INTRODUCTION

Reactive oxygen species (ROS) plays a significant role in regulation of cell growth, energy production and intercellular signaling and over production of ROS results in loss of detoxification capacity of the body cell which causes extensive damage to DNA, protein, carbohydrate, and lipids [1,2].

ROS are inevitably generated due to the incomplete reduction of O2 in electron transfer reactions as byproducts of biological reactions. Antioxidants, the substances which have the capacity to eliminate the pro-oxidants and scavenge free radicals play a protective role against cell damage caused by oxidative stress. Natural antioxidants isolated from plant material have an excellent protective efficacy against such damages[3,4]. Numerous medicinal plants and their crude extracts have been reported for their hepatoprotective and antioxidant activity [5]; very insignificant number of drugs have come out from all those works till date. Silymarin, a plant-based hepatoprotective drug isolated from Silybum marianum (L.) get major attention for its curative efficacy and become both scientifically and commercially acclaimed. However, this drug is not adequate in all respect to give oxidative stress release. On the other hand, synthetic drugs cause severe side effects. It has found in several studies that plant derived antioxidants scavenge free radicals. The study of free radical scavenging activity and the chemistry of free radical is complicated. Therefore, it is necessary to search the natural plants products [6-9].

Oxidative stress plays a significant role in regulation of cell growth, energy production and intercellular signaling and over production of ROS results in loss of detoxification capacity of the body cell which causes extensive damage to DNA, protein, carbohydrate, and lipids [1,2].

RESULTS

The in vivo study revealed that the PF3 most effectively protected the liver from damage by CCl4. The findings were also confirmed by histopathological observations. High-performance liquid chromatography result was clearly indicated that the fraction was pure.

CONCLUSION

Ethyl acetate fraction (Pajanelia fraction 3) from P. longifolia may be considered as a liver protective agent.

Keywords: Liver protective activity, Biochemical parameters, High-performance liquid chromatography study, Histopathological observations, Pajanelia longifolia (Willd.) K. Schuman.

METHODS

Plant material

P. longifolia (Willd.) K. Schuman was collected from Cachar District of Southern Assam part of Northeast India. The plant was identified at the Assam University Herbarium, Silchar, and a voucher specimen (H-17) was deposited for future reference. The air-dried and powdered bark (500 g) was defatted at room temperature with n-hexane followed by extraction with ethanol (1500 ml). Qualitative phytochemical screening of the crude extracts was performed following the method [11].

Thin layer chromatography (TLC) analysis and column chromatography of crude extract

The sample was dissolved in minimum amount of solvent and absorbed on silica gel (60-120 mesh) and allowed to dry. The column was eluted with n-hexane, and then the polarity was gradually increased using ethyl acetate and acetone. Eluents were collected in 500 ml reagent bottle.
(Qualigen), and the progress of the separation was monitored by thin layer chromatography, using the solvent system hexane, ethyl acetate (9:1, 9.5:0.5, 8.5:1.5, and 4:1) and hexane, ethyl acetate, methanol (9:0.5:0.5, 8:1:1, and 7.5:2:0.5) a total of 38 fractions were collected. Fraction (10, 16 and 21) after preparative thin layer chromatography gave a single spot on TLC using different solvent system with different ratios.

Purity of fractions
The percentage of purity of fractions were checked by analytical high-performance liquid chromatography (HPLC) (Perkin Elmer, S-200, Volume: 1.000000 ul, Sample Amount: 1.0000, Channel: A, A/D mV Range: 1000, End Sampling Rate: 10.000 pts/s, Sample Time: 20.00 min, Area Reject: 0.000000, Dilution Factor: 1.00, Cycle: 1).

Methodology for bioactivity analysis
Animal
Swiss albino mice (24-26 g) of either sex were used as animal model. The animals were maintained under controlled conditions throughout the period of experimentation. They were provided with standard laboratory diet. Ethical committee approval was sorted before the experiment.

Chemicals
All chemicals were of analytical grade and purchased from Merck India Ltd., Mumbai, India. Silymarin received from Ranbaxy India Ltd. as gift sample.

CCl\textsubscript{4} induced hepatotoxicity
The hepatic injury was induced using CCl\textsubscript{4} [12] with slight modifications. On the basis of acute toxicity study, the dose concentration of 150 mg/kg body weight per orally (b.w. p.o.) (F1, F2 and F3) was selected. Animals were divided into six groups with six animals each as follows, Group I: Normal control, received liquid paraffin (1 ml/kg b.w. p.o.) for 5 days. Group II: Negative control, treated with CCl\textsubscript{4} (0.5 ml/kg b.w. p.o.) for 5 days. Group III: Positive control treated with silymarin (50 mg/kg b.w. p.o.) and then after 30 minutes, 0.5 ml/kg b.w. of CCl\textsubscript{4} intoxicated intraperitoneally for 5 days. Group IV, V and VI treated with (F1, F2 and F3 150mg/kg b.w. p.o., respectively, and then after 30 minutes, 0.5 ml/kg b.w. of CCl\textsubscript{4} intoxicated intraperitoneally for 5 days. On the 6\textsuperscript{th} day, the animals were sacrificed to assess the liver function and histological changes.

