as well as CCl_4- and paracetamol-induced hepatic damage (Baheti et al 2006), is known.

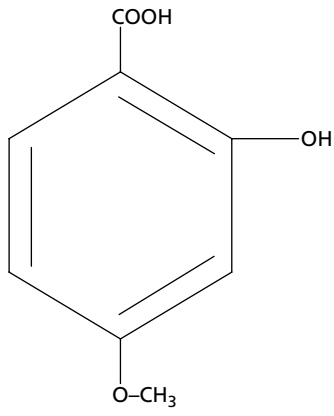

Figure 1 Structure of 2-hydroxy-4-methoxy benzoic acid.

The studies so far have been on hepatotoxicity induced by drugs. Ethanol is being abused by society, which leads to alcoholic liver disease, and the mechanism of action of ethanol is different from that of CCl_4- and paracetamol-induced liver injury. Since *H. indicus* has a protective role on liver damage induced by the above drugs, its active principle, HMBA, is expected to significantly inhibit the development of liver injury in ethanol administration in rats. It is expected to reduce the severity of liver damage in terms of body weight, hepatic marker enzymes, such as aspartate transaminase, alanine transaminase, alkaline phosphatase, γ-glutamyl transpeptidase and lactate dehydrogenase, lipid peroxidative markers, such as TBARS, hydroperoxides (LOOH) and conjugated dienes (CDs), antioxidants, such as SOD, CAT, GPx, GSH, vitamin C and α-tocopherol and histological changes in hepatotoxicity in rats induced by ethanol. Thus, due to the wide pharmacological actions of *H. indicus* and recent interest (Sarasan et al 1994), as well as HMBA being the active principle of *H. indicus* that when given in low doses can be markedly effective rather than the whole extract, the present study was undertaken to establish the antioxidant and hepatoprotective effect of HMBA on an animal model of ethanol-induced liver damage.

Materials and Methods

Drugs and chemicals

2-Hydroxy-4-methoxy benzoic acid, the active principle of *H. indicus*, was purchased from Sigma-Aldrich Co (USA) and had a purity of 99.0%. All other chemicals and solvents were of certified analytical grade and purchased from S.D. Fine Chemicals (Mumbai and Himedia Laboratories Pvt. Ltd, Mumbai, India).

Animals

All the animal handling and experimental procedures were approved by the institutional animal ethics committee, Annamalai University (Registered number: 160/1999/CPC-ESA) and animals were cared for in accordance with the principles, and guidelines of Indian National Law on animal care and use.

Male Wistar rats, 90 days old, 130–180 g, bred in the Central Animal House, Rajah Muthiah Medical College, were used for the study. The rats were given free access to water and normal laboratory pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd, Bangalore, India), consisting of protein (22.21%), fat (3.32%) and fibre (3.11%), balanced with carbohydrates (> 67%), vitamins and minerals.

Study design

The rats were divided into four groups of ten each. Groups 1 and 2 received normal diet and isocaloric glucose from 27% glucose solution (1 mL contains 1.08 cal) daily by intragastric intubation. Liver cell damage was induced in rats of groups 3 and 4 by administering 20% ethanol (5.0 g kg^{-1} daily) (Enomoto et al 1999) (1 mL contains 1.08 cal) as an aqueous solution using an intragastric tube daily for 30 days. At the end of this period the rats were treated as follows for the next 30 days. Group 1 rats continued to receive standard pellet diet and isocaloric glucose from 27% glucose solution in 1% carboxymethylcellulose (CMC) and served as control. Group 2 rats continued to receive standard pellet diet, isocaloric glucose from 27% glucose solution and were administered HMBA (200 μg kg^{-1} daily) (Alam et al 1994) in 1% CMC by intragastric intubation every day. Group 3 rats continued to receive standard pellet diet, 20% ethanol and 1% CMC daily. Group 4 rats continued to receive standard pellet diet, 20% ethanol and HMBA (200 μg kg^{-1} daily) in 1% CMC every day. The total experimental duration was 60 days.

