EVALUATION OF HEPATOPROTECTIVE AND ANTIOXIDANT ACTIVITY OF FLEMINGIA STROBILIFERA R.Br. AGAINST EXPERIMENTALLY INDUCED LIVER INJURY IN RATS

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ABSTRACT
The present study was carried out to evaluate the hepatoprotective and antioxidant effect of the Chloroform extract of Flemingia Strobilifera R.Br. leaf (CEFS) in wistar albino rats. Antioxidant was studied using 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) assay. Protective action of CEFS leaf extract was evaluated using animal model of hepatotoxicity induced by ethanol-Carbon tetrachloride (CCl4). Liver marker enzymes were assayed in serum and antioxidant status was assessed in liver tissue. Histopathology was also studied. CEFS leaf did not demonstrated in vitro scavenging of DPPH radicals. Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) and total bilirubin were increased and the levels of total protein were decreased in ethanol-CCl4 treated rats. CEFS leaf at both the doses did not decreased the elevated levels of all of these biochemical parameters and did not restore the normalcy of total protein significantly. Lipid peroxidation (LPO) was increased significant in liver tissue in the ethanol-CCl4 treated rats while the activities of reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) were decreased. CEFS leaf at both the doses did not decreased the elevated levels of lipid peroxide and did not restored the normalcy of GSH, CAT and SOD. Histopathology's also showing similar results. From this study it can be concluded that the CEFS leaf did not showed significant hepatoprotective and antioxidant action.

Keywords: CEFS leaf  Flavonoid ethanol CCl4 Hepatoprotective Antioxidant.

INTRODUCTION
Liver is the key organ for detoxication and disposition of endogenous substances. It is continuously and widely exposed to xenobiotics, hepatotoxins, and chemotherapeutic agents that lead to impairment of its functions1. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages2. Hepatotoxicity is one of very common ailment resulting into serious dehilities ranging from severe metabolic disorders to even mortality. Hepatotoxicity in most cases is due to free radical. Free radicals are fundamental to many biochemical processes and represent an essential part of aerobic life and metabolism3. Reactive oxygen species mediated oxidative damage to macromolecules such as lipids, proteins and DNA has been implicated in the pathogenesis of major diseases like cancer, rheumatoid arthritis, degeneration process of aging and cardiovascular disease etc. Antioxidants have been reported to prevent oxidative damage caused by free radicals by interfering with the oxidation process through radical scavenging and chelating metal ions4.

Lever disease is still a worldwide health problem. Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effect. In the absence of a reliable liver protective drug in modern medicine there are a number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders. In view of severe undesirable side effects of synthetic agents, there is growing focus to follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity5.

Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticanergic etc6. F. strobilifera R.Br, an important medicinal plant, is commonly known as Kusrunt found in Sind, Rajputana, Bengal, South India and Andaman’s7. Previous chemical studies showed that flavonoids, flavonoid glycosides, chalcones, epoxchromenes and pterocarpens were the main constituents found in this genus of Flemingia Strobilifera R.Br.8,9.
Phytochemical evaluation
CEFS were subjected to qualitative analysis for various phytoconstituent like alkaloids, glycosides, saponins, phytosterols, phenolic compound, tannins, proteins and amino acids.

Acute toxicity studies
The acute toxicity was determined on virgin female albino wistar rats by fixed dose method of OECD Guide line no 420 given by CPCSEA. Groups of 6 rats were administered test drug by oral route in the range of 2000-300 mg/kg and mortality was observed after 24 hr. The safe dose was found to be 300 mg/kg body weight. For the study two doses were selected, 30 mg/kg body weight and 60 mg/kg body weight (1/10th, 1/5th of the maximum safe dose).

Assessment of in-vitro antioxidant activity
DPPH assay was evaluated using the method[15]. In brief, 200μl of methanol / vehicle buffer / positive control/various concentration of CEFS leaf solution and 50μl of DPPH (0.659mM) solution were incubated at 25°C for 20minutes. Following incubation the absorbance was read at 510 nm. A control reaction was carried out without the test sample. The % inhibitions were calculated as follows.

