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Research article

Hemidesmus indicus PROTECTS AGAINST ETHANOL-INDUCED LIVER TOXICITY

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Abstract: Alcoholic liver disease (ALD) is one of the most common diseases in modern society. A large number of studies are in progress aiming to identify natural substances that would be effective in reducing the severity of ALD. Although there are currently a number of drugs on the market, their long-term use can have numerous side effects. *Hemidesmus indicus* is an indigenous Ayurvedic medicinal plant used in soft drinks in India. In this study, we examined the effects of its ethanolic root extract on experimental liver damage in order to evaluate its hepatoprotective effects against hepatotoxicity induced in rats by ethanol at a dosage of 5 g/kg body weight for 60 days. The *H. indicus* root extract was given at a dose of 500 mg/kg body weight for the last 30 days of the experiment. The animals were monitored for food intake and weight gain. The liver was analysed for the degree of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) and antioxidant status using the activities of glutathione-depedendant enzymes. The degree of liver damage was analysed using serum marker enzyme activities, the total protein, albumin, globulin,

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Abbreviations used: A/G ratio – albumin/globulin ratio; ALD – alcoholic liver disease; ALP – alkaline phosphatase; ALT – alanine aminotransferase; AST – aspartate aminotransferase; CMC – carboxy methyl cellulose; FT-IR – fourier transform infrared; GGT – γ-glutamyl transpeptidase; GPx – glutathione peroxidase; GR – glutathione reductase; GSH – reduced glutathione; GST – glutathione-S-transferase; HMBA – 2-hydroxy 4-methoxy benzoic acid; LDH – lactate dehydrogenase; ROS – reactive oxygen species; TBARS – thiobarbituric acid reactive substances

ceruloplasmin and liver glycogen contents, and the A/G ratio. The Fourier transform infrared spectra (FT-IR) of the liver tissues were recorded in the region of 4000-400 cm^{-1}. The ethanol-fed rats showed significantly elevated liver marker enzyme activities, lipid peroxidation levels and reduced antioxidant levels as compared to the control rats. Oral administration of *H. indicus* for the latter 30 days resulted in an increased food intake and weight gain, decreased TBARS levels, near normal levels of glutathione-dependent enzymes, increased total protein, albumin, globulin and liver glycogen contents, an increased A/G ratio, and decreased liver marker enzyme activities and ceruloplasmin levels. The relative intensity of the liver FT-IR bands for the experimental groups were found to be altered significantly ($p < 0.05$) compared to the control samples. For the group that had *H. indicus* co-administered with ethanol, the intensity of the bands was near normal. Moreover, the results of the FT-IR study correlated with our biochemical results.

Key words: FT-IR spectroscopy, Lipid peroxidation, *Hemidesmus indicus*, Rat liver

INTRODUCTION

One of the most common causes of chronic liver disease is alcohol consumption [1]. Free radicals and oxidative stress have been implicated in the pathogenesis of ethanol-induced liver injury in humans and experimental animals [2, 3]. The most extensively studied aspect of free radical-induced liver injury is lipid peroxidation. Free radical-mediated lipid peroxidation is considered to be a primary mechanism of cell membrane destruction and cell damage [4]. One of the most thoroughly investigated examples is lipid peroxidation stimulated by hepatotoxin-ethanol. There appears to be increasing evidence that alcohol toxicity may be associated with increased oxidative stress and free radical-associated injury [5]. The 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 [3]. Glutathione is a tripeptide present in all mammalian cells. It participates in many metabolic processes, of which the protection of cells against free radicals and toxic metabolites is one of the most important.

Fourier transform infrared (FTIR) microspectroscopy is considered an ideal tool for comparative studies. Infrared spectroscopy is a widely used method for analyzing molecular structure and structural interactions in biological systems. It measures the absorption of vibrating molecules, which result from the energy transitions of the vibrating dipoles. Very small alterations in bond lengths and angles can be detected by this technique, and so FT-IR has emerged as a powerful tool to investigate the structural changes of molecules in detail. With the development of sophisticated Fourier transform infrared spectrometers, there have been very rapid advances in the applications of IR spectroscopy to the study of biological molecules. Although the same information was obtained

from spectra recorded with dispersive instruments (IR) and interferometers, the FT-IR technique enables the rapid and reproducible recording of high resolution, low-noise spectra, even in aqueous media. The data acquisition process is automated. The data obtained is stored in digitally encoded formats that facilitate spectral interpretation with the aid of post-aquisation data manipulation algorithms. This property of the technique provides the accurate detection of small changes even in weak absorption bands [6]. Moreover, infrared spectroscopy has been used as a powerful method for the study of molecular structures and intermolecular interactions in biological tissues and cells [7]. Several authors have used infrared spectroscopy on biological substances like the muscles and liver. Severcan *et al.* [8] monitored liver microsomal membrane lipid peroxidation in diabetic rats by FTIR. Galin *et al.* [9] also investigated the changes in the primary, secondary and tertiary structures of nucleic acids in rats exposed to gamma radiation via FT-IR spectroscopic studies.

