Many studies have shown that reactive oxygen species including oxygen free radicals are causative factors in the etiology of degenerative diseases, including some hepatopathies. In many in vitro and in vivo studies, several classical antioxidants have been shown to protect hepatocytes against lipid peroxidation or inflammation, thereby preventing the occurrence of hepatic necrosis (Hsiao & all, 2001).

Lichens have been used by various ethnic groups from the time of early civilization. Irrespective of the advances in medical sciences, tribal people still utilize these plants. The lichens are utilized for different purposes depending on their nutritive, medicinal and cosmetic properties. They have been appreciated in traditional medicine but their importance has largely been ignored by the modern pharmaceutical industry because of the difficulties in establishing axenic cultures and conditions for rapid growth. These factors preclude their routine use in most conventional screening processes (Upreti & Chatterjee, 2007). With this background information an attempt was made to get scientific evidences for these traditional reports on lichens in herbal formulation.

In the present study antioxidative potential of four cultured species of lichens Arthothelium awasthii, Heterodermia podocarpa, Parmotrema tinctorum and Usnea ghattensis has been described. Of these, U. ghattensis extract demonstrated maximum antioxidant activity by various in vitro assays, the extract of cultured lichen U. ghattensis was selected for the further study to investigate the hepatoprotective activity.

Materials and methods

Animals

To determine the hepatoprotective activity of lichen extract, adult (6-8 weeks old)
albino mice of either sexes bred in the animal house of Agharkar Research Institute, Pune, were used for the preparation of liver slices. The approval for this work, using animals, was taken from the Institutional Animal Ethical Committee of Agharkar Research Institute, Pune. The \textit{in vitro} mice liver slice culture method of Wormser \& all (1990) was used for the determination of hepatoprotective activity of the lichen extract against ethanol-induced hepatotoxicity.

**Preparation of liver slices**

Adult albino mice of either sexes, weighing 18-20 gm were taken and dissected after cervical dislocation. The liver lobes were removed and transferred in prewarmed Krebs-Ringers-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (KRH) medium. Liver was sliced into small pieces (4-6 mg) of about $0.5 \times 0.5 \times 0.5$ mm using a prep blade. Slices were incubated for 1 h in a capped Erlenmeyer flask containing approx. 30 ml of KRH medium at 37$^\circ$C in a waterbath shaker.

During this incubation, the slices were washed by carefully removing the medium and replacing it with fresh medium every 10 min. All media used for incubation and rinsing were prewarmed. Slices were divided into small portions (20-22 slices) of 100-120 mg wet weight. Slices were incubated for 1 h in 2 ml KRH medium in 20 ml capped beakers at 37$^\circ$C in a rotary waterbath shaker. The cultures were aerated in an Erlenmeyer flask with oxygen every 10 min by removing the cap.

**Table: 4.1. Composition of KRH medium.**

<table>
<thead>
<tr>
<th>Component</th>
<th>mM concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>2.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>118.0</td>
</tr>
<tr>
<td>KCl</td>
<td>2.85</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>2.5</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.15</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>1.18</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Make up to 1000 ml, pH adjusted to 7.4 by 1 N NaOH</td>
</tr>
</tbody>
</table>

**Experiment design**
Liver slices were divided into three sets:

**Set 1**, control, slices incubated in KRH medium,

**Set 2**, slices incubated in 112 mM ethanol,

**Set 3**, slices incubated in 112 mM ethanol + lichen extract.

The 50% release of liver marker enzyme in slices treated with ethanol or in combination with lichen extract was determined by following the method of Invitox Protocol no. 42 (1992). The Dry methanol extract with concentrations 10, 50, 100, and 200 μg was added in the KRH medium before transferring the preincubated liver slices in the medium. Duplicate cultures were set up for each concentration to minimize the errors. Cultures were incubated for 2 h at 37°C. Trolox is a water-soluble vitamin E analogue, and it is soluble in the KRH medium and used as a standard antioxidant to compare with the response of methanol lichen extract.