Biochemical assay
Serum of Swiss albino mice was used for biochemical analysis, such as serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) [13]. The bilirubin content [14] and the serum alkaline phosphatase (SALP) [15].

Preparation of liver homogenate
The liver homogenate was prepared as per the method [16].

Measurement of antioxidant enzymatic and nonenzymatic levels
Measurement of antioxidant enzymatic and non-enzymatic levels was done according to standard procedures. Catalase (CAT) [17], superoxide dismutase (SOD) [18], glutathione peroxidase (GPx) [17], reduced glutathione (GSH) [19], and lipid peroxidation (LPO) [20].

Histopathological observation
Liver tissues were fixed in 10% formalin and were graded with ethanol. The paraffin embedded liver tissue were cut into thin sections, stained with hematoxylin-eosin dye and observed under microscope (BX41, OLYMPUS) to note the changes in the liver tissue.

Statistical analysis
The data presented here were expressed as mean±standard error. The results were analyzed by one way ANOVA with Tukey–Kramer multiple comparisons test. The level of significance was accepted at p<0.001, p<0.01 and p<0.05.

RESULTS
Preliminary phytochemical screening
The preliminary phytochemical screening of ethyl acetate extract revealed the presence of secondary metabolites such as alkaloids, steroids, tannin, reducing sugar, and flavonoids.

Column chromatography of the extract
Column chromatography was carried out with ethyl acetate bark extract in a usual manner using solvents on the basis of their polarity and after collecting appropriate fractions the preparative TLC was done. Fractions (10, 13, 16, 19, 21, 28 and 32) were subjected to preparative TLC with different solvent systems to check the purity of fractions. Preparative TLC of fractions 10 (Pajanelia fraction-1 [PF1]), fraction 16 (PF2) and fraction 21 (PF3) with different solvent systems exhibited one spot.

Purity of fraction
From analytical HPLC study of PF3 exhibited single spot in TLC which is existing in fairly pure form (Fig. 1).

Bioactivity analysis
Biochemical assay
Mice treated with a single dose of CCl\textsubscript{4} developed significant (p<0.001, control vs. toxic group) hepatic damage as observed from elevated levels of SGOT (81.17±2.18), SGPT (94.74±1.31), SALP (134.25±2.19), and serum bilirubin (3.82±0.02). Treatment with 150 mg/kg b.w. p.o. dose of fraction PF1, PF2 and PF3 exhibited significant (p<0.001 compared to toxic group and p<0.001, p<0.01 and p<0.05 compared to control group) liver protective activity in serum enzyme and bilirubin levels of Swiss albino mice. Fraction PF3 exhibited maximum level of (significant at p<0.001 compared to toxic group) liver protective effect in SGOT (29.22±1.97), SGPT (24.72±2.89), SALP (61.79±2.11) and serum bilirubin (0.52±0.01) compared to the other two fractions (PF1 and PF2). The protective efficacy of fraction PF3 was high compared to that of protective efficacy exhibited by standard drug silymarin (Table 1). The results were also supported by histopathological observations.

Measurement of antioxidant enzymatic and nonenzymatic levels
Single dose of CCl\textsubscript{4} developed significant damage in hepatic cells of Swiss albino mice as observed from elevated levels of LPO (0.72±0.03) and decreased levels of SOD (0.52±0.01) unit/mg protein, CAT (3.82±0.02) and decreased levels of SOD (0.52±0.01) mg protein, CAT (3.82±0.02).

Table 1: Protective effect of isolated fractions on serum biochemical parameters against CCl\textsubscript{4} induced hepatotoxicity in Swiss albino mice

<table>
<thead>
<tr>
<th>Doses</th>
<th>SGOT (IU/L)</th>
<th>SGPT (IU/L)</th>
<th>SALP (IU/L)</th>
<th>Bilirubin (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>26.61±1.55</td>
<td>18.85±2.05</td>
<td>41.67±2.11</td>
<td>0.31±0.01</td>
</tr>
<tr>
<td>Group II</td>
<td>81.17±2.18</td>
<td>94.74±1.31</td>
<td>134.25±2.19</td>
<td>3.82±0.02</td>
</tr>
<tr>
<td>Group III</td>
<td>43.09±1.62</td>
<td>24.08±1.12</td>
<td>81.79±0.85</td>
<td>0.74±0.01</td>
</tr>
<tr>
<td>Group IV</td>
<td>74.18±1.69</td>
<td>61.54±1.27</td>
<td>72.17±0.77</td>
<td>1.03±0.03</td>
</tr>
<tr>
<td>Group V</td>
<td>54.72±3.01</td>
<td>37.19±1.55</td>
<td>63.16±1.10</td>
<td>1.16±1.10</td>
</tr>
<tr>
<td>Group VI</td>
<td>29.22±1.97</td>
<td>24.72±2.89</td>
<td>61.72±2.89</td>
<td>0.52±2.11</td>
</tr>
</tbody>
</table>

n=6 animal, values are given as mean±SE, values are statistically significant at p<0.001, p<0.01 and p<0.05 level of significance. Composed with control p<0.001, Compared with control p<0.01, Compared with control p<0.05, Compared with standard p<0.001, Compared with toxic p<0.001.