Biological macromolecules are classified as proteins, lipids, nucleic acids and polysaccharides. One of the remarkable characteristics of living organisms is how many macromolecules they utilize, and what different and important roles these play in their physiological systems. The functioning of all biological macromolecules depends on their shape and three-dimensional structures. They provide us a very clear example and a very sensitive expression of the relationship between molecular structures and the chemical and physical properties of a substance [7]. These biochemicals are highly sensitive to ethanol, and ethanol-induced impairment in biochemical synthesis has been reported by many investigators [10].

Medicinal plants that are commonly included in Ayurvedic recipes for liver ailments have recently drawn much attention, as they seem to have hepatoprotective properties, and there is no reliable hepatoprotective drug available in modern medicine. The research conducted on several natural plant products used as heptoprotective agents is well documented [11].

Hemidesmus indicus (Asclepiadaceae) is a widely distributed medicinal plant in India. It has been used as a traditional medicine in the treatment of biliousness, blood diseases, diarrhea, respiratory disorders, skin diseases, syphilis, fever, bronchitis, asthma, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite, burning sensations and rheumatism [8]. Jain and Singh [9] reported that *H. indicus* is employed in traditional medicine for gastric ailments. Its extract mainly consists of essential oils and phytosterols, like hemidesmol, hemidesterol and saponins. The active principle of *H. indicus* is 2-hydroxy 4-methoxy benzoic acid (HMBA) [12]. It is also known to have antioxidant properties [13]. Although the protective effect of *H. indicus* against rifampicin and isoniazid-induced hepatotoxicity in rats [14] and CCl_4- and paracetamol-induced hepatic damage [15] are known, the effect of *H. indicus* is not yet clear in the case of ethanol-induced hepatoxicity. Due to the wide pharmacological actions of *H. indicus* and recent interest [16], this study was undertaken to establish the hepatoprotective effect

of *H. indicus* on an animal model of ethanol-induced liver damage using a Fourier transform infrared spectrometer.

MATERIALS AND METHODS

Chemicals

All the chemicals used for the assays were of analytical grade. The solvents were distilled before use.

Hemidesmus indicus

The plant material (*H. indicus*) was collected from a local market, and identified and authenticated by the Department of Botany of Annamalai University. The roots were cleaned, shade-dried and disintegrated. An ethanolic extract was prepared [17] using a soxlet apparatus, and screened for its hepatoprotective property. The ethanolic extract (yield: 9.2%) was dark brown in color and sticky in consistency, and gave positive results for flavonoids, terpenoids, tannins, coumarins and glycoside, and negative results for alkaloids, anthraquinones, lactones/esters, proteins/amino acids and saponins [18].

Animals

Male Wister rats with body weights of 130-180 g, bred in the Central Animal House, Rajah Muthiah Medical College, were used for the study. The animal handling and experimental procedures were approved by the Institutional Animal Ethics committee, Annamalai University (Registered number: 160/1999/ CPCESA), and the animals were treated in accordance with the principles and guidelines of Indian National Law on animal care and use. The animals were fed *ad libitum* with water and a normal laboratory pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Bangalore, India), consisting of protein (22.21%), fat (3.32%) and fiber (3.11%), balanced with carbohydrates (> 67%), vitamins and minerals.

Study design

The animals were divided into four groups of 10 each. Groups 1 and 2 received a normal diet and isocaloric glucose from a 40% glucose solution in 1% carboxy methyl cellulose (CMC) daily by intragastric intubation. Liver cell damage was induced in the rats of groups 3 and 4 by administering 20% ethanol (5.0 g/kg b.w./day) [19] as an aqueous solution using an intragastric tube daily for 30 days. At the end of this period, the animals were treated as follows for a further 30 days. The group 1 animals continued to receive the standard pellet diet and isocaloric glucose from the 40% glucose solution in 1% CMC; this group served as the control. The group 2 animals continued to receive the standard pellet diet and isocaloric glucose from the 40% glucose solution, and were also given the ethanolic root extract of *H. indicus* (500 mg/kg b.w./day p.o.) [20] in 1% CMC by intragastric intubation every day. The group 3 animals continued to receive the standard pellet diet, 20% ethanol and 1% CMC daily.

