Abstract

*Helminthostachys zeylanica* (Linn.) Hook. F (Ophioglossaceae), locally known as “Pazhutharakali” is a medicinal pteridophyte, used traditionally for the treatment of jaundice in India. In the present study, the ethanolic extract of *H. zeylanica* rhizome (HZ) was used for *in vitro* and *in vivo* evaluation of its antihepatotoxicity. *In vitro* primary rat hepatocyte culture studies with HZ+Ethanol showed significant hepatoprotection as evidenced by increased cell viability, lowering of hepatic enzyme levels (Alanine transaminase (ALT), Aspartate transaminase (AST)). *In vitro* FeCl$_2$–ascorbic acid induced lipid peroxidation studies revealed the free radical scavenging effect of HZ. *In vivo* ethanol induced hepatotoxicity studies with HZ in wistar rats substantiates the above results as indicated by decreased levels of ALT, AST, ALP, total cholesterol (TC), total lipids (TL), triglycerides (TG), iron, (Fe) and copper (Cu) in serum. HZ treatment could also reduce liver tissue malondialdehyde (MDA) levels along with the enhancement in glutathione (GSH) levels. The histopathological studies of liver samples also supported the biochemical findings, confirming its antihepatotoxic property. Total phenolic content of HZ was estimated as 95.78mg/g. Thus the observed hepatoprotective action of *H. zeylanica* may be due to its antioxidant activity as indicated by decreased lipid peroxidation via increased glutathione levels, thereby hastening the elimination of ethanol and acetaldehyde from the blood. The results of the current investigation thus indicated good correlation between the *in vivo* and *in vitro* studies and also substantiate its traditional claim for hepatoprotection.

*Keywords: Anti-lipid peroxidant effects; Ethanol; Primary rat hepatocyte culture; Helminthostachys zeylanica; Alcoholic liver disease (ALD)*


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1. Introduction

In spite of remarkable strides in mainstream modern medicine to unravel complicated metabolic processes of the liver, no single therapeutic agent has been found to date, that can provide a lasting remedy for patients with hepatic disorders. With lack of effective treatment for liver diseases, researchers are turning towards ethnic drugs of herbal origin used traditionally, considering their efficacy, safety and lesser side effects as described by Suja et al. (2003 a). Several plants of the pteridophyte group, used by tribal and folk medical practitioners are a promising source of effective hepatoprotective agents. The impairment of liver function is generally caused by xenobiotics, excessive exposure to various hazardous chemical agents and protozoal or viral infections.

Alcoholic liver disease (ALD) is one of the most serious consequences of chronic alcohol abuse. Chronic alcohol intake produces a variety of physiological and physical changes in the human body, especially the liver which leads to serious consequences on the health of the individual and in majority of cases, it is life threatening. Hence it is the urgent need of the hour for the prevention of ALD and to develop inexpensive natural agents which possess hepatoprotective effects and therefore it has become the focus of research in recent years.

*Helminthostachys zeylanica* (Linnaeus) Hook. f (Ophioglossaceae) is an endangered medicinal pteridophyte, growing in swampy places in the Western Ghats of South India, up to an altitude of 3000 ft. It is locally known as “Pazhutharakali,” due to the resemblance of its rhizome to the feet of the centipede (Pazhuthara-centipede, Kal-feet). Dan and Shanavaskhan (1991) reported that Kattunaikan tribe of Malappuram district; Kerala, India uses the rhizome of this plant to treat snake bites. Prakash (1998) reported that *H. zeylanica* is used traditionally for the treatment of jaundice. It is used as an aperient, febrifuge, intoxicant, anodyne and to treat sciatica, boils, ulcers and malaria. The aboriginals of Nicobar Islands are known to use the rhizome of this plant for treating impotency as reported by Dagar and Dagar (1987). Suja et al. (2002) have scientifically confirmed the aphrodisiac properties of *H. zeylanica* in Swiss albino mice. Pandey and Bhargavan (1980) reported the antiviral properties of *H. zeylanica*. The hepatoprotective effect of *H. zeylanica* against carbon tetrachloride (CCl₄)-induced liver damage in Wistar rats was earlier reported by Suja et al. (2004 a). In the present investigation, we report for the first time, the hepatoprotective effect of the ethanolic extract of *Helminthostachys zeylanica* on ethanol-induced liver damage in Wistar rats.