The rats were fasted overnight, anaesthetized with an intramuscular injection of ketamine hydrochloride (30 mg kg^{-1}), and were sacrificed by cervical decapitation. Blood samples were collected in heparinized test tubes and plain tubes and then centrifuged for the separation of plasma and serum, respectively. The erythrocytes were washed with 0.9% saline three times and subsequently distilled water was added for haemolysis. The haemolysate was then used for estimating the total haemoglobin content. Liver tissues were immediately homogenized and used for various biochemical estimations. For histopathological study, three rats from each group were perfused with formalin (10%) and the tissues were separated and stored in 10% formalin. They were later sectioned using a microtome, dehydrated in graded alcohol, embedded in paraffin section and stained with haemotoxylin and eosin.

Estimation of liver marker enzymes

Serum aspartate aminotransferase (AST; EC 2.6.1.1) and serum alanine aminotransferase (ALT; EC 2.6.1.2) were assayed using the diagnostic kit based on the method of Reitman & Frankel (1957). Serum alkaline phosphatase (ALP; EC 3.1.2.3.1) was estimated using the diagnostic kit based on Kind & King's method (Kind & King 1954; King 1965). The serum γ-glutamyl transpeptidase (GGT; EC 2.3.2.2) was assayed according to the method of Rosalki & Rau (1972). The activity of lactate dehydrogenase (LDH; EC 1.1.1.27) was estimated by the method of King (1965).

Lipid peroxidation and enzyme assays

Assay for lipid peroxidation was carried out by measuring TBARS in the tissues by the method of Ohkawa et al (1979) and Yagi (1978). The pink chromogen produced by the reaction of malondialdehyde, a secondary product of lipid peroxidation, with thiobarbituric acid was estimated at 532 nm. LOOH were estimated by the procedure of Jiang et al (1992). This method is based on the rapid peroxide-mediated oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) under acidic conditions in the presence of xylenol orange. The Fe^{3+}–xylenol orange complex was measured spectrophotometrically at 560 nm. CDs were estimated by the method of Recknagel & Glende (1984). Lipid peroxidation is associated with the rearrangement of double bonds in polyunsaturated fatty acids, leading to the formation of CDs, which absorb light at 233 nm. The oxidation index of the lipid sample at 233 nm and 215 nm was computed, which reflected the diene content and the extent of peroxidation.

SOD (EC 1.15.1.1), was assayed by the method of Kakkar et al (1984). The assay was based on the 50% inhibition of the formation of NADH-phenazinemethosulfate-nitrobluetetrazolium (NBT) formazan at 520 nm. The activity of CAT (EC 1.11.1.6) was assayed by the method of Sinha (1972) based on the conversion of dichromate in acetic acid to perchromic acid and then to chromic acetate, when heated in the presence of hydrogen peroxide. The chromic acetate formed was measured at 620 nm. The activity of GPx (EC 1.11.1.9) was assayed by the method of Rotruck et al (1973). A known amount of enzyme preparation was incubated with H_2O_2 in the presence of GSH for a specified time period. The amount of H_2O_2 utilized was determined by the method of Ellman (1959). The enzyme activity was expressed as μmol of GSH consumed per min per mg protein.

GSH in the tissues was assayed by the method of Ellman (1959). GSH estimation was based on the development of yellow colour when 5, 5-dithiobis (2-nitro benzoic acid) dinitrobisbenzoic acid was added to compounds containing a sulfhydryl group. Ascorbic acid was measured according to the method of Omaye et al (1979). α-Tocopherol in plasma and tissues was estimated by the method of Desai (1971). Protein was estimated by the method of Lowry et al (1951) using bovine serum albumin as the standard.

Statistical analysis

Data were analysed by one-way analysis of variance followed by Duncan's multiple range test (DMRT) using a commercially available statistics software package (SPSS for Windows, V. 13.0, Chicago, USA). Results were presented as means ± s.d. $P < 0.05$ was regarded as statistically significant.

Results

Biochemical changes

Table 1 shows the average weight gain, food intake and liver–body weight ratio of control and experimental rats during the experimental period. The food intake and weight gained were significantly reduced in alcohol-administered rats and the liver–body weight ratio was significantly increased as compared with control rats. Rats co-administered HMBA along with ethanol from the 31st day showed significant weight gain, increased food intake and decreased liver–body weight ratio ($P < 0.05$) as compared with untreated ethanol-fed rats.