**Hepatoprotective effect *in vitro***

After completion of the incubation period, liver slices were homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.4) giving a concentration of 100 mg/ml and centrifuged at 10000 rpm for 3 min at 4°C. Liver marker enzyme lactate dehydrogenase (LDH) and antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were estimated in the medium and in the supernatant. Measurement of lipid peroxidation and glutathione (GSH) content were also done. Effective concentration, EC₅₀, of lichen extract was estimated. It is the dose that inhibits the LDH release by 50% with respect to control.

**Measurement of lactate dehydrogenase activity**

Lactate dehydrogenase (LDH) activity was measured spectrophotometrically at 340 nm according to the method of Racher (1998). Cuvette containing 10 mM Tris - Cl buffer, pH 7.2 (2 ml), 10 mg/ml NADH and pyruvate, and 70 μl of sample were added to this reaction mixture. Commercially available LDH (Sigma) was used as the standard.
Measurement of superoxide dismutase activity

The superoxide dismutase (SOD) activity was assayed spectrophotometrically according to Marklund & Marklund (1974) by means of inhibition of pyrogallol autoxidation. Reaction mixture containing 50 mM Tris-cacodylic acid buffer, pH 8.2, 1 mM diethylenetriaminepentaacetic acid, 0.2 mM air-equilibrated pyrogallol, iron in a trace amount, and 100 μl liver tissue homogenate was added to this mixture. Changes in absorbance at 420 nm were recorded at 30 sec intervals for 5 min. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol autoxidation with standard SOD. Data were expressed as SOD units/min/mg protein as compared with the standard.

Measurement of catalase activity

Catalase (CAT) activity was measured by the method of Aebi (1983). Liver tissue homogenate (10%, w/v) were centrifuged (10000 rpm) at 4°C for 10 min. 1 ml of the supernatant was added to a quartz cuvette, containing 30 mM H$_2$O$_2$ solution prepared in 50 mM potassium phosphate buffer (pH 7.0) and water. Changes in absorbance at 240 nm were recorded at 15 sec intervals for 1 min. One unit was defined as the amount of the enzyme that converts 1 mol substrate to product in 1 sec.

Measurement of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was measured according to the method of Rotruck & all (1973). Briefly, 2 ml of 80 mM sodium phosphate buffer (pH 7.0) contained 1 mM ethylenediamine tetraacetic acid, 1 mM sodium azide, 0.4 mM glutathione (GSH), and 100 μl of the liver tissue homogenate. The reaction was started by the addition of 2.5 mM H$_2$O$_2$. The change in absorbance at 255 nm was recorded at 30 sec intervals for 3 min. The values were expressed as μg of GSH consumed/min/mg protein.

Measurement of lipid peroxidation
Lipid peroxidation was estimated by the method of McMillan & all (1998) in terms of thiobarbituric acid reactive substances (TBARS) formed in liver tissue homogenate, with some modification. Briefly, liver tissue homogenate was incubated with 1:1 ratio of 0.67% thiobarbituric acid (TBA) at 100°C for 15 min. The mixture was allowed to cool at room temperature, and absorbance was read at 532 nm by a UV-Vis spectrophotometer. TBARS formation was quantified with a standard curve using known amounts of malondialdehyde (MDA).

**Estimation of glutathione**

Glutathione (GSH) content was determined after deproteinization according to Ellman (1959). Proteins were precipitated with metaphosphoric acid (MPA) and sodium chloride. Then filtrate was added to a cuvette containing 0.5 M phosphate buffer, pH 7.5, and the color was developed by the addition of 5, 5′-dithiobis (2- nitrobenzoic acid). The absorbance was read at 420 nm after 1 h.

**Estimation of proteins**

Protein in the tissue homogenate was estimated according to the method of Bradford (1976) as described in previous Chapter 1.