2. Main Research

2.1. Materials and methods

2.1.1. Plant material and preparation of the extract

The rhizomes of *H. zeylanica* were collected from Kollam district of Kerala, India and authenticated by the plant taxonomist of the Institute. A voucher specimen (TBGT 57043 dated 25 June 2011) had been deposited at the Herbarium of the Institute. The rhizomes were shade dried and powdered. The powder (100 gm) was then extracted with 1000 ml ethanol, using a Soxhlet apparatus and the solvent removed by evaporation, at low temperature under reduced pressure on a rotary evaporator. The crude extract was referred to as HZ (yield: 0.9% w/w with respect to the dried
plant material). For administration, HZ was suspended in 0.5 % Tween–80 to required concentrations.

2.1.2. Animals
Wistar albino male rats, (150 -200 gms) and Swiss albino male mice, (20–25 gms) obtained from the Institute Animal House were used. They were housed under standard conditions and fed commercial rat feed (Lipton India Ltd, Mumbai, India) and water ad libitum. All experiments involving animals were done, according to guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, after getting the approval of the Institute’s Animal Ethics Committee.

2.1.3. Reagents
Ethanol, dithio-bis-2-nitrobenzoic acid (DTNB), glutathione (GSH), thiobarbituric acid (TBA), malondialdehyde (MDA), KCl-Tris-HCl buffer, Tris - HCl buffer, ascorbic acid (AA), FeCl₂ butanol, formaldehyde, Folin's reagent, penicillin/ streptomycin, MTT(3,4, 5 – dimethyl thiazol – 2-yl -2, 5-diphenyl tetrazolium bromide),Ca^{2+} - free Hank’s balanced salt solution (HBSS), Mg SO₄, Mg Cl₂, Ca Cl₂, heparin, HEPES Buffer, foetal bovine serum (FBS), collagen type I and collagenase type IV were obtained from HiMedia, Mumbai, India. Silymarin and gallic acid, were procured from Sigma – Aldrich, USA. AST, ALT, SB, ALP, cholesterol and total lipid kits were purchased from Crest Diagnostics Pvt Ltd, India. All other reagents used for the study were of analytical grade.

2.1.4. Phytochemical analysis of HZ
2.1.4.1. Preliminary phytochemical analysis of HZ
Preliminary phytochemical analysis of HZ was carried out as per the standard methods of Harborne (1984).

2.1.4.2. Estimation of total phenolics of HZ
The total phenolic content in HZ was determined by Folin Ciocalteu's method as described by Singleton and Rossi (1965). A standard curve was prepared using different concentrations of gallic acid (10, 25, 50, 100, 110, 125, 150, 200 µg/ml) prepared in ethanol. To the above standard solutions of gallic acid (1 ml each), 5 ml of Folin Ciocalteu’s reagent (1:10 dilution in distilled water) was added and after 8 min, 4 ml of 7.5% sodium carbonate was added. These solutions were incubated at room temperature for 2 h and their absorbance at 765 nm was measured on UV-Visible spectrometer (UV-1650PC, Shimadzu, Japan). For the test solution, 1 ml of HZ (10 mg in 10 ml EtOH) was mixed with 5 ml of Folin Ciocalteu’s reagent and 4 ml of 7.5% sodium carbonate was added after 8 min and incubated at room temperature for 2 h and its absorbance was also read at 765 nm. The gallic acid equivalent (GAE) was plotted and the total phenolic content of HZ was calculated in terms of GAE.

