ABSTRACT
The present study has been carried out to evaluate the *In vitro* hepatoprotective activity of methanolic extract of *Drynaria quercifolia* L. rhizome. For cytotoxicity screening, primary hepatocytes monolayer cultures were treated with CCl₄ and different concentrations of extracts of *Drynaria quercifolia* L. rhizome. Protection against CCl₄ was determined by MTT assay. The result showed that dose dependent increase in percentage of viability at the doses of 100, 250, 500µg/ml against CCl₄. *In vitro* hepatoprotective activity of plant extract was also evaluated by analyzed various parameters such as MDA, GSH, SGOT, SGPT and protein in carbon tetrachloride induced hepatocytes. CCl₄ induced cell damage was well manifested by significant increase of MDA, SGOT, and SGPT and decrease of GSH and protein. Plant extracts (100, 250, 500µg/ml) along with CCl₄ treated hepatocytes reversed all the above parameters near to normal. Result of this study demonstrates that the *Drynaria quercifolia* rhizome having good hepatoprotective potential which may be due to the presence of various phytochemicals.

KEYWORDS: Carbon tetrachloride, *Drynaria quercifolia*, Phytochemical, Hepatocytes, Methanol extract.

INTRODUCTION
The liver plays an astonishing array of vital functions in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathway to growth, fight against disease, nutrient supply, and energy provision and reproduction. It functions as a centre of metabolism of nutrients such as carbohydrate, protein and lipids and
excretion of waste metabolites. Therefore, maintenance of healthy liver is essential for the overall well being of an individual.[1] Liver diseases are among the most serious ailment. Liver disease includes acute or chronic hepatitis (Inflammatory liver diseases), hepatosis (non inflammatory diseases) cirrhosis (degenerative disorder resulting in fibrosis of the liver) etc., an injury to it or impairment of its function may lead to many complication.[2] Liver disease are mainly caused by toxic chemicals such as consumption of alcohol, high doses of paracetomol, carbon tetrachloride, chemotherapeutic agents, peroxidised ion. Most of the chemicals damage liver mainly by inducing lipid peroxidation and other oxidative damage.[3] Liver cells can be damaged in a variety of ways like cell can become inflamed, bile flow can be obstructed, cholesterol or triglycerides can accumulated and blood flow to the liver may be compromised.

Carbon tetrachloride is a colorless, non-flammable liquid with a characteristic odor.[4] Carbon tetrachloride was used in fire extinguishers which are useful for fighting fires near electrical equipment because it does not conduct electricity. It is known to cause damage to the liver, lungs, kidneys, adrenals and central nervous system in humans and experimental animals. Possible mechanisms of its toxicity have been reviewed.[5] Various hepatoprotective agents have been studied to observe the beneficial effects against the chemically induced liver injury produced by carbon tetrachloride.

Herbal based therapeutic for liver disorder has been in use in India for a long time and has been popularized world over by leading pharmaceutical. Despite the significant popularity of several herbal medicines in general and for liver disease in particular they are still unrespectable treatment modalities for liver disease. In India more than 87 plants are used in 33 patented and proprietary multi ingredients plant formulation. [6] In spite of the tremendous advance made significant and hepatoprotective agents is available in modern therapeutics. Therefore important has been safe given globally develop plant based hepatoprotective drugs effective against a variety of liver diseases.

*Drynaria quercifolia* (Asvakatri) belongs to the Family of Polypodiaceae is found throughout India, especially in the plains or very low down in the mountains, on trees or rocks. South China, Malaysia and Tropical Australia.[7] It is used in medicinal system by different groups of people to treat various kinds of health problems including urinary tract infection.[8] The rhizome paste is applied for treatment of diarrhea, typhoid, cholera, chronic jaundice, fever, headach and skin disease. Whole plant is anthelmintic, expectorant and tonic.[9,10] It is also
used in the treatment of chest disease, cough, hectic fever, dyspepsia, loss of appetite, chronic jaundice and cutaneous infection.\cite{11} Drynaria quercifolia along with the other combination of herbs is used to treat pain from traumatic injury, such as sprains and contusions with bruising and swelling.\cite{12} Hence the present study has been designed to evaluate the \textit{in vitro} hepatoprotective activity of \textit{Drynaria quercifolia} L. rhizome.