**In vitro toxicity study**

In the liver slice culture system release of lactate dehydrogenase (LDH) was used as a marker to study the hepatotoxicity of ethyl alcohol. The toxicity of cultured lichen extract and Trolox, a water-soluble vitamin E analogue, was determined. Liver slices were divided into three sets (100 mg wet weight) and treated with fixed concentrations of 10, 50, 100, and 200 mg of (1) Trolox, (2) lichen extract only, and (3) ethanol. These sets were incubated for 2 h at 37°C. After completion of incubation period, % of LDH release was calculated. No toxic effect of Trolox or lichen extract was detected even at doses of 100 mg and 200 mg.

**Histopathology of liver slice**
Histopathology of liver slices was done by fixing the liver slices in 4% formaldehyde buffer. Sections of 10 μm size were cut by a freeze microtome and stained with haematoxyline/eosin (Luna, 1968) and examined under a light microscope.

**Analysis of antioxidant component in the extract of cultured lichen *Usnea ghattensis***

Antioxidant component in the methanol extract of cultured lichen *U. ghattensis* was determined using the method recently published by Espin & all (2000). Thin layer chromatography (TLC) plates, coated with silica gel (Silica gel 60 F254, Merck) were each spotted with 20 µl of the lichen extracts at a concentration of 2.5%. The plates were then developed in a solvent system ethyl acetate : methanol : water (10 : 2 : 1; v/v/v). After drying, one of the developed plates was first observed under UV light at a wavelength of 365 nm and sprayed with 0.4 mM DPPH radical in methanol. Furthermore, the developed TLC plates were sprayed separately with spray 1 solution (1% solution of iron (III) chloride in water) mixed immediately before use with an equal volume of a 1% solution of potassium hexacyanoferrate (III) in water (Barton’s reagent) which gave a blue colour in the presence of phenolic compounds, and spray 2 solution (2% iron (III) chloride in ethanol), which, when heated at 105°C for 5-10 min, gave either a blue colour, indicating the presence of phenolics with trihydroxy groups, or a green colour, indicating phenols with dihydroxy groups, or a red/brown colour, indicating the presence of other phenolics.

**Statistical analysis**

Experimental results are the mean (± SEM) of three parallel measurements. Student’s ‘t’ test was used to determine the statistical significance between the sets of liver slices.

**Results**
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Hepatoprotective activity of *Usnea ghattensis* culture in vitro

The results on the hepatoprotective activity of cultured lichen *Usnea ghattensis* using *in vitro* mice liver slice culture model are presented below.

Effect of lichen extract on lactate dehydrogenase and lipid peroxidation activity in mice liver slice

The effects of various concentrations of lichen extract on the lactate dehydrogenase (LDH) activity of mice liver tissue homogenate have been presented in fig. 4.1. The LDH activity was significantly decreased (50%) in the liver slice treated with 112 mM ethanol compared to control (untreated) or treated with Trolox 50 μg/100 mg tissue weight. LDH activity in liver tissue increased with the increase in extract concentration. It seems that the LDH activity is dose dependent.

The LDH release and lipid peroxidation of mice liver tissue under various time courses are presented in fig. 4.2. Continuous LDH release (50%) was observed up to 2 h by the liver tissue treated with ethanol only, whereas the LDH release by the control (untreated) was constant (below 10%). However, the extract-treated liver tissue showed 20% LDH release up to 1 h incubation and further decreased equivalent to the control level at 2 h incubation.

Ethanol is known to generate oxidative stress in cells, which can be measured from the extent of lipid peroxidation in liver tissue. The lipid peroxidation of the liver tissue under various time courses was measured in terms of TBARS and is expressed as μM malondialdehyde (MDA) formed. The results are presented in fig. 4.2.