The group 4 animals continued to receive the standard pellet diet and 20% ethanol, and also the ethanolic extract of *H. indicus* (500 mg/kg b.w./day p.o.) in 1% CMC every day. The total duration of the experiment was 60 days.
The animals were fasted overnight, anaesthetized with an intramuscular injection of ketamine hydrochloride (30 mg/kg b.w.) and killed by cervical decapitation. Blood samples were collected in plain tubes, and then centrifuged for the separation of serum. The liver tissues were immediately homogenised and used for the various biochemical assays. For the histopathological study, three animals from each group were perfused with formalin (10%), and their tissues were separated and stored in 10% formalin. They were later sectioned using a microtome, dehydrated in graded alcohol, embedded in paraffin sections, and stained with haemotoxylin and eosin.

Lipid peroxidation and enzyme assays

The assay for lipid peroxidation was carried out by measuring the levels of thiobarbituric acid reactive substances (TBARS) in the tissues by the method of Ohkawa [21] and Yagi [22]. The pink chromogen produced by the reaction of malondialdehyde, a secondary product of lipid peroxidation with thiobarbituric acid, was estimated at 532 nm. Serum aspartate aminotransferase (AST, EC 2.6.1.1) and serum alanine aminotransferase (ALT, EC 2.6.1.2) were assayed using a diagnostic kit based on the method of Reitman and Frankel [23]. The serum alkaline phosphatase activity (ALP, EC 3.1.2.3.1) was estimated using a diagnostic kit based on Kind and King's method [24, 25]. The serum γ-glutamyl transpeptidase activity (GGT, EC 2.3.2.2) was assayed according to the method of Rosalki and Rau [26]. The activity of lactate dehydrogenase (LDH, EC 1.1.1.27) was estimated by the method of King [25]. The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was assayed by the method of Rotruck *et al.* [27]. 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 [28]. The enzyme activity was expressed as μmoles of GSH consumed/min/mg protein. The reduced glutathione (GSH) content in the tissues was assayed by the method of Ellman [28]. GSH estimation was based on the development of a yellow color when 5,5-dithiobis (2-nitro benzoic acid) di-nitrobisbenzoic acid was added to compounds containing a sulphydryl group. The activity of GR (EC. 1.6.4.2) was assayed by the method of Rotruck *et al.* [27] and Carlberg and Mannervik [29]. Glutathione-S-transferase (GST) was assayed by the method of Habig and Jakoby [30]. Protein contents were estimated by the method of Lowry *et al.* [31] using bovine serum albumin as the standard. The serum total protein and albumin were estimated by Biuret's method [32]. The serum globulin concentration was calculated using the following formula after the estimation of the total protein and albumin contents: Globulin = Total protein – Albumin. The serum ceruloplasmin level was estimated by the method of Ravin [33]. The liver glycogen content was estimated by the method of Morales *et al.* [34].

Fourier transform infrared spectroscopy analysis

The whole liver tissue samples from each group of rats were isolated and homogenized with a 0.2 M phosphate buffer, pH 7.4, and centrifuged at 100,000 × g for 10 min. The membrane-rich parts of these homogenates were lyophilized and made into a fine powder to be used for FT-IR analysis [35].

5 mg of membrane-rich sample was mixed with 100 mg of dried potassium bromide, and again lyophilized in order to remove bound water, which might interfere with the measurement of the amide band. This was then subjected to a pressure of 5 x 10^6 pa, and made into a clear pellet of 13 mm diameter and 1 mm thickness. The absorbance spectra were recorded using the Spectrum RX I FT-IR System (Nicolet Instrument Corporation, Madison, USA). For each spectrum, 8 scans were recorded, at a spectral resolution of 4 cm^{-1}. The frequencies for all the sharp bands were accurate to 0.01 cm^{-1}. The spectrometer was continuously purged with dry nitrogen. The absorption intensity of the peak was calculated using the baseline method. Each observation was confirmed by taking at least three replicates. The spectra were recorded in the range 4000-400 cm^{-1} [36]. Peak normalization was done with respect to 1654 cm^{-1}. The average band intensity was calculated for the band 3012 cm^{-1}.