2.1.5. In vitro evaluation of ethyl alcohol - induced hepatotoxicity
2.1.5.1. Isolation of rat hepatocytes
Rat hepatocytes were isolated using the in situ collagenase liver perfusion technique as described by Seglen (1973).The hepatocytes were suspended in calcium free Hank’s balanced salt solution (HBSS) supplemented with HEPES (15mM), 10% FBS, penicillin (100 IU/ml) streptomycin (100µg/ml), insulin (10⁻⁸ M) and plated in collagen type I precoated culture plates. After plating, the cells were incubated at 37°C (95 % humidity, 5% CO₂). Hepatocyte attachment to the culture
dish was completed at 2 h after plating, and the medium was changed to remove unattached or dead cells. At 18h after plating, hepatocytes were treated with ethanol (hepatotoxic agent) and test sample as indicated in the following assay.

2.1.5.2. Ethanol intoxication and drug treatment regimen in primary rat hepatocyte culture
Hepatocytes were incubated (in triplicate) for 24 h with ethanol (96µl/ml)/HZ (25, 50, 100 µg/ml)/silymarin (25µg/ml) at 37°C. After incubation, cell viability was tested using MTT assay and releases of hepatic marker enzymes were also estimated.

2.1.5.3. Determination of cell viability in primary culture of rat hepatocytes
MTT assay is based on the ability of viable cells to reduce MTT from a yellow water-soluble dye to dark blue insoluble formazan product according to Mosmann (1983). MTT (5 mg/ml) was dissolved in phosphate-buffered saline (PBS) and added to the hepatocyte culture to make a final concentration of 100µl/ml DMSO. Optical density was assessed using a microplate reader at 570/620 nm.

2.1.5.4. Determination of hepatic marker enzymes
AST, ALT in the culture medium were assayed using standard kits purchased from Crest Diagnostics, Mumbai, India.

2.1.6. In vitro anti-lipid peroxidation studies
The anti-lipid peroxidant effect of HZ was studied as per the modified method described by Suja et al. (2004 b). Protein content was determined by the method of Lowry et al. (1951). Briefly, 2 g of rat liver tissue was sliced and homogenized with 150 mM KCl-Tris-HCl buffer (pH 7.2). The reaction mixture was composed of 0.25 ml liver homogenate, Tris - HCl buffer (pH 7.2), 0.1 mM ascorbic acid (AA), 4mM FeCl2 and 0.005 ml of various concentrations of HZ. The mixture was incubated at 37 °C for 1 h in capped tubes. Then, 0.1 N HCl, Sodium dodecyl sulphate (SDS, 9.8%), 0.9 ml distilled water and 2 ml of thiobarbituric acid (TBA, 0.6%) were added to each tube and vigorously shaken. The tubes were placed in a boiling water bath at 100 °C for 30 min. After cooling, 5 ml of butanol was added and centrifuged at 3000 rpm for 25 min. The absorbance of the supernatant was measured at 532 nm. The experiment was repeated twice.

2.1.7. In vivo evaluation of ethyl alcohol induced hepatotoxicity
The methodology of Jafri et al. (1999) was adopted for this study. Alcohol has a calorific value of about 7 calories of dietary carbohydrates, so in moderate quantities, it is a food in itself except for vitamins, minerals etc. Hence throughout the experiment, some quantity of glucose (isocaloric to the amount of alcohol) was also administered to all the animals of the control group, in order to equalise the calories due to alcohol in the other groups. Dietary linoleic acid is essential for development of experimentally induced liver damage. Corn oil is known to contain 55-60% linoleic acids. Nanji et al. (1989) has reported about the occurrence of severe pathological changes in rats fed ethanol with corn oil. Hence throughout the experiment, corn oil (10ml/kg/day) was also administered to all animals of all groups.