**MATERIALS AND METHODS**

**Chemicals**
Carbon tetrachloride, Thiobarbituric acid, 2, 4-Dinitro phenyl hydrazine, and glutathione were purchased from Sigma chemical, Mumbai. All other reagents and chemicals used in this study were of analytical grade with high purity.

**Collection and identification of plant materials**
The material used in the present study is the rhizome of \textit{Drynaria quercifolia}. The rhizome was collected from the Kollimalai, Namakkal Dt, Tamil Nadu, India. The rhizome is covered with small brown coloured hair like structures they were remove using sterile scalpel and washed with sterile distilled water and cut in to small pieces and dried in shade and made in to fine powered, using blender. The powder was used for these studies.

**Preparation of extracts**
The powder material of \textit{Drynaria quercifolia} was macerated with methanol at room temperature for 3 days. After 3 days, the supernatant was transferred into china dish. The supernatant was completely removed by keeping the china dish over a boiling water bath at 60°C. A semi solid extract was obtained after complete elimination of alcohol. The obtained residue was kept in the refrigerator for further use. The extract was made up to a known volume in distilled water just before the experimental process.

**Cytotoxicity Screening**
The screening of hepatoprotective activity was based on the protection of rat hepatocytes against CCl4-induced damage determined by estimating mitochondrial synthesis using the tetrazolium assay. The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based.\cite{13}
Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to $1.0 \times 10^5$ cells/ml using medium containing 10% new born calf serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100µl of different drug concentrations (100, 250, 500µg/ml) were added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO$_2$ atmosphere, and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, the drug solutions in the wells were discarded and 50µl of MTT in DMEM was added to each well. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO$_2$ atmosphere. The supernatant was removed and 50µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 540nm.

In vitro hepatoprotective activity

Fasting Wister male adult rats weighing 280-300 g were used. Liver cells were isolated by a modified procedure of Seglen (1979). The isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10% newborn calf serum antibiotics, $10^{-6}$ dexamethasone and $10^{-8}$ M bovine insulin. The cell suspension was incubated at 37°C for 30 minutes in a humified incubator under 5% CO$_2$. After incubation of 24 hours, the hepatocytes were exposed to the fresh medium containing CCl$_4$ (1%) along with/without various concentrations of (100, 250, 500µg/ml) plant extract or medium alone. After 60 min of CCl$_4$ intoxication, concentration of MDA, GSH, Protein, SGOT, SGPT in the medium were measured as an indication of hepatocytes necrosis using diagnostic kits.

Statistical Analysis

The results were presented as Mean ± SD. Data was statistically analyzed using student “t” test. P values set as lower than 0.05 were considered as statistically significant.

RESULTS

In the present study, we have screened the cytotoxicity and in vitro hepatoprotective activity of Drynaria quercifolia rhizome against carbon tetrachloride induced hepatotoxicity. The observations made on control and experimental groups were compared as follows.

Table 1 represents the % of viability of control and experimental hepatocytes. Microculture
Tetrazolium (MTT) assay confirmed that cells which are exposed only with toxicant \( \text{CCl}_4 \) showed decreased percentage of viability (39.78%) when compared to control while the cells which are pretreated with extract showed an increase in percentage of viability and the results were highly significant (P<0.05) when compared to \( \text{CCl}_4 \) intoxicated cells. The percentage viability was 65.23, 78.45 and 87.43 for 100µg, 250µg and 500µg of plant extracts respectively. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity which was interpreted as a measure of viability cell number. The assay has therefore been adopted for use with cultures of exponentially growing cells.[17]