Administration of ethanol produced severe liver damage, as indicated by marked increase in the activity of AST, ALT,

Table 1 Effect of HMBA on body weight and liver weight to body weight ratio of control and ethanol-administered rats

Group	Body weight		Net gain (g)	Average food intake (g)	Liver wt × 100/body wt
	Day 1	Day 60			
Control	145.11 ± 6.34	226.07 ± 10.93	80.97 ± 4.61[c]	9.18 ± 0.25[c]	2.77 ± 0.14[a]
HMBA	141.02 ± 5.80	224.10 ± 7.03	83.07 ± 1.56[c]	8.96 ± 0.35[c]	2.74 ± 0.18[a]
Ethanol	140.54 ± 7.33	152.61 ± 6.53	12.07 ± 0.96[a]	7.55 ± 0.26[a]	5.19 ± 0.38[c]
Ethanol + HMBA	142.00 ± 3.94	193.74 ± 3.37	51.73 ± 3.36[b]	8.76 ± 0.45[b]	3.30 ± 0.09[b]

Values are means ± s.d. for 10 rats in each group. [a–c]Values not sharing a common superscript letter within each row differ significantly at $P < 0.05$ (DMRT).

Table 2 Effect of HMBA on hepatic marker enzymes of control and ethanol-administered rats

Group	AST (IU L^{-1})	ALT (IU L^{-1})	ALP (IU L^{-1})	GGT (IU L^{-1})	LDH (IU L^{-1})
Control	75.66 ± 3.39[a]	26.89 ± 1.28[a]	102.77 ± 22.11[a]	2.27 ± 0.66[a]	120.17 ± 3.08[a]
HMBA	75.73 ± 3.30[a]	26.57 ± 2.58[a]	117.18 ± 11.43[a]	2.24 ± 0.15[a]	127.07 ± 3.26[b]
Ethanol	127.91 ± 7.13[c]	58.11 ± 1.85[b]	158.39 ± 5.43[b]	7.33 ± 1.23[c]	332.95 ± 8.55[d]
Ethanol + HMBA	105.00 ± 6.12[b]	26.92 ± 1.89[a]	109.74 ± 15.62[a]	3.54 ± 0.31[b]	173.37 ± 4.45[c]

AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; LDH, lactate dehydrogenase. Values are means ± s.d. for 10 rats in each group. [a–d]Values not sharing a common superscript letter within each row differ significantly at $P < 0.05$ (DMRT).

ALP, GGT and LDH (Table 2). However, on HMBA co-administration, the activity was significantly decreased ($P < 0.05$) as compared with rats fed ethanol alone.

Table 3 shows the levels of TBARS, LOOH and CDs of control and experimental rats. Lipid peroxidation, indicated by TBARS, LOOH and CDs, was significantly higher in plasma, erythrocytes and liver of ethanol-administered rats as compared with normal control rats. TBARS, LOOH and CD levels were lowered significantly in the plasma, erythrocytes and liver cells of ethanol-administered rats treated with HMBA ($P < 0.05$).

Table 4 shows the activity of SOD, CAT and GPx in the plasma, erythrocytes and liver of control and experimental rats. SOD, CAT and GPx activity in the plasma, erythrocytes and liver of rats on ethanol supplementation (group 3) were significantly lower than the control rats (group 1). Treatment with HMBA to ethanol administered rats (group 4) significantly elevated SOD, CAT and GPx activity ($P < 0.05$) as compared with those rats receiving ethanol treatment alone (group 3).

The concentration of GSH was significantly lower in plasma, erythrocytes and liver cells of rats receiving ethanol (group 3) as compared with control rats (group 1) (Table 5). Treatment with HMBA to ethanol-administered rats (group 4) significantly elevated GSH levels ($P < 0.05$) as compared with those receiving ethanol alone (group 3).

Table 5 shows the levels of vitamin C and vitamin E in plasma and liver of control and experimental rats. Vitamin C and vitamin E levels in plasma and liver of rats on ethanol supplementation were significantly decreased compared with the control rats. Treatment with HMBA to ethanol-administered rats significantly increased the vitamin C and vitamin E levels ($P < 0.05$) as compared with those rats receiving ethanol supplementation alone.