Statistical analysis

The data was analyzed via the one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a commercially available statistics software package (SPSS® for Windows, V. 13.0, Chicago, USA). All the groups were compared simultaneously by DMRT. In the Tabs below, if a superscript letter for two entries is the same, it indicates that there is no significant difference between the two. If the superscript letters are different, then the two are significantly different ($p < 0.05$). The results are presented as means ± SD. p values < 0.05 were regarded as statistically significant.

RESULTS

Biochemical changes

Tab. 1 shows the average weight gain, average food intake and liver to body weight ratio for the control and experimental rats during the experimental period. The average food intake and the average weight gain were significantly reduced in the rats receiving ethanol, and their liver to body weight ratio was significantly increased as compared to the results for the control animals. Rats that had *H. indicus* co-administered along with ethanol from the 31st day showed a significant weight gain, an increased food intake and a decreased liver to body weight ratio as compared to the untreated ethanol-fed rats.

In Tab. 2, it can be seen that the administration of ethanol produced severe liver damage as indicated by the marked increase in the serum AST, ALT, ALP, GGT and LDH activities. However, upon *H. indicus* co-administration, there was a significant reversal in these values.

Tab. 1. The effect of *H. indicus* on the body weight and liver to body weight ratio of the control and ethanol-administered rats.

Groups	Body weight		Net gain [g]	Average food intake [g]	Liver wt x 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}
H. indicus	142.04 ± 6.84	224.46 ± 12.33	82.42 ± 5.51^{c}	9.27 ± 0.25^{c}	2.81 ± 0.23^{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 + *H. indicus*	144.39 ± 6.20	198.94 ± 3.46	54.56 ± 5.67^{b}	8.71 ± 0.22^{b}	3.18 ± 0.07^{b}

The values are the means ± SD for the 10 rats in each group. Values not sharing a common superscript letter within each column differ significantly at $p < 0.05$ (DMRT).

Tab. 2. The effect of *H. indicus* on the hepatic marker enzymes of the control and ethanol-administered rats.

Groups	AST (IU/l)	ALT (IU/l)	ALP (IU/l)	GGT (IU/l)	LDH (IU/l)
Control	75.66 ± 3.39^{a}	26.89 ± 1.28^{b}	102.77 ± 22.11^{a}	2.27 ± 0.66^{a}	120.17 ± 3.08^{a}
H. indicus	80.69 ± 2.50^{a}	21.41 ± 0.56^{a}	111.80 ± 14.09^{a}	2.03 ± 0.46^{a}	130.02 ± 3.34^{b}
Ethanol	127.91 ± 7.13^{c}	58.11 ± 1.85^{c}	158.39 ± 5.43^{b}	7.33 ± 1.23^{c}	332.95 ± 8.55^{d}
Ethanol + *H. indicus*	97.56 ± 4.49^{b}	27.81 ± 2.16^{b}	108.64 ± 23.15^{a}	3.11 ± 0.06^{b}	159.58 ± 4.10^{c}

The values are the means ± SD for the 10 rats in each group. Values not sharing a common superscript letter within each column differ significantly at $p < 0.05$ (DMRT).

Tab. 3. The effect of *H. indicus* on the hepatic TBARS, GSH, GPx, GR and GST of the control and ethanol-administered rats.

Groups	TBARS (mmol/mg tissue)	GSH (mmol/mg protein)	GPx (U*/mg protein)	GR (U**/mg tissue)	GST (U***/mg tissue)
Control	0.73 ± 0.05^{a}	17.64 ± 0.37^{c}	13.22 ± 1.06^{b}	21.81 ± 2.44^{b}	7.50 ± 0.87^{c}
H. indicus	0.66 ± 0.22^{a}	18.50 ± 0.34^{d}	15.02 ± 1.20^{b}	21.27 ± 1.99^{b}	7.22 ± 0.62^{c}
Ethanol	1.92 ± 0.06^{c}	10.02 ± 0.39^{a}	6.00 ± 0.48^{a}	11.78 ± 1.00^{a}	3.54 ± 0.31^{a}
Ethanol + *H. indicus*	0.83 ± 0.04^{b}	15.15 ± 0.32^{b}	12.50 ± 1.00^{b}	20.84 ± 1.49^{b}	5.60 ± 0.69^{b}

*µmoles of glutathione utilized/minute, **µmoles of NADPH oxidised/minute, ***µmoles of GSH-CDNB conjugate formed/minute. The values are the means ± SD for the 10 rats in each group. Values not sharing a common superscript letter within each column differ significantly at $p < 0.05$ (DMRT).