The animals were divided into six groups of 10 rats each. Group 1 served as control and received 0.5% Tween-80 in distilled water as vehicle (30ml/kg/day), corn oil (10ml/kg/day) and glucose, isocaloric to the amount of alcohol in three divided doses. Group 2 received ethyl alcohol (36.6%
v/v 30ml/kg/day) and corn oil (10ml/kg/day) in three divided doses. Groups 3, 4 and 5 received HZ (100, 200 and 300 mg/kg respectively) along with ethyl alcohol (36.6% v/v 30ml/kg/day) and corn oil (10ml/kg/day) in three divided doses. Group 6 received Silymarin (Sigma Chemical Company, USA) at a dose of 100 mg/kg p.o., along with ethyl alcohol (36.6% v/v 30ml/kg/day) and corn oil (10ml/kg/day) in three divided doses. All the animals received their respective treatments for 20 days.

On the 21st day of the ethyl alcohol induced hepatotoxicity study, all the animals were sacrificed by mild CO₂ inhalation. Blood samples were collected and serum separated for evaluating the biochemical parameters. The serum was then used for the assay of marker enzymes, namely AST, ALT, ALP, besides bilirubin, cholesterol, total lipids and triglycerides, iron and copper according to standard methods (Reitman and Frankel, 1957; Malloy and Evelyn, 1937; Zlatkis et al., 1953; Kind and King, 1954; Foster and Dunn, 1973; Annino, 1976; Yee and Goodwin, 1974).

2.1.7.1. Estimation of reduced glutathione (GSH)
The liver samples were treated with DTNB and the yellow coloured complex developed was read at 412 nm as reported in the studies of Moron et al. (1979).

2.1.7.2. MDA estimation
The liver tissue was homogenized and suspended in thiobarbituric acid (TBA) and the optical density of the clear pink supernatant was read at 532 nm, after centrifugation as described by Fong et al. (1973).

2.1.7.3. Histopathological studies
The liver samples obtained from the control and treated groups of the above experiments were preserved in 10% buffered formalin and processed for routine paraffin block preparation. Using a rotary microtome (American Optical Co., USA), sections of thickness about 7µm were cut and stained with hematoxylin and eosin. Mounting of the specimen was done by using Distrene phthalate xylene (DPX) as reported by Mukherjee (1995). The slides were examined under the microscope for histopathological changes such as spotty necrosis, fatty changes, ballooning degeneration and infiltration of Kupffer cells and lymphocytes.

2.1.8. Behavioural and toxic effects
Toxicity studies were carried out by acute toxic class method as described in OECD (Organisation of Economic Co-operation and Development) Guideline No. 423. Swiss albino mice of either sex weighing 18-22 g were randomly distributed to 8 different groups with six animals in each group. The animals were fasted overnight and HZ was administered orally at dose levels of 100, 200, 400, 800, 1600, 3200, 6400 and 12,800 mg/kg. The animals were closely observed continuously for 1 h for any gross behavioural changes like drowsiness, restlessness, writhing, convolution, piloerection, symptoms of toxicity and mortality if any, and then intermittently for the next 6 h and then again, 24 h after dosing with HZ and for 72h for mortality if any as described by Ecobichon (1997).

2.1.9. Statistical analysis
The significance of differences among the various treated groups and control group were analysed by means of one way analysis of variance (ANOVA) followed by Duncan’s multiple range test. Means
bearing same superscripts do not differ significantly (P ≤ 0.001, P ≤ 0.01). Method described by Raghava (1987).

2.2. Results

2.2.1. Phytochemical analysis of HZ
Preliminary phytochemical screening of HZ gave positive tests for the presence of flavonoids, triterpenoids, saponins, tannins and glycosides. Total phenol content was estimated as 95.78 mg/g.

2.2.2. In vitro evaluation of ethyl alcohol induced hepatotoxicity
2.2.2.1 Determination of hepatic marker enzymes release and cell viability in primary culture of rat hepatocytes
In primary cultures of rat hepatocytes, ethanol treatment showed decrease in cell viability (30%). HZ (100 µg/ml) treatment showed increase in cell viability up to 50% and silymarin (25µg/ml) up to 51%. When rat hepatocytes were incubated with ethanol there was a marked elevation in the hepatic marker enzyme levels, which was restored to almost normal values on treatment with HZ (100 µg/ml) or Silymarin (25µg/ml) (Figs 1 A-C).