Histopathological changes

Histopathological changes in the liver are shown in Figure 2. The liver samples of alcohol-administered rats showed feathery degeneration, micro- and macrocellular fatty changes, periportal fibrosis and vascular congestion. On treatment with HMBA, the liver showed normal histology with mild congestion of the central vein. Control rats treated with HMBA demonstrated normal liver histology.

Discussion

Increasing evidence supports the hypothesis that ethanol-induced tissue damage may be a consequence of oxidative stress and nutritional deficiencies (Thomson et al 1970). Our studies are in agreement with this hypothesis, showing decreased food intake and increased oxidative stress in ethanol-administered rats. Alcohol is rich in calories (7.1 kcal g^{-1}) and devoid of nutrients. Thus, isocaloric substitution of carbohydrates by ethanol resulted in a decreased weight gain. On the other hand, alcohol also reduces the absorption of foodstuffs and nutrients from the intestine (Pirola & Lieber 1975). Since body weight is considered to be a putative indicator of health, the increased weight gain in HMBA-co-administered rats suggested the beneficial protective effect of HMBA against ethanol. Other causes are mitochondrial insufficiency in fatty acid oxidation secondary to chronic alcohol consumption and acetaldehyde toxicity (Lieber 1991). The ratio between liver weight and the total body weight showed significant decrease in ethanol-fed rats supplemented with HMBA as compared with those of the unsupplemented ethanol-fed rats, which may be because the compound may

Table 3 Effect of HMBA on plasma, erythrocyte and hepatic lipid peroxidative markers of control and ethanol-administered rats

Group	Plasma			Erythrocytes			Liver		
	TBARS (nmol (mg protein)$^{-1}$)	LOOH $\times 10^{-5}$ (mmol (mg protein)$^{-1}$)	CDs (nmol (mg protein)$^{-1}$)	TBARS (nmol (mg Hb)$^{-1}$)	LOOH (mmol (mg Hb)$^{-1}$)	CDs (nmol (mg Hb)$^{-1}$)	TBARS (mmol (mg tissue)$^{-1}$)	LOOH (mmol (mg tissue)$^{-1}$)	CDs (mmol) (mg tissue)$^{-1}$)
Control	1.56 ± 0.03^{a}	9.57 ± 0.62^{a}	0.67 ± 0.02^{a}	1.52 ± 0.06^{a}	6.87 ± 0.11^{a}	6.07 ± 0.11^{a}	0.73 ± 0.05^{a}	64.96 ± 4.13^{a}	52.32 ± 1.13^{a}
HMBA	1.48 ± 0.02^{a}	8.71 ± 0.44^{a}	0.65 ± 0.03^{a}	1.43 ± 0.14^{a}	6.57 ± 0.17^{a}	5.59 ± 0.28^{a}	0.69 ± 0.03^{a}	62.24 ± 1.50^{a}	49.57 ± 2.14^{a}
Ethanol	3.13 ± 0.25^{c}	16.27 ± 0.74^{c}	1.39 ± 0.06^{c}	3.08 ± 0.23^{c}	13.25 ± 1.02^{c}	10.73 ± 0.96^{c}	1.92 ± 0.06^{c}	90.47 ± 3.25^{b}	86.88 ± 3.22^{c}
Ethanol + HMBA	2.02 ± 0.06^{b}	11.73 ± 0.91^{b}	0.82 ± 0.03^{b}	2.24 ± 0.14^{b}	9.83 ± 0.41^{b}	7.63 ± 0.23^{b}	0.95 ± 0.02^{b}	66.98 ± 2.10^{a}	64.35 ± 2.27^{b}

TBARS, thiobarbituric acid reactive substances; LOOH, lipid hydroperoxides; CDs, conjugated dienes. Values are means ± s.d. for 10 rats in each group. $^{a–c}$Values not sharing a common superscript letter within each row differ significantly at $P < 0.05$ (DMRT).