Group I - Control, Group II - Toxic (CCl\textsubscript{4}), Group III - Standard (Silymarin), Group IV - 150 mg/kg b.w. p.o. dose of PF1; Group V - 150 mg/kg b.w. p.o. dose of PF2; Group VI - 150 mg/kg b.w. p.o. dose of PF3. SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase, SALP: Serum alkaline phosphatase, SE: Standard error, CCl\textsubscript{4}: Carbon tetrachloride, PF: Pajanelia fraction.
(0.24±0.01 unit/mg protein), GPx (26.68±1.01 unit/mg protein), and GSH (14.34±0.31 unit/mg protein) values over those of the control group LPO (0.66±0.01 nmoles thiobarbituric acid reactive substances (TBARS)/mg protein) and SOD, CAT, GPx and GSH (1.92±0.00, 0.78±0.02, 87.36±1.12 and 64.87±1.51 unit/mg protein, respectively). Pretreatment with fraction F1, F2 and F3 at a concentration of 150 mg/kg b.w. p.o. conferred significant (p<0.001 and p<0.01 compared to control, p<0.001 compared to toxic and p<0.01 compared to standard) antioxidant activity in enzymatic and nonenzymatic levels in Swiss albino mice by lowering the elevated levels of LPO and by increasing the decreased levels of SOD, CAT, GPx, and GSH. Fraction PF3 exhibited a maximum level (p<0.001 compared to toxic) of antioxidant activity. LPO (0.96±0.02 nmoles TBARS/mg protein) and SOD, CAT GPx and GSH (0.92±0.01, 0.49±0.02, 42.84±2.70 and 47.73±1.44 unit/mg protein, respectively) and 300 mg/kg b.w. p.o. dose exhibited least level (p<0.001 compared to toxic, standard and control, respectively) of antioxidant activity. Silymarin (50 mg/kg b.w. p.o.) also significantly altered the levels of LPO (0.76±0.02 nmoles TBARS/mg protein), SOD (1.84±0.01 unit/mg protein), CAT (0.71±5±0.01 unit/mg protein), GPx (68.73±1.96 unit/mg protein), and GSH (66.76±0.87 unit/mg protein) as compared with the CC group (Table 2).
The protective efficacy of fractions was further analyzed by histopathological study where it was found that 150 mg/kg b.w. dose of PF3 exhibited a maximum level of healing of necrosis which was nearly similar to the control group (Fig. 2) where normal hepatocytes were found.

**DISCUSSION**

The hepatic cells consist of higher concentration of GOT and GPT in cytoplasm and GOT particularly exists in mitochondria. Due to the necrosis or membrane damage the hepatospecific enzymes are released in circulation and therefore, it can be measured by measuring the serum enzyme levels. High concentration of bilirubin in the serum is an indication for increased erythrocyte degeneration rate. On the other hand, ALP level in serum is related to the function of hepatic cells. Increased ALP level in serum is due to increase its synthesis in increase of bilirubin pressure. Mammalian cells contain antioxidant enzymes, including CAT, SOD, and GPx. For the maintenance of the bodies redox balance the body cells tightly maintained the levels of these enzymes and also non-enzymes such as LPO and GSH [21]. Free radicals are detoxified by antioxidant enzymes and are converted to more stable molecules. The activity of CAT enzyme results in the conversion of hydrogen peroxide to water and oxygen.

Melondialdehyde is a breakdown product of LPO, and therefore, it is a useful index of LPO. In another case, water and oxidized glutathione were produced by the combined activity of GSH and hydrogen peroxide, where GPx helps to this conjugation [22]. A significant increase in serum enzymes and bilirubin levels and a significant decrease in antioxidant enzymatic and nonenzymatic levels were recorded after 5th day of CCl4 intoxication, indicating considerable hepatocellular injury. Isolated fractions PF1, PF2 and PF3 from bark of the selected plant were exhibited significant activity in a used dose dependent manner. The fractions were subsequently recovered the serum enzymes, bilirubin and antioxidant enzymatic and nonenzymatic levels toward normal condition. PF3 from *P. longifolia* (Willd.) K. Schuman exhibited maximum level of liver protective as well as antioxidant activity at a dose of 150 mg/kg b.w. which was similar and in some cases better than standard drug Silymarin (50 mg/kg b.w.). From this study, it is evident that the fraction PF3 has drug curricular activity. Fraction PF3 has showed better protective activity compare to standard drug silymarin. Hence, fraction PF3 can be considered for further studies.

**CONCLUSION**

PF3 from ethyl acetate extract of *P. longifolia* (Willd.) K. Schuman can be considered as an effective liver protective as well as oxidative stress reducing agent as it ameliorates almost to normalcy the damage caused by CCl4 to hepatic function.

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