Percentage inhibition = (1- absorbance of test/absorbance of control) × 100

Assessment of hepatoprotective activity
Female Albino wistar rats weighing 150– 200 g were maintained in animal house and they were divided in to 5 groups of 6 animals in each. The weight range of the animals was equally distributed throughout the groups. They were acclimatized to housing conditions for at least one week prior to use. The animals of group I were treated with vehicle for 21 days and animals in group II to group V were treated 40%/v/v ethanol (2ml/100g body wt. p.o) for 21 days. On 20th day they were injected with CCl4 (1:1 in groundnut oil, 0.1 ml/kg body wt. s.c.)[16]. In addition to this, the following treatments were given to the animals of group III, IV and V from day 15th to 21st day. Group III were treated with Silymarin 100 mg/kg Group IV and V were treated with CEFS leaf 30 mg/kg and 60 mg/kg respectively. The blood was obtained from all animals by puncturing retro-orbital plexus.

The blood sample was allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and utilized for the estimation of various biochemical parameters namely SGOT[12], SGPT[12], ALP[13], total bilirubin[14] and total protein[15]. After collection of blood samples the rats in different groups were sacrificed and their livers were excised immediately and washed in ice cold normal saline, followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10%/w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation[16]. A part of homogenate after precipitating proteins with Trichloroacetic acid (TCA) was used for estimation of glutathione[17]. The rest of the homogenate was centrifuged at 1500 rpm for 15 min at 40°C. The supernatant thus obtained was used for estimation of SOD[18] and CAT activities[19].

Histopathological examination
Liver pieces were preserved in 10% formaldehyde solution. The pieces of liver processed and embedded in paraffin wax. Sections of about 4–6 microns were made and stained with hematoxylin and eosin and photographed.

Analysis of results
The results were expressed as mean ± SEM and were analyzed for statistically significant difference using one-way ANOVA followed by Tukey's Kramer post hoc test. P values < 0.05 were considered significant.

RESULTS

Preliminary phytochemical investigation
The Preliminary phytochemical investigation of the CEFS leaf showed moderate presence of flavanoids.

In-vitro antioxidant activity
The CEFS leaves did not showed antioxidant activity in DPPH free radical scavenging model. Only 19.33 inhibitions were noted with 200 μg/ml of CEFS leaf. Thus IC50 value could not be determined for DPPH radical inhibition assay (Table 1)

Serum biochemical parameters
The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin were significantly (P<0.001) increased and the levels of total protein were significantly (P<0.001) decreased in ethanol-CCl4 treated rats when compared to control group (Table 2). Administering CEFS leaf (30 and 60mg /kg) did not reduced the elevated levels of AST, ALT ALP and total bilirubin levels as well as not restore the levels of total protein towards normalcy when compared to ethanol-CCl4 treated rats.

Hepatic Oxidative Stress parameters
Malondialdehyde (MDA) level was significantly (P<0.001) increased and the levels of GSH, CAT and SOD were significantly (P<0.001) decreased in ethanol-CCl4 treated rats when compared to control group. Administering CEFS leaf (30 and 60 mg/kg) did not decreased the elevated levels of malondialdehyde (MDA) content as well as not rises the antioxidant levels. (Table 3).

Histopathology
Histopathological studies also provided supportive evidence for the biochemical analysis. The CEFS treated group showed the fatty change and lymphocytic infiltrate around portal triad compared to group II (Fig. 1). Fatty change, structure of focal necrosis with portal tractsitis and congestion were observed in group treated with ethanol-CCl4. Treatment with silymarin exhibited almost normal architecture (Fig. 1).