Table 4 Effect of HMBA on plasma, erythrocytes and hepatic SOD, CAT and GPx of control and ethanol-administered rats

Group	Plasma			Erythrocytes			Liver		
	SOD (U* (mg protein)$^{-1}$)	CAT (U** (mg protein)$^{-1}$)	GPx (U*** (g protein)$^{-1}$)	SOD (U* (mg Hb)$^{-1}$)	CAT (U** (mg Hb)$^{-1}$)	GPx (U*** (mg Hb)$^{-1}$)	SOD (U* (mg protein)$^{-1}$)	CAT (U** (mg protein)$^{-1}$)	GPx (U*** (mg protein)$^{-1}$)
Control	2.60 ± 0.07^{b}	3.54 ± 0.32^{c}	22.70 ± 1.43^{c}	2.03 ± 0.06^{b}	154.21 ± 2.56^{c}	14.37 ± 0.42^{c}	5.75 ± 0.15^{b}	75.31 ± 1.72^{b}	13.22 ± 1.06^{c}
HMBA	2.52 ± 0.09^{b}	3.94 ± 0.14^{d}	23.35 ± 0.83^{c}	2.20 ± 0.39^{b}	154.80 ± 4.63^{c}	15.10 ± 0.41^{d}	6.02 ± 0.12^{c}	73.56 ± 1.67^{b}	12.70 ± 1.02^{c}
Ethanol	1.59 ± 0.08^{a}	1.76 ± 0.03^{a}	13.15 ± 0.49^{a}	1.29 ± 0.37^{a}	96.75 ± 2.90^{a}	7.55 ± 0.20^{a}	2.07 ± 0.19^{a}	52.34 ± 2.67^{a}	6.00 ± 0.48^{a}
Ethanol + HMBA	2.58 ± 0.22^{b}	2.52 ± 0.38^{b}	18.38 ± 1.30^{b}	2.00 ± 0.06^{b}	141.89 ± 2.87^{b}	10.58 ± 0.58^{b}	5.68 ± 0.10^{b}	69.64 ± 2.68^{b}	11.46 ± 1.01^{b}

SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase. Values are means ± s.d. for 10 rats in each group. $^{a–d}$Values not sharing a common superscript letter within each row differ significantly at $P < 0.05$ (DMRT). *Enzyme required for 50% inhibition of nitroblue tetrazolium (NBT) reduction per minute; **μmol of hydrogen peroxide utilized per minute; ***μmol of glutathione utilized per minute.

Table 5 Effect of HMBA on plasma and hepatic GSH, vitamin C and vitamin E levels and erythrocyte GSH of control and ethanol-administered rats

Group	Plasma			Liver			Erythrocytes
	GSH (mmol (mg protein)$^{-1}$)	Vit C (mg dL^{-1})	Vit E (mg dL^{-1})	GSH (mmol (mg protein)$^{-1}$)	Vit C (mg/100 g tissue)	Vit E (mg/100 g tissue)	GSH (mmol (mg Hb)$^{-1}$)
Control	35.22 ± 0.61^{c}	2.06 ± 0.04^{c}	2.68 ± 0.17^{c}	17.64 ± 0.37^{c}	0.84 ± 0.10^{c}	5.46 ± 0.16^{c}	3.62 ± 0.19^{c}
HMBA	37.14 ± 0.64^{d}	2.07 ± 0.05^{c}	2.79 ± 0.10^{c}	18.02 ± 0.34^{c}	0.89 ± 0.04^{c}	5.35 ± 0.10^{c}	3.55 ± 0.27^{bc}
Ethanol	15.06 ± 0.71^{a}	1.25 ± 0.15^{a}	1.28 ± 0.17^{a}	10.02 ± 0.39^{a}	0.51 ± 0.05^{a}	3.39 ± 0.11^{a}	1.52 ± 0.14^{a}
Ethanol + HMBA	30.36 ± 1.38^{b}	1.61 ± 0.09^{b}	1.91 ± 0.14^{b}	16.20 ± 0.34^{b}	0.64 ± 0.01^{b}	4.18 ± 0.23^{b}	3.26 ± 0.22^{b}

GSH, reduced glutathione. Values are means ± s.d. for 10 rats in each group. [a–d]Values not sharing a common superscript letter within each row differ significantly at $P < 0.05$ (DMRT)