Tab. 3 shows the levels of hepatic TBARS and activities of GSH, GPx, GR and GST for the livers of the control and experimental animals. Lipid peroxidation as indicated by TBARS was significantly higher in the livers of the rats that had ethanol administered to them than in those of the normal control rats. Upon *H. indicus* supplementation to the ethanol-fed rats, the TBARS level was lowered significantly in the liver as compared to the level for the unsupplemented ethanol-fed rats. The GSH, GPx, GR and GST activities in the livers of the rats receiving ethanol were significantly lower than those for the

control rats. Treatment of ethanol-administered rats with the *H. indicus* extract significantly elevated the glutathione-dependent enzyme activities as compared with those for animals receiving ethanol alone.

Tab. 4 shows the levels of serum total protein, albumin, globulin, ceruloplasmin and liver glycogen contents and the A/G ratio. The serum total protein, albumin, globulin and liver glycogen contents and the A/G ratio were significantly decreased, and the serum ceruloplasmin level was significantly increased in ethanol-fed animals. Upon treatment with *H. indicus*, there was a significant increase in the total protein, albumin, globulin and liver glycogen contents and the A/G ratio, and a decrease in the serum ceruloplasmin level as compared with those animals receiving ethanol alone.

Tab. 4. The effect of *H. indicus* on the serum total protein, albumin, globulin, ceruloplasmin and liver glycogen contents and the A/G ratio of the control and ethanol-administered rats.

Groups	Control	*H. indicus*	Ethanol	Ethanol + *H. indicus*
Total protein (g/dl)	6.44 ± 0.14^{b}	6.29 ± 0.17^{b}	5.55 ± 0.11^{a}	6.65 ± 0.19^{b}
Albumin (g/dl)	3.75 ± 0.26^{b}	3.55 ± 0.08^{b}	2.80 ± 0.11^{a}	3.60 ± 0.32^{b}
Globulin (g/dl)	2.71 ± 0.15^{a}	2.73 ± 0.17^{a}	2.75 ± 0.08^{a}	3.05 ± 0.18^{b}
A/G Ratio	1.36 ± 0.03^{c}	1.30 ± 0.09^{c}	1.01 ± 0.05^{a}	1.18 ± 0.17^{b}
Cereloplasmin (mg/dl)	17.53 ± 0.45^{a}	16.54 ± 0.42^{a}	32.90 ± 0.84^{d}	21.27 ± 0.54^{c}
Glycogen (mg/g of tissue)	25.26 ± 0.65^{d}	23.83 ± 0.61^{c}	17.14 ± 0.44^{a}	20.59 ± 0.53^{b}

The values are the means ± SD for the 10 rats in each group. Values not sharing a common superscript letter within each row differ significantly at $p < 0.05$ (DMRT).

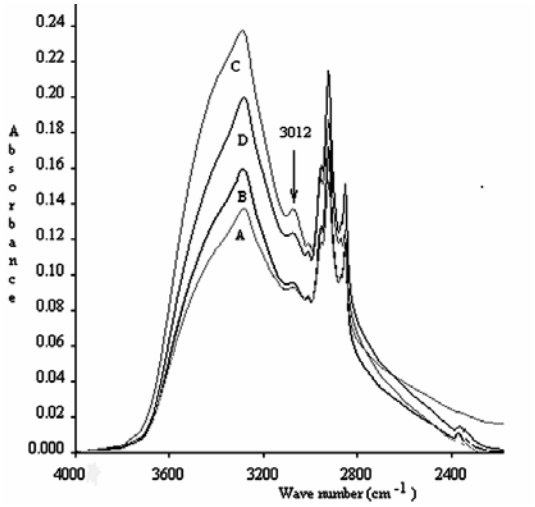

Fig. 1. Representative FT-IR spectra of rat liver tissues. A – Control, B – Control + *H. indicus*, C – Ethanol and D – Ethanol + *H. indicus*.

FT-IR spectral changes

Fig. 1 shows the representative FT-IR spectra of the control (A), *H. indicus*-treated (B), ethanol-treated (C) and *H. indicus* + ethanol-treated (D) rat liver tissues in the CH region (4000-2400 cm^{-1}). The FT-IR spectra revealed significant differences in the absorbance intensities between the control, ethanol- and *H. indicus*-supplemented tissues. The average band intensities for the band at 3012 cm^{-1} for the control and experimental groups are shown in Tab. 5.