The continuous increase of MDA (up to 0.07 μM) was observed up to 2 h incubation in the liver slice treated with ethanol alone, whereas in the control (untreated), the liver tissue showed a linear increase in MDA formation (below 0.02 μM) up to 1.5 h. However, the formation of MDA gradually decreased, and finally a concentration below 0.005 μM MDA was obtained at 2 h incubation of liver tissue treated with lichen extract along with ethanol.
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The above results indicated that LDH activity and the lipid peroxidation are dose-time dependent.

**Effect of lichen extract on the antioxidant status of liver slice culture**

Antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and the nonenzymatic antioxidant glutathione (GSH) protect cells from oxidative stress of highly reactive free radicals. These enzymes are induced on the generation of free radicals in cells. Activities of SOD, CAT, GPx, and GSH were checked in liver slice culture treated with ethanol, ethanol + Trolox, and extract + ethanol. The activities were checked every 30 min up to 2 h. The results are presented in table 4.2.

The activities of the antioxidant enzymes SOD, CAT, and GPx were linearly increased up to 2 h in the liver tissue treated with ethanol, whereas SOD and GPx linearly increased up to 1 h incubation except CAT, which decreases after 30 min incubation in the liver tissue treated with Trolox along with ethanol. However, the liver tissue treated with lichen extract with ethanol showed a linear increase in the activities of SOD, CAT, and GPx up to 1 h, and thereafter, the activities were decreased.

As far as GSH content is concerned, there was a continuous increase in the GSH content observed in the liver tissue treated with ethanol only, whereas GSH content was increased up to 1 h and thereafter decreased in the tissue treated with extract or Trolox with ethanol. The results indicated that the antioxidative status of liver slice is time dependent.

**Histological observations of liver slice cultures**
The degeneration of hepatocytes in the liver tissue treated with ethanol was prominent, whereas the extract-treated liver tissue showed very less fatty degeneration up to 30 min and thereafter started regeneration of hepatocytes (Fig. 4.3).

**Bioactive components in the lichen extract**

As far as the bioactive component in the lichen extract is concerned, the qualitative analysis by HPLC and TLC, showed the presence of lichen substances usnic acid and norstictic acid along with trihydroxy phenolic groups in the extract.

**Discussion**

The development of hepatic disease in response to ethanol exposure associated with metabolic imbalance in the liver leads to the formation of reactive oxygen species (ROS). Inadequate removal of ROS may cause cell damage by attacking membrane lipids and proteins and inactivating antioxidant enzymes thus mediating several forms of tissue damage (Hirnwich & all, 1982; Frienhel & all, 1985; Halliwell & Guttridge, 1990; Datla & all, 2000).

The lipid peroxidation in terms of malondealdehyde (MDA) formation was increased with the increasing of ethanol exposure up to 2 h in the liver tissue treated with ethanol. However, the administration of lichen extract in presence of ethanol significantly decreased the MDA formation in the liver tissue. These results would indicate that the extract could hinder their interaction with polyester fatty acids and could abolish the enhancement of the lipid peroxidation process leading to MDA formation (Gupta & all, 2006 a, b).

In the present study, also it has been found that the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were linearly increased up to 2 h in the liver tissue treated with ethanol. The results are in agreement with the results those reported that an increase in Mn-SOD, which was found after a short-term exposure to ethanol in a cell culture model using human hepatoma cells (Perera & all, 1995). This increase in SOD activity diminished
after repeated ethanol administration. Further, the prolonged ethanol exposure may suppress the cellular adaptive response to oxidative stress (Thome & all, 1997).

The relative contribution of CAT and GPx in decomposition of endogenous hydrogen peroxide is dictated by tissue specificity. GPx has a more important contribution in the liver (Jones & all, 1981), whereas CAT predominates in the renal tissue (Freeman & Crapo, 1982). Further, these results are in agreement with those that reported an increase of CAT and GPx activities in the kidney after ethanol treatment (Dinu & all, 2005). Therefore, an increase in CAT activity would indicate an enhanced ethanol toleration of the liver.