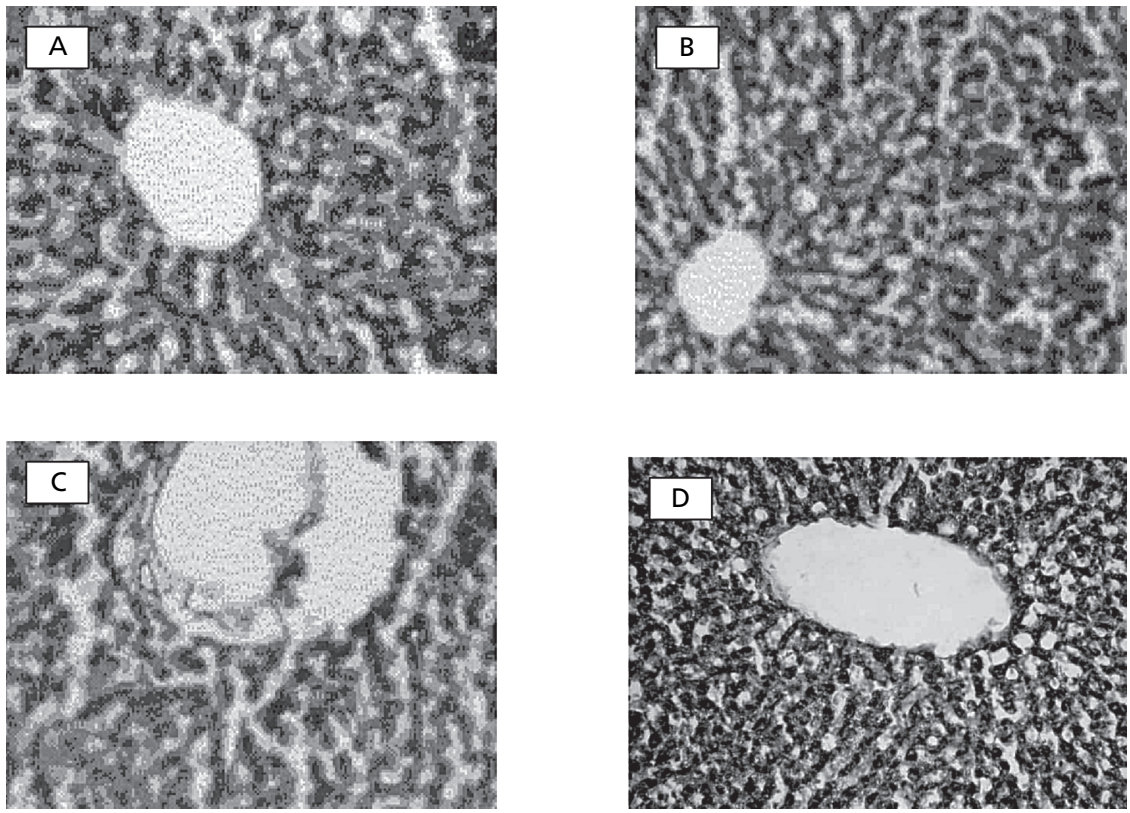

Figure 2 Representative photomicrographs of histopathological changes due to HMBA treatment in the liver of control and experimental rats. A. Control rat liver showing central vein and hepatocytes arranged in the form of cords (H&E × 20). B. Liver of control rats treated with HMBA showing normal histology (H&E × 20). C. Ethanol-administered rat liver showing feathery degeneration, micro- and macrocellular fatty changes, periportal fibrosis and vascular congestion (H&E × 20). D. Ethanol + HMBA-treated rat liver (H&E × 20); normal histology was observed, although central vein shows congestion. Photomicrographs were taken using a Sanyo (Aver Version 300) digital camera.

increases the elimination of ethanol directly from the intestines without absorption.

Liver damage after ethanol ingestion is a well-known phenomenon, and an obvious sign of hepatic injury is the leakage of cellular enzymes into plasma (Baldi et al 1993). The increased levels of serum enzymes, such as AST, ALT, ALP, GGT and LDH, observed in alcohol-administered rats, may indicate the increased permeability, damage or necrosis of hepatocytes (Goldberg & Watts 1965). HMBA supplementation showed a marked hepatoprotective effect, which correlates with the results of previous research done with *H. indicus* (Baheti et al 2006), as demonstrated by the reversal of the changes produced by ethanol.