Tab. 5. The effect of *H. indicus* on the average band intensity for the peak at 3012 cm^{-1} for normal and ethanol-administered rat liver tissue, as yielded by the FT-IR study.

Groups	Average band intensity
Control	0.032 ± 0.003^a
H. indicus	0.036 ± 0.004^a
Ethanol	0.126 ± 0.003^b
Ethanol + *H. indicus*	0.053 ± 0.005^c

The values are expressed as the means ± S.D. for six of the rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

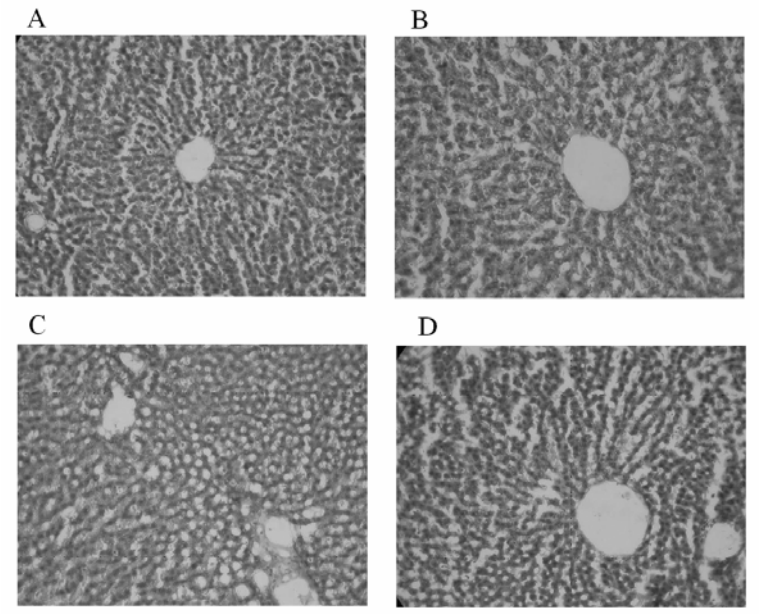

Fig. 2. Representative photomicrographs of histopathological changes due to *H. indicus* treatment in the liver of control and experimental rats. A – Control animal liver showing the central vein and hepatocytes arranged in the form of cords: H&E x 20. B – Liver tissue from a control rat treated with *H. indicus* showing normal histology: H&E x 20. C – Ethanol-administered rat liver: H&E x 20. Feathery degeneration, micro- and macrovesicular fatty changes, periportal fibrosis and vascular congestion in the liver. D – Ethanol + *H. indicus*-treated rat liver: H&E x 20. Normal histology was observed; however, the central vein shows congestion.

Histopathological changes

The histopathological changes to the liver tissue are shown in Fig. 2. The liver samples of the rats that received ethanol showed feathery degeneration, micro- and macrovesicular fatty changes, periportal fibrosis and vascular congestion. In the rats that were treated with *H. indicus* and ethanol, the liver showed normal histology with mild congestion of the central vein. The control rats treated with *H. indicus* had normal liver histology.

DISCUSSION

Weight gain was significantly reduced in rats that were given ethanol for a long period of time compared to the untreated controls. The waste of energy by the microsomal ethanol-oxidizing system during ethanol metabolism could be one of the causes for the decreased weight gain [37]. Other causes are mitochondrial insufficiency in fatty acid oxidation secondary to chronic alcohol consumption and acetaldehyde toxicity [38]. A significant improvement in the body weight gain was observed upon *H. indicus* treatment; this may be due to the inhibitory effect of *H. indicus* on liver microsomes. The ratio between the liver weight and total body weight significantly decreased in ethanol-fed rats receiving the *H. indicus* extract as compared to those of the unsupplemented ethanol-fed rats; this may be because the extract increases the elimination of ethanol directly from the intestines without absorption or because *H. indicus* consumption prevents fat accumulation in the liver. Liver damage after ethanol ingestion is a well-known phenomenon, and the obvious sign of hepatic injury is the leakage of cellular enzymes into the plasma [39]. The increased levels of serum enzymes such as AST, ALT, ALP, GGT and LDH observed in the ethanol-administered rats may indicate the increased permeability, damage and/or necrosis of hepatocytes [40]. *H. indicus* extract supplementation showed a marked hepatoprotective effect which correlates with the results of previous researchers [15]. This is evidenced by the reversal of the changes produced by ethanol. Moreover, the observed decrease in the activities of these enzymes shows that *H. indicus*, to a certain extent, preserves the structural integrity of the liver from the toxic effects of ethanol.