Table 1: Determination of % of Viability in experimental hepatocytes

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration</th>
<th>% of viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I - Control(Untreated)</td>
<td>---</td>
<td>100</td>
</tr>
<tr>
<td>Group II - ( \text{CCl}_4 ) (0.015mg)</td>
<td>1%</td>
<td>43.21</td>
</tr>
<tr>
<td>Group III - ( \text{CCl}_4 + \text{Drynaria quercifolia} )</td>
<td>100µg/ml</td>
<td>65.23*</td>
</tr>
<tr>
<td>Group IV - ( \text{CCl}_4 + \text{Drynaria quercifolia} )</td>
<td>250 µg/ml</td>
<td>78.45*</td>
</tr>
<tr>
<td>Group V - ( \text{CCl}_4 + \text{Drynaria quercifolia} )</td>
<td>500µg/ml</td>
<td>87.43*</td>
</tr>
</tbody>
</table>

Values were expressed as mean±SD for triplicate in each group.

*Significantly different from Group II (P<0.05)

**In vitro hepatoprotective activity**

Table 2 shows the level of MDA and GSH of control and experimental hepatocytes. MDA is a secondary product of lipid peroxidation which is used as an indicator of tissue damage by series of chain reactions.[18] Hepatotoxic compounds like \( \text{CCl}_4 \) are known to cause marked elevation in enzyme activities. In the present study, MDA level was significantly increased (P<0.05) in group II than group I whereas the hepatocytes pretreated with \textit{Drynaria quercifolia} (100µg, 250µg and 500µg) attenuated the increased content of MDA in hepatocytes.

Glutathione is a ubiquitous thiol-containing tripeptide, which plays a central role in cell biology. It is implicated in the cellular defense against xenobiotics and naturally occurring deleterious compounds, such as free radicals and hydroperoxides. Glutathione status is a highly sensitive indicator of cell functionality and viability. Glutathione is responsible for the regulation of lipidperoxidation.[19] The toxic activation of \( \text{CCl}_4 \) via the CYP2E1 (Cytochrome P450 2E1) pathway, the detoxification pathway involves GSH conjugation of the trichloromethyl radical, a CYP2E1-mediated \( \text{CCl}_4 \) metabolite. Previous studies on the mechanism of \( \text{CCl}_4 \)-exposed hepatotoxicity have shown that GSH plays a key role in
detoxifying the reactive toxic metabolites of CCl₄ and that liver necrosis begins when the GSH stores are markedly depleted. GSH is largely mediated through the activity of GST, and forms with the toxic metabolites of CCl₄. GSH plays a crucial protective role as a scavenger of free radicals that combine with non-protein thiols at the GSH reactive center to abolish free radical toxicity.

GSH depletion is linked to a number of diseases states including cancer, cardiovascular diseases. It is implicated in the cellular defense against xenobiotics naturally occurring deleterious components, such as free radical and hydroperoxides. Thus the GSH concentration in the liver cells is important. Our result shows that a treatment with Drynaria quercifolia significantly inhibited lipid peroxidation and significantly reduces CCl₄-exposed hepatic GSH depletion. This was attributed to the decreased bioactivation of CCl₄ caused by the Drynaria quercifolia treatment.

Table 2: Effect of Drynaria quercifolia rhizome on MDA and GSH in experimental hepatocytes

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA(nmol)</th>
<th>GSH(mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.99±0.44</td>
<td>4.21±0.08</td>
</tr>
<tr>
<td>II</td>
<td>1.44±0.44*</td>
<td>2.15±0.04*</td>
</tr>
<tr>
<td>III</td>
<td>2.59±0.31**</td>
<td>4.04±0.01**</td>
</tr>
<tr>
<td>IV</td>
<td>2.59±0.31**</td>
<td>4.17±0.02**</td>
</tr>
<tr>
<td>V</td>
<td>2.59±0.13**</td>
<td>4.25±0.05**</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD for triplicate in each group.
* Significantly different from Group I (P<0.05)
** Significantly different from Group II (P<0.05)

Table 3 shows the level of SGOT, SGPT and protein of control and experimental hepatocytes. SGOT, and SGPT activities were increased and protein levels was decreased in group II when compared to group I. Plant extract treated cells (Group III, IV, V) reversed the level of SGOT, SGPT and protein than Group II (p<0.05).