Oxidation of polyunsaturated fatty acids (lipid peroxidation) of membranes is a common process in living organisms, since they are the target of oxygen-derived free radicals produced during mitochondrial electron transport (Porter et al 1995). Increased lipid peroxidation associated with chronic ethanol administration, as an indicator of oxidative stress, has been often determined in both animal models and human

clinical trials. Excess lipid peroxidation, as measured by formation of TBARS, LOOH or CDs, has been found in most studies (Nordmann 1994). In agreement with these findings, ethanol-administered rats showed increased levels of lipid peroxidation markers, such as TBARS, LOOH and CDs in the circulation and tissues. The increased peroxidation can result in changes in the cellular metabolism of hepatic and extrahepatic tissues. Products of lipid peroxidation formed in the primary site reaching the other organs and tissues via the blood stream provoke lipid peroxidation there and consequently cause cellular and tissue damage (Ikuo et al 1991). Increased accumulation of lipid peroxidation products in cells can result in cellular dehydration, whole cell deformity and cell death (Winrow et al 1993).

Lipid peroxidation is an important cause of alcoholic liver disease. Free radical generation and lipid peroxidation products play a pivotal role in the mechanism by which ethanol may exert its toxic effects on the liver and other extrahepatic tissues (Nordmann 1994). Our HMBA-co-administered rats showed significantly decreased levels of these lipid peroxidative markers as compared with alcoholic rats. HMBA also exhibited its potent antioxidant activity by decreasing lipid peroxidation in control rats administered with HMBA as compared with normal controls. In this context, Mary et al (2003) have also reported that *H. indicus* has antioxidant activity in in-vitro studies. Decreased lipid peroxidation on HMBA administration suggests the decreased impact of reactive oxygen species (ROS) on lipid membrane, thus increased protection against alcohol-induced liver injury. The inhibition of lipid peroxidation by HMBA, therefore, may be one of the mechanisms by which HMBA exerts its protection against ethanol-mediated tissue injury. Some studies have shown that treatment with silymarin protects the liver, probably through decreasing lipid peroxidation (Pares et al 1998). HMBA may also have a similar mode of action.

Free radical scavenging enzymes, such as SOD, CAT and GPx, are the first line of defence against oxidative injury. SOD scavenges excess superoxide anions and converts them to H_2O_2. The primary role of catalase is to scavenge H_2O_2 that has been generated by free radicals or by SOD and convert it to water. GPx works in tandem with CAT to scavenge excess H_2O_2, as well as other free radicals, in response to oxidative stress. The equilibrium between these enzymes is important for the effective removal of oxidative stress in intracellular organelles. This antioxidant defence system is significantly altered on alcohol administration. Our results showed decreased activity of SOD, CAT and GPx in chronic ethanol-administered rats. It has been reported that ethanol impairs the antioxidant system of the tissues in proportion to the amount of ethanol ingestion (Scott et al 2000). Biphasic fluxes of these enzymes' activity are common; an increase or decrease may relate to the presence of excess ROS. The decreased activity of enzymic antioxidants observed in ethanol-administered rat erythrocytes and tissues may be a consequence of irreversible inactivation of enzyme proteins from increased free radical production, resulting from ethanol metabolism (Santiard et al 1995). Lowered activity of these enzymes will result in the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of cell membrane integrity and membrane function (Krishnakantha & Lokesh 1993). There was a significant increase in the activity of these enzymes on HMBA co-administration. HMBA is reported to scavenge superoxide radicals and H_2O_2 (Alam et al 1994). Due to these properties, it was expected that HMBA might decrease the workload of enzymic antioxidants and reduce the free-radical-mediated inactivation of enzyme proteins and thereby maintain the activity of enzymic antioxidants.