Reduced glutathione (GSH) offers one of the mechanisms for the scavenging of toxic free radicals. As far as GSH content in the liver tissue after ethanol treatment is concerned, a continuous increase in the GSH content up to 2 h was observed in the liver tissue. In this study, the results are consistent with the reports that an increase in GSH content could be expected to prepare the hepatocytes against a potential oxidative damage (Scharf & all, 2003; Alia & all, 2006).

The results on the administration of the lichen extract or Trolox in the ethanol-induced toxicity in the liver revealed significant depletion in the lipid peroxidation, antioxidative enzymes SOD, CAT, and GPx, and the nonenzymatic antioxidant GSH in this study. This would indicate that the methanol extract of cultured lichen U. ghattensis has antioxidant and hepatoprotective potentials.

In conclusion, the results of the present study suggest that the U. ghattensis extract could prevent oxidative liver damage. However, further comprehensive pharmacological investigations will be needed to elucidate the mechanism of this hepatoprotective effect.
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Figure: 4.1. Effect of lichen extract on lactate dehydrogenase (LDH) activity in mice liver slice in vitro. Results are the mean (± SEM) of three consecutive readings. * p < 0.001, when compared to normal, ** p < 0.05 when compared with that treated with ethanol.

Figure: 4.2. Time course of lipid peroxidation of liver tissue and lactate dehydrogenase (LDH) release in the presence of ethanol and the methanolic extract of lichen U. ghattachis. Values are the mean (± SEM) of three experiments.
Table 4.2. Effect of cultured lichen *U. ghattensis* extract on the antioxidant status of liver slice culture in vitro against ethanol-induced hepatotoxicity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Time at which the activity was measured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 h</td>
</tr>
<tr>
<td>GSH</td>
<td>Normal</td>
<td>7.40 ± 0.23</td>
</tr>
<tr>
<td>(µg/mg tissue)</td>
<td>Ethanol</td>
<td>14.60 ± 0.33*</td>
</tr>
<tr>
<td></td>
<td>Trolox + ethanol</td>
<td>7.02 ± 1.01</td>
</tr>
<tr>
<td></td>
<td>Extract + ethanol</td>
<td>7.02 ± 0.41</td>
</tr>
<tr>
<td>GPx</td>
<td>Normal</td>
<td>0.040 ± 0.005</td>
</tr>
<tr>
<td>(unit/min/mg protein)</td>
<td>Ethanol</td>
<td>0.044 ± 0.004*</td>
</tr>
<tr>
<td></td>
<td>Trolox + ethanol</td>
<td>0.028 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>Extract + ethanol</td>
<td>0.018 ± 0.003</td>
</tr>
<tr>
<td>CAT</td>
<td>Normal</td>
<td>0.190 ± 0.05</td>
</tr>
<tr>
<td>(unit/min/mg protein)</td>
<td>Ethanol</td>
<td>0.813 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>Trolox + ethanol</td>
<td>0.836 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>Extract + ethanol</td>
<td>0.366 ± 0.006</td>
</tr>
<tr>
<td>SOD</td>
<td>Normal</td>
<td>0.009 ± 0.01</td>
</tr>
<tr>
<td>(unit/min/mg protein)</td>
<td>Ethanol</td>
<td>0.024 ± 0.004*</td>
</tr>
<tr>
<td></td>
<td>Trolox + ethanol</td>
<td>0.015 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Extract + ethanol</td>
<td>0.011 ± 0.001</td>
</tr>
</tbody>
</table>

Values are the mean (± SEM) of three parallel sets of culture in each observation. GSH: Glutathione, GPx: Glutathione peroxidase, CAT: Catalase, SOD: Superoxide dismutase *p < 0.001 as compared with normal group.
Figure: 4.3. Representative photomicrograph of histopathological changes in mice liver slices. (A) normal control mice liver slice, (B) ethanol treated liver slice shows degenerated hepatocytes, (C) and (D) are ethanol plus lichen extract treated liver slices after 0.5 and 2 h.