<table>
<thead>
<tr>
<th>Table 1: DPPH free radical scavenging activity</th>
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<tr>
<td>Treatment</td>
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</tr>
<tr>
<td>Gallic acid</td>
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IC50 values were calculated by log probit analysis. *Activity was not observed in the concentration range tested, hence IC50 not calculated.
Table 2: Effects of CEFS leaf on serum Biochemical markers

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SGOT U/L</th>
<th>SGPT U/L</th>
<th>ALP IU/L</th>
<th>Total Bilirubin mg/dl</th>
<th>Total protein g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Control</td>
<td>86.28±3.261</td>
<td>37.92±1.876</td>
<td>202.71±8.572</td>
<td>0.292±0.014</td>
<td>6.94±0.323</td>
</tr>
<tr>
<td>Ethanol+CCl4 (2 ml/100gm + 0.1 ml/kg s.c.)</td>
<td>295.70±10.785*</td>
<td>182.45±6.124*</td>
<td>308.21±14.410*</td>
<td>0.714±0.035*</td>
<td>4.91±0.245*</td>
</tr>
<tr>
<td>Silymarin (100 mg/kg) CEFS</td>
<td>116.41±5.820***</td>
<td>67.99±2.993***</td>
<td>214.19±8.723***</td>
<td>0.405±0.020***</td>
<td>6.82±0.328***</td>
</tr>
<tr>
<td>CEFS (30 mg/kg)</td>
<td>272.71±14.571**</td>
<td>170.28±7.914**</td>
<td>272.62±14.281**</td>
<td>0.657±0.035**</td>
<td>5.54±0.248**</td>
</tr>
<tr>
<td>CEFS (60 mg/kg)</td>
<td>254.24±13.262**</td>
<td>160.52±8.676**</td>
<td>260.23±13.961**</td>
<td>0.615±0.031**</td>
<td>5.90±0.25**</td>
</tr>
</tbody>
</table>

All values are expressed as MEAN±SEM, one way analysis of variance (ANOVA) followed by Tukey’s Kramer post hoc analysis. *P<0.001 vs. vehicle control. **P>0.05, ***P<0.001 vs. Ethanol+CCl4

Table 3: Effects of CEFS leaf on Hepatic oxidative stress.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>MDA (nM/g)</th>
<th>GSH (µM/g)</th>
<th>CAT (µM/min/g)</th>
<th>SOD (unit/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Control</td>
<td>199.47±8.472</td>
<td>15.22±0.966</td>
<td>197.42±8.972</td>
<td>93.73±3.685</td>
</tr>
<tr>
<td>Ethanol+CCl4 (2.0ml/100gm + 0.1 ml/kg s.c.)</td>
<td>435.10±19.785*</td>
<td>7.31±0.365*</td>
<td>119.12±4.956*</td>
<td>36.70±1.735*</td>
</tr>
<tr>
<td>Silymarin (100 mg/kg) CEFS</td>
<td>233.60±10.68***</td>
<td>13.51±0.675***</td>
<td>162.07±8.843***</td>
<td>84.52±3.226***</td>
</tr>
<tr>
<td>CEFS (30 mg/kg)</td>
<td>390.21±18.56**</td>
<td>8.52±0.371**</td>
<td>135.21±5.010**</td>
<td>41.29±1.814**</td>
</tr>
<tr>
<td>CEFS (60 mg/kg)</td>
<td>372.21±17.261**</td>
<td>9.25±0.379**</td>
<td>145.72±6.386**</td>
<td>45.18±2.109**</td>
</tr>
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</table>

All values are expressed as MEAN±SEM, one way analysis of variance (ANOVA) followed by Tukey’s Kramer post hoc analysis. *P<0.001 vs. vehicle control. **P>0.05, ***P<0.001 vs. Ethanol+CCl4.
content can be deemed as a useful index of the severity of cellular of advanced chronic liver diseases. Hence decline in total protein production of liver cells. The lowered level of total proteins synthesis has been advanced as a contributory hepatoprotective dysfunction in chronic liver diseases. Stimulation of protein activation mixed function oxidase system. This eventually leads to by enhanced production of active metabolites by the ethanol activated mixed function oxidase system. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxic is, the index of its protective effects. Hepatocellular necrosis or membrane damage leads to very high levels of serum GPT and SGPT released from liver to circulation. Among the two, GPT is a better index of liver injury, since SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner, thus liver GPT represents 90% of total enzyme present in the body.