In the assessment of liver damage by carbon tetrachloride, the determination of enzyme activities such as aspartate aminotransferase (AST/SGOT) and alanine aminotransferase (ALT/SGPT) is largely used. Activities of AST and ALT are the most frequently utilized indicators of hepatocellular injury. Necrosis or membrane damage releases the enzymes into circulation; and therefore, they can be measured in hepatocytes. ALT is more specific to the
liver, and is thus a better parameter for detecting liver injury. Elevated levels of hepatocyte enzymes are indicative of cellular leakage and loss of functional integrity of the cell membrane in the liver.[25] In the present study, the CCl₄ treated hepatocytes showed a significant (p<0.05) elevation (Table- 3) in the activities of ALT and AST as compared to the normal control hepatocyte, thereby indicating oxidative damage. Co-treatment with *Drynaria quercifolia* at doses of 100, 250 and 500µg/ml, significantly prevented the rise in the levels of the marker enzymes. The diminished rise of hepatocyte enzymes in the *Drynaria quercifolia* treated groups is a clear manifestation of the hepatoprotective effect of the *Drynaria quercifolia* rhizome. Diminution of total protein by CCl₄ is a further indication of liver damage.[26] Plant extract has increased the level of protein towards the respective normal level, which indicates hepatoprotective activity. Stimulation of protein synthesis has been advanced as a contributary hepatoprotective mechanism which accelerates the regeneration process and the production of liver cells.[27, 28]

**Table 3: Effect of *Drynaria quercifolia* rhizome on SGOT, SGPT activities and protein level in experimental hepatocytes**

<table>
<thead>
<tr>
<th>Groups</th>
<th>SGOT (IU/dl)</th>
<th>SGPT (IU/dl)</th>
<th>Protein(gm\dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>38.8±7.77</td>
<td>15.5±4.38</td>
<td>7.28±0.68</td>
</tr>
<tr>
<td>II</td>
<td>88.7±15.69*</td>
<td>33.1±14.17*</td>
<td>2.92±5.16*</td>
</tr>
<tr>
<td>III</td>
<td>27.6±7.91**</td>
<td>19.6±7.95**</td>
<td>6.14±0.54**</td>
</tr>
<tr>
<td>IV</td>
<td>27.6±7.91**</td>
<td>24.8±19.3**</td>
<td>6.19±0.26**</td>
</tr>
<tr>
<td>V</td>
<td>19.6±9.02**</td>
<td>18.6±8.05**</td>
<td>7.68±0.45**</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD for triplicate in each group P<0.005

* Significantly different from Group I (P<0.05)

**Significantly different from Group II (P<0.05)

**CONCLUSION**
A number of pharmacological and chemical agents act as hepatotoxin and produce variety of liver ailments. Carbon tetrachloride intoxication in rats is an experimental model widely used to study necrotic and steatonic changes in hepatic tissue. Accordingly, cytotoxicity screening and *In vitro* hepatoprotective activity of *Drynaria quercifolia* rhizome on CCl₄ induced hepatotoxicity was investigated in freshly isolated rat hepatocytes. Cytotoxic screening indicated that the *Drynaria quercifolia* rhizome significantly reduced the toxicity against CCl₄. The biochemical parameters such as MDA, SGOT, and SGPT were elevated significantly (p< 0.05) and decrease the level of glutathione and protein were recorded.
significantly (p<0.05) on CCl₄ treatment. However, co-treatment with *Drynaria quercifolia* rhizome on CCl₄ induced toxicity in hepatocytes reduced the oxidative stress by decreased lipid peroxidation, restored the liver markers and improved the level of non-enzymatic antioxidant GSH and protein. This indicates that the lipid peroxidation and hepatotoxicity elicited by CCl₄ intoxication had been reversed due to the effect of *Drynaria quercifolia*. Thus, the potential hepatoprotective activity of *Drynaria quercifolia* may be due to the presence of various phytochemical such as flavonoids and alkaloid compounds. Further investigation is needed to confirm through *in vivo* experiment. Over all the *Drynaria quercifolia* rhizome as a source of natural hepatoprotective agent that can be important in oxidative stress mediated diseases diabetic, cancer, arthritis etc.

REFERENCES