Fig. 1. Effect of ethanolic extract of H. zeylanica (HZ) and Silymarin treatment on ethanol (EA) induced cytotoxicity in primary cultured rat hepatocytes (A) AST; (B) ALT and (C) cell viability. Significant P ≤0.01, n =6, ANOVA followed by Duncan’s multiple range test
2.2.3. In vivo evaluation of ethanol (EA) induced hepatotoxicity

Ethanol fed rats developed significant hepatocellular damage as evident from a significant elevation in the levels of serum marker enzymes like AST, ALP and AKP. There was also a significant increase in bilirubin, cholesterol, total lipids and triglycerides in serum of rats treated with ethanol as compared to normal control rats. Treatment of ethanol fed rats with HZ (100, 200 and 300 mg/kg) caused significant reduction of these parameters almost comparable to that of silymarin treated and normal groups (Table 1 and Fig 2). The effect produced by administration of HZ (200 mg/kg) was significant in our findings. Serum and copper levels were significantly increased in ethanol intoxicated rats, when compared with control animals. HZ treatment (200 and 300 mg/kg) could drastically decrease the levels of the unbound serum iron and copper to normal levels comparable to Silymarin treatment (Fig 3).

**Table 1** Effect of ethanolic extract of rhizome of *H. zeylanica* (HZ) on serum/hepatic parameters in ethanol (EA) induced hepatic damage in Wistar rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Parameters</th>
<th>Hepatic parameters</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AST (IU/L)</td>
<td>ALT (IU/L)</td>
</tr>
<tr>
<td></td>
<td>SAKP KA units/100ml</td>
<td>Bilirubin (mg%)</td>
</tr>
<tr>
<td></td>
<td>MDA (nmol/mg wet liv)</td>
<td>GSH (nmol/mg protein)</td>
</tr>
<tr>
<td>Normal control</td>
<td>57.00±2.05c</td>
<td>35.50±2.33b</td>
</tr>
<tr>
<td></td>
<td>7.70±2.22a</td>
<td>0.16±0.01a</td>
</tr>
<tr>
<td></td>
<td>0.641±0.001c</td>
<td></td>
</tr>
<tr>
<td>Ethanol (EA) control</td>
<td>84.16±2.05e</td>
<td>50.66±1.9d</td>
</tr>
<tr>
<td></td>
<td>10.1±2.13d</td>
<td>0.81±0.03e</td>
</tr>
<tr>
<td></td>
<td>0.211±0.002a</td>
<td>0.545±0.002b</td>
</tr>
<tr>
<td>EA+HZ (100mg/kg)</td>
<td>64.00±2.8d</td>
<td>40.22±2.07c</td>
</tr>
<tr>
<td></td>
<td>9.40±2.42c</td>
<td>0.50±0.05d</td>
</tr>
<tr>
<td></td>
<td>0.637±0.007b</td>
<td>0.683±0.003c</td>
</tr>
<tr>
<td>EA+HZ (200mg/kg)</td>
<td>55.76±2.04c</td>
<td>37.00±3.68b</td>
</tr>
<tr>
<td></td>
<td>8.70±2.3b</td>
<td>0.35±0.04c</td>
</tr>
<tr>
<td></td>
<td>0.683±0.003c</td>
<td></td>
</tr>
<tr>
<td>EA+HZ (300mg/kg)</td>
<td>52.31±3.46b</td>
<td>36.22±2.31b</td>
</tr>
<tr>
<td></td>
<td>8.27±2.33b</td>
<td>0.31±0.03c</td>
</tr>
<tr>
<td></td>
<td>0.721±0.003d</td>
<td></td>
</tr>
<tr>
<td>EA+Silymarin (100mg/kg)</td>
<td>48.10±2.38a</td>
<td>33.38±2.88a</td>
</tr>
<tr>
<td></td>
<td>7.78±2.56a</td>
<td>0.28±0.05b</td>
</tr>
<tr>
<td></td>
<td>0.872±0.002c</td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean ±SD, n=6, analysis of variance (ANOVA) followed by Duncan's multiple range test. Means bearing same superscripts do not differ significantly, (P≤0.01, P≤0.001)
Fig. 2. Effect of ethanolic extract of rhizome of *H. zeylanica* (HZ) on serum lipid parameters in ethanol (EA) induced hepatic damage in Wistar rats. Significant P ≤0.01, n =6, ANOVA followed by Duncan’s multiple range test