Albumin and globulins are two key components of serum proteins. As albumin is synthesized in the liver, it can be used as a biomarker to monitor liver function [41]. Lower contents of serum total proteins, especially albumin, were seen in the ethanol-administered rats [42], demonstrating the decreased functional ability of the livers of the rats that had received ethanol. In addition, the lower A/G ratio (< 1) is a sign of poor health. A significant increase in the serum total protein, albumin and A/G ratio was observed in the rats that had *H. indicus* co-administered. This stabilization of serum protein levels is a clear indication of *H. indicus* being related to an improvement in the functional status of the liver cells.

Ceruloplasmin is an important enzyme that oxidises iron from the ferrous to the ferric state. It has been demonstrated that iron-catalysed lipid peroxidation requires both Fe (II) and Fe (III), and the maximum rate occurs when the ratio is approximately one [43]. The serum ceruloplasmin levels are reported to increase under diseased conditions, leading to the generation of oxygen products such as superoxide radicals and hydrogen peroxide [44]. The observed increase in serum ceruloplasmin in the ethanol-fed rats may be caused by increased lipid peroxide levels. Administration of *H. indicus* to ethanol-fed rats restored the serum ceruloplasmin concentration to near normal levels, indicating the beneficial effects of *H. indicus*.

Ethanol-induced stress mainly disturbs the rate of carbohydrate metabolism, altering the glycogen profile. The level of glycogen, a reserve energy source, is decreased during ethanol treatment [45]. A fall in the glycogen profile in the liver tissue indicates the possibility of glycogenolysis, and an extensive utilization of energy stores. This stepped-up utilization is to meet the extra demands of energy necessitated by the quick and brisk movement which the animal shows in its behavioral response during the initial period of ethanol treatment. Treatment with *H. indicus* restored the liver glycogen levels to near normal, indicating the advantageous effect of *H. indicus.*

Oxidation of the polyunsaturated fatty acids (lipid peroxidation) present in the membranes is a common process in living organisms, since they are the targets of oxygen-derived free radicals produced during mitochondrial electron transport [46]. Increased lipid peroxidation associated with chronic ethanol administration is an indicator of oxidative stress, and has been determined in both animal models and human clinical trials. Excess lipid peroxidation, as measured by the formation of TBARS, has been found in most studies [47]. In agreement with these findings, the ethanol-administered rats showed increased levels of lipid peroxidation markers such as TBARS in their tissues. Increased peroxidation can result in changes in the cellular metabolisms of the hepatic and extrahepatic tissues. Moreover, the products of lipid peroxidation formed in the primary site can reach other organs and tissues via the bloodstream, provoking lipid peroxidation there and consequently causing cellular and tissue damage [48]. Increased accumulation of lipid peroxidation products in the cells can result in cellular dehydration, whole cell deformity, and cell death [49].

Lipid peroxidation is an important cause of alcoholic liver disease [50]. Free radical generation and lipid peroxidation products play a pivotal role in the mechanism by which ethanol exerts its toxic effects on the liver and other extrahepatic tissues [46]. In our study, *H. indicus* co-administered rats showed significantly decreased levels of these lipid peroxidative markers as compared to the ethanol-fed rats. In this context, Mary *et al.* [51] reported the antioxidant property of *H. indicus* based on *in vitro* studies. Decreased lipid peroxidation upon *H. indicus* extract administration suggests the decreased impact of ROS on the membranes, and thereby increased protection against ethanol-induced liver injury. Thus, the inhibition of lipid peroxidation by *H. indicus* may be one of the

mechanisms by which *H. indicus* exerts its protection against ethanol-mediated tissue injury. Some studies have shown that treatment with silymarin protects the liver, probably by decreasing lipid peroxidation [52]. *H. indicus* may have a similar mode of action.