The elevated levels of serum marker enzymes are indicative of cellular leakage and loss of functional integrity of cellular membrane in liver. ALP activities on the other hand are related to functioning of hepatocytes, its increase in serum is due to increased synthesis in the presence of increased biliary pressure.

In the present study, Treatment with CEFS leaf (30 and 60mg/kg p.o.) did not suppressed the elevated serum levels of SGOT and SGPT towards the respective normal value this clearly indicates that the plant extract has not stabilizes the plasma membrane as well as not helped in healing of the hepatic tissue damage.

Serum ALP and total bilirubin levels are also related to the status and function of hepatic cells. Increase in serum ALP is due to increased synthesis, in presence of increased biliary pressure. The CEFS leaf (30 and 60mg/kg p.o.) did not able to improve the secretary mechanism of hepatic cells and did not reduced the elevated levels of ALP and total bilirubin. The site specific oxidative damage of some of the susceptible amino acids of proteins is regarded as the major cause of metabolic dysfunction during pathogenesis. Hypoalbuninaemia is most frequent in the presence of advanced chronic liver diseases. Hence decline in total protein content can be deemed as a useful index of the severity of cellular dysfunction in chronic liver diseases. Stimulation of protein synthesis has been advanced as a contributory hepatoprotective mechanism, which accelerates the regeneration process and the production of liver cells. The lowered level of total proteins recorded in the serum as well as liver of ethanol-CCl4 treated rats reveals the severity of hepatopathy. CEFS leaves has failed to restore the normalcy of total protein level.

Lipid peroxidation has been postulated to be the destructive process in liver injury due to ethanol-CCl4 administration. The increase in MDA levels suggest enhancement of lipid peroxidation which is leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with CEFS leaves did not reduced the levels of lipid peroxidation.

Glutathione (GSH) is one of the most abundant naturally occurring tripeptide, non-enzymatic biological antioxidant present in liver. Its functions are concerned with the removal of free radicals such as H2O2 and superoxide radicals, maintenance of membrane protein, detoxification of foreign chemicals and biotransformation of drugs.

In the present study, the decreased level of GSH has been associated with an enhanced level of lipid peroxidation in ethanol-CCl4 intoxicated groups of rats. Treatment with CEFS leaf has not increased the level of glutathione. Serum activities of superoxide dismutase (SOD) and catalase (CAT) are the most sensitive enzymatic index in liver injury caused by ROS and oxidative stress. SOD is one of the most abundant intracellular antioxidant enzymes present in all aerobic cells and it has an antioxic effect against ROS. CAT is a haemoprotein; it protects the cells from the accumulation of H2O2 by dismutating it to form H2O and O2. Therefore reduction in the activities of these enzymes may indicate the toxic effects of ROS produced by toxicants. In the present study, it was observed that CEFS leaves did not cause a significant rise in hepatic SOD and CAT activities. This suggests that CEFS leaf has not diminished the ROS generated that may lessen the oxidative damage to the hepatocytes and which may improve the activities of the liver antioxidant enzymes.

Preliminary phytochemical screening of the CEFS leaf revealed the moderate presence of flavonoids. In the present state of knowledge of the chemical constituents of CEFS leaf, it is not attributing for its hepatoprotective and antioxidant effect since the flavonoids is found moderately in leaves as shown by phytochemical investigations. CEFS leaf was unable to block the bioactivation of ethanol-CCl4 by cytochrome P450 enzyme and did not inhibit the lipid peroxidation and also not prevented the formation of free radical or scavenges the free radicals.

It can be concluded that the data obtained in the present study suggest that the CEFS leaf do not have significant hepatoprotective and antioxidant activities on ethanol-CCl4 induced hepatic damage in rats.

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