Fig. 3. Effect of ethanolic extract of *H. zeylanica* (HZ) and Silymarin treatment on Serum Fe and Cu levels. Significant P values ≤0.01, n =6, ANOVA followed by Duncan’s multiple range test

2.2.3.1. Estimation of reduced glutathione (GSH)
There was a marked decrease in the level of GSH, the key antioxidant in the ethanol treated group when compared with the normal control group. GSH depletion was inhibited by HZ (100, 200 and 300 mg/kg) and silymarin treated groups (Table 1).

2.2.3.2. MDA estimation
The ethanol treated group showed significant increase in MDA level, when compared with normal control. HZ treatment showed a decrease in MDA level in a dose dependent manner. Silymarin (100 mg/kg) significantly prevented the rise in MDA level which was almost comparable to the normal control group (Table 1).

2.2.3.3. Histopathological studies

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Histopathological studies basically supported the results obtained from the biochemical estimations. Liver slices of unchallenged control rats showed normal hepatic architecture with distinct hepatic cells, sinusoidal space and central vein, whereas rats treated with ethyl alcohol showed moderate to marked fatty changes, liver cell degeneration, spotty necrosis, hepatocyte swelling and active Kupffer cells. The histological pattern of liver of rats treated with HZ showed a marked degree of protection against ethanol induced alterations and they were almost similar to those from silymarin and unchallenged control rats (Fig 4-9).

**Figs. 4-9** Histopathological architecture of normal and ethanol treated rat liver samples. **Fig. 4.** Section of normal rat liver (normal control) showing normal hepatic cells with prominent nuclei and cytoplasm. **Fig. 5.** Section of ethanol intoxicated rat liver showing spotty necrosis, Kupffer cells, gross necrosis, extensive vacuolization, anucleate hepatocytes and inflammatory infiltration of...
lymphocytes. **Fig. 6.** Section of HZ (100 mg/kg)+ethanol treated rat liver showing binucleate hepatocytes, reduced necrosis and almost normal hepatic architecture. **Fig. 7.** Section of HZ (200 mg/kg)+ethanol treated rat liver showing reduced cell membrane damage. **Fig. 8.** Section of HZ (300 mg/kg)+ethanol treated rat liver showing less vacuolisation, necrosis and Kupffer cells compared to toxin group. **Fig. 9.** Section of Silymarin (100 mg/kg)+ethanol treated rat liver showing almost normal histological architecture

2.2.4. *In vitro* anti-lipid peroxidation studies

*In vitro* anti-lipid peroxidation studies revealed a significant increase in MDA levels in FeCl₂-AA control groups, compared to normal controls. HZ treatment significantly reduced MDA levels in rat liver microsomes at 100 to 300 μg/ml concentrations (Table 2.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>HZ (µg/ml)</th>
<th>Malondialdehyde(MDA) (n mol/g wet liver)</th>
<th>MDA Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>1.50±0.60a</td>
<td>-</td>
</tr>
<tr>
<td>FeCl₂-AA</td>
<td>-</td>
<td>5.75±0.02d</td>
<td>-</td>
</tr>
<tr>
<td>FeCl₂-AA+ <em>H. zeylanica</em></td>
<td>50</td>
<td>2.26 ±0.01c</td>
<td>60.70</td>
</tr>
<tr>
<td>FeCl₂-AA+ <em>H. zeylanica</em></td>
<td>100</td>
<td>1.95±0.01b</td>
<td>66.08</td>
</tr>
<tr>
<td>FeCl₂-AA+ <em>H. zeylanica</em></td>
<td>200</td>
<td>1.71±0.03ab</td>
<td>70.20</td>
</tr>
<tr>
<td>FeCl₂-AA+ <em>Silymarin</em></td>
<td>100</td>
<td>1.43±0.03a</td>
<td>75.13</td>
</tr>
</tbody>
</table>