Glutathione is a major non-protein thiol in living organisms. It plays a central role in co-ordinating the antioxidant defense processes 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 glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST). We observed decreased levels of reduced glutathione (GSH), GPx, GR and GST in the livers of the ethanol-fed rats, representing increased utilization to counter the excessive 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 the reduced glutathione content. Moreover, the generation of large quantities of acetaldehyde during ethanol metabolism will ultimately deplete cellular GSH pools, the -SH group being utilized for conjugation. Perturbation in the redox status of GSH can not only impair the cellular defense against toxic compounds, but also result in enhanced oxidative stress and oxidative injury [53]. Lowered GR activity results in the accumulation of highly reactive free radicals leading to deleterious effects such as loss of cell membrane integrity and function. Decreased activities of GST *in vitro* and *in vivo* make the organism more vulnerable to alkylating agents [54, 55]. Our observation also reveals that ethanol decreases the activity of GST towards the substrate CDNB in the rat liver, which correlates with our earlier reports [56].

H. indicus co-administered rats showed significantly improved GSH, GPx, GR and GST levels as compared to ethanol-fed rats. The results obtained suggest that the maintenance of cellular reduced glutathione levels by *H. indicus* was mainly due to the inactivation of ROS via its radical scavenging effects, sparing the antioxidant enzymes such as SOD [57] and CAT. Restoration of GSH, GPX, GR and GST levels has been shown to inhibit alcohol toxicity [58, 59]. Therefore, it may be presumed that the effects of *H. indicus* act as a normalization mechanism whereby adequate levels of glutathione and its dependent enzymes are maintained for the detoxification of xenobiotics.

A number of reports show that a phenolic OH is essential for both antioxidant activity and free radical kinetics [60]. A model compound with an orthosubstituted hydroxyl group to the aromatic ring seems to be adequate for the antioxidant and H_2O_2- or DPPH-scavenging activity of phenolic acids [61]. The phenolic compounds act as effective donors or oxygen acceptors in the presence of H_2O_2 [62, 63]. 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 [64]. As the active principle of *H. indicus* is

HMBA, which also has a hydroxyl group in the ortho position of the phenolic ring, the extract may be a potent antioxidant.

Infrared spectra reflect the total chemical composition of cells, and some of the spectral bands can be assigned to distinct functional groups or chemical substructures. The increasing use of FT-IR spectroscopy demonstrates that this technique is a valuable tool because of its ability to monitor simultaneously protein, lipid and poly-saccharide components.

The FT-IR spectrum of a natural membrane is very complex and consists of several bands. The main absorptions observed in the CH stretching region in the spectra are labeled in Fig. 1. The olefinic band (3012 cm^{-1}) is one of the weakest bands in the spectrum; it arises from the unsaturated lipids. It is known that unsaturated lipids are more prone to lipid peroxidation [65]. The intensity, or more accurately the area of the absorption bands is directly related to the concentration of the molecules [66, 67] Therefore, the intensity of this band can be used as an index of lipid peroxidation. In this study, the olefinic band (3012 cm^{-1}) was investigated for monitoring the lipid peroxidation status of rat liver microsomal membranes. The stronger bands in the CH stretching region (bands 2957, 2924 and 2853cm^{-1}) are mainly due to saturated lipids [68-70], and these bands are not discussed here. The mean values of the intensity and statistical significance of these values were calculated and compared. The average band intensity of 3012 cm^{-1} was increased in the ethanol-fed animals, which may be due to the loss of unsaturation during lipid peroxidation reactions, and to the presence of double bonds in the lipid peroxidation products such as malondialdehyde, lipid aldehydes, and alkyl radicals. Hence, the intensity of the olefinic band was increased due to the accumulation of end products of lipid peroxidation. Our results are in accordance with the study of Liu *et al.* [71]. The average band intensity reverted to near normal when the ethanol-fed animals were treated with the *H. indicus* extract, which may be due to the antioxidant properties of the extract.

Moreover, alcohol administration produces a spectrum of histological abnormalities in the liver, as described earlier [72]. The liver histology of the ethanol-fed rats showed pathomorphological alterations (Fig. 2). These changes were predominant in the centrilobular region having reduced oxygen perfusion. Hepatic damage may be partially attributed to cytochrome P_{450}-dependent enzyme activities in liver; that tends to be present in greatest concentration near the central vein and lowest near the peripheral sites [73]. Treatment with *H. indicus* reduced the histological changes produced by ethanol and significantly reversed the alcohol-induced liver changes.

The ability of *H. indicus* to enhance the levels of antioxidants along with its antilipid-peroxidative activity suggest that this extract might be potentially useful in counteracting free radical-mediated injuries involved in the development of tissue damage caused by ethanol abuse. In this investigation, the FT-IR spectra patterns showed a remarkable recovery from the adverse effects of ethanol intoxication, upon *Hemidesmus indicus* supplementation.

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