The second line of defence consists of the non-enzymic scavengers, such as GSH, ascorbic acid and α-tocopherol, which scavenge residual free radicals escaping decomposition by the antioxidant enzymes. Moreover, enzymic antioxidants are inactivated by the excessive levels of free radicals and hence the presence of non-enzymic antioxidants is presumably essential for the removal of these radicals (Allen 1991). Glutathione is a major non-protein thiol in living organisms, which plays a central role in coordinating the antioxidant defence process in our body. Glutathione reacts directly with ROS and electrophilic metabolites, protects essential thiol groups from oxidation and serves as a substrate for several enzymes, including GPx.

We observed lower levels of plasma, erythrocyte and hepatic GSH in alcoholic rats which represents the increased utilization to counter the increased oxidative stress. The shortage of NADPH (due to the increased oxidation of ethanol by MEOS, which uses NADPH as a cofactor) suppresses the reduction of oxidized glutathione by glutathione reductase and subsequently decreases glutathione content. Generation of large quantities of acetaldehyde during ethanol metabolism ultimately will deplete cellular GSH pools by forming S-conjugation with the –SH group. Perturbation in the redox status of GSH can not only impair cell defence against toxic compounds, but also results in enhanced oxidative stress and oxidative injury (Reed 1990).

HMBA co-administered rats showed significantly improved GSH levels as compared with alcoholic rats. This suggests that the maintenance of GSH by HMBA was mainly due to inactivation of ROS via its radical scavenging effects, sparing antioxidant enzymes such as SOD (Ravishankara et al 2002) and CAT. Restoration of GSH levels has been shown to inhibit alcohol toxicity (Garcia-Ruiz et al 1995; Iimuro 2000). Therefore, it was presumed that the effects of HMBA might be related to a normalization mechanism by maintaining adequate levels of GSH for detoxification of xenobiotics.

Antioxidant systems other than GSH may also play a role in preventing lipid peroxidation under experimental and clinical conditions. α-Tocopherol and ascorbic acid are naturally occurring free radical scavengers (Yu 1994). Both ascorbic acid and α-tocopherol are known to be decreased in liver diseases, particularly in alcoholics (Bjorneboe 1987). In this context, thiol compounds, such as GSH, might be involved in regenerating α-tocopherol from its radical form (Wefers & Sies 1988). The observed decrease in the levels of α-tocopherol and ascorbic acid may be due to their increased utilization for scavenging ethanol- or oxygen-derived radicals.

A number of reports show that the phenolic OH is essential for both antioxidant activity and free radical kinetics (Priyadarsini et al 2003). A model compound with an ortho-substituted hydroxyl group to the aromatic ring seems to be adequate for antioxidant and H_2O_2- or DPPH-scavenging

activity of phenolic acids (Sroka & Cisowski 2003). The phenolic compounds act as effective donors or oxygen acceptors in the presence of H_2O_2 (Adak et al 1996; Navas Diaz et al 1998). It has been reported that compounds with a hydroxyl group in the ortho position of the phenolic ring have peroxyl radical- and superoxide-scavenging properties (Alanko et al 1999). So HMBA may decrease the workload of the antioxidants, such as SOD, CAT and GPx, etc. This may be the reason for the optimum activity of these antioxidants in the HMBA + ethanol-treated rats. The presence of a hydroxyl group in the phenolic ring of HMBA may be a reason for its antioxidant properties.

Moreover, alcohol administration produces a spectrum of histological abnormalities in the liver, as described earlier (MacSween & Burt 1986). Liver histology of ethanol administered rats showed pathomorphologic alterations (Figure 2). These changes were predominant in the centrilobular region, having reduced oxygen perfusion. Hepatic damage may be partially attributed to cytochrome P450-dependent enzyme activity in liver that tends to be present in greatest concentration near the central vein and lower near the peripheral sites (Sarkar et al 1995). Treatment with HMBA reduced the histological changes produced by ethanol and significantly lessened the alcohol-induced liver changes, which correlates with the biochemical findings.

Conclusions

The above data confirms the hepatoprotective effect of HMBA as it counteracts the free radical-mediated injuries involved in the development of tissue damage caused by alcohol abuse by enhancing the antioxidant status. Hence, it merits further development for exploitation as a therapeutic agent. Multiple mechanisms may interplay in its antioxidant and hepatoprotective efficacy and further research on the mechanism of action of 2-hydroxy-4-methoxy benzoic acid (HMBA) is to be explored.

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