Values are the mean ±SD, n=6, analysis of variance (ANOVA) followed by Duncan’s multiple range test. Means bearing same superscripts do not differ significantly, (P≤0.01)

2.2.5. *Behavioural and toxic effects*

In the toxicity study, no mortality occurred within 24 h with the 8 doses of HZ tested. The LD₅₀ was therefore greater than 12,800 mg/kg p.o. in mice (Data not shown).

3. **Discussion**

Alcoholic liver disease (ALD) is a major health and economic problem in the world and the treatment of alcoholic liver diseases still remains a challenge for the scientific community as reported by Kerr et al. (2000). According to Kai (1995), ALD is a common consequence of prolonged and heavy alcohol intake which encompasses acute inflammation, a wide spectrum of lesions, which progresses in to the most characteristic being alcoholic steatosis (fatty liver), alcoholic hepatitis, ultimately to alcoholic fibrosis and cirrhosis. The close relation of ethanol and liver is due to the fact that more than 80% of ingested alcohol is metabolised in the liver without feedback mechanism. Rang et al. (2003) reported that in liver, alcohol is first metabolized to acetaldehyde and then converted to acetic acid. Acetaldehyde is extremely reactive and toxic which...
binds to phospholipids, amino acid residues and sulphhydryl groups resulted in the development of liver damage. According to Shaw et al. (1995) radical mediated oxidative stress and the induction of cytochrome P 450 by ethanol plays a critical role in the pathogenesis of ethanol toxicity resulting in the damage of plasma membrane receptors. Therefore, some natural products with antioxidant activity could attract great attention as potential functional ingredients to protect alcohol induced liver injury.

In the present study, in vitro alcohol intoxication on hepatocytes caused cell membrane damage and consequent increase in serum marker enzymes in the culture medium which is ameliorated by the treatment of HZ or Silymarin. Zimmerman (1970) discovered that disturbances in the transport function of the hepatocytes, resulted in hepatic injury which leads to leakage of enzymes from cells due to altered permeability of membranes. Our in vitro studies confirmed the hepatoprotective activity of HZ against ethanol in primary culture of rat hepatocytes which is almost comparable to the reference compound Silymarin.

The in vivo pathogenicity of alcohol intoxicated rat liver is evident from increasing levels of AST, ALT and AKP. Pre treatment of rats with HZ significantly reverted these biochemical parameters in a dose dependent manner and thus protected from hepatocellular injury caused by ethanol, almost comparable to Silymarin. Our results indicated that HZ at all the doses tested significantly reverted the increased serum parameters induced by ethanol, indicating improvement of functional status of the liver. The histopathological findings also substantiated the biochemical data.

Ethanol ingestion in rats causes an increase in serum iron and copper level due to the hepatic dysfunction, impaired biliary secretion and generation of toxic hydroxyl radicals. The increased serum iron level indicates the generation of toxic hydroxyl radicals. Copper plays a pathogenic role in primary biliary cirrhosis, which catalyses the transformation of superoxide radical into highly reactive hydroxyl radical which initiates lipid peroxidation and oxidative damage. HZ could drastically decrease the levels of iron and copper may be through the chelating properties of the antioxidant flavonoids present in it. Similar results were reported by Singh et al. (2003) with the treatment of Tinospora cordifolia.

Peroxy radicals are important agents that mediate lipid peroxidation thereby damaging cell membrane as reported by Suja et al. (2003) b). The free radicals generated from ethanol and induction of cytochrome P450 caused peroxidation of cell membrane and resulted in the production of MDA. Oh et al. (1998) reported that MDA has been shown to react with critical biomolecules such as nucleic acids, thus damaging the cells. Tuma et al. (1996) discovered that acetaldehyde and MDA can react together in a synergistic manner and generate highly toxic hybrid adducts (MAA-adducts) which plays an important role in the pathogenesis of alcoholic liver injury. According to Oh et al. (1998), it is the enhancement in the activities of the hepatic antioxidant defence system may be one of the protective mechanisms of the body against such oxidative damage. GSH depletion is an important mechanism in the sensitisation of liver to alcohol induced injury as reported by Yuan et al. (2007). HZ improved the antioxidant defence system by increasing reduced glutathione (GSH) and decreasing MDA level comparable to Silymarin. According to Prakash (2008) binding of acetaldehyde, a metabolite of ethanol with GSH may contribute to reduction in the levels of GSH and may lead to increased lipid peroxidation with concomitant changes in membrane permeability and cellular damage. Our results are in line with this report..
in vitro antilipid peroxidation studies with FeCl₂-AA mixture also supported the above findings and indicated good correlation between the in vivo and in vitro studies. Gopumadha van et al. (2008) reported that hepatomegaly is a common finding after chronic ethanol ingestion and is mainly due to an increase in fat, protein and water. Similar findings were reported by Lieber, (2000) in which elevation of total lipids after ethanol ingestion was mainly due to the increase of triglycerides and cholesterol which leads to fatty liver. This may be due to the activation of enzyme HMG Co-A reductase, the rate-limiting step in cholesterol biosynthesis. Treatments with HZ significantly reduced the cholesterol, lipid and triglyceride levels, suggesting that HZ prevented ethanol-induced hyperlipidaemia, probably due to its hepatoprotective activity.

Phytochemical evaluation of the dried rhizomes of *H. zeylanica* by Chen et al. (2003) showed the presence of cyclized geranyl stilbenes and 3-hydroxyl acetophenone; Huang et al. (2003) reported flavonoid, Ugonins and Dan and Shanavaskhan (1991) reported calcium, phosphorous, iron, carotene and ascorbic acid. According to Lin et al. (2009) Ugonins, carotene and ascorbic acid are potent antioxidants, especially Ugonin K which has neuroprotective properties in human neuroblastoma cells. Murthy et al. (2005) has already been reported that beta carotene from algal source showed potent anti hepato toxic effects. Tradtrantip et al. (2006) discovered 3-hydroxy acetophenone, one of the constituents of HZ which acts as a choleretic agent through the stimulation of exocytosis and increase in paracellular permeability. Suja et al. (2004 a) had earlier reported the choleretic activities of *H. zeylanica in vivo*.

4. Conclusion

In conclusion, the major mechanism behind the hepatoprotective potential of HZ may be due to the combined synergistic effect of its phytochemical constituents like triterpenoids, saponins, tannins glycosides, antioxidant flavonoids like Ugonins and micronutrients like carotene, ascorbic acid etc which minimise the deleterious effects of free radicals through accelerated detoxification, strengthening the antioxidant defence system via increasing the glutathione availability and enhancing choleretic activity.

The efficacy of any hepatoprotective drug depends on its ability to ameliorate the harmful effects in liver and to maintain the normal hepatic physiological mechanism which has been disturbed by the hepatotoxins. Our investigation provided convincing data for the justification of the traditional/tribal claim of *Helminthostachys* in treating liver diseases. Thus *Helminthostachys zeylanica* can be ranked as a promising candidate for the development of phytomedicine for liver diseases. Future studies are contemplated in the direction of deciphering the exact mechanism of action responsible for the hepatoprotective effect of *H. zeylanica* and its clinical utility in humans.

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