ABSTRACT

Objective: We investigated the liver protective activity of 2,3-6 trimethyloct-6-enal from the methanol extract of *Pajanelia longifolia* (Willd.) K. Schuman. The liver protective activity of 2,3,6 trimethyloct-6-enal was evaluated against paracetamol (2 mg/kg body weight per orally) induced liver toxicity in Swiss albino mice.

Methods: Considering the Spectral data (IR spectrum, 1H NMR spectrum and 13C NMR spectrum) the predictable structure of 2,3,6 trimethyloct-6-enal was elucidated. To study the liver protective activity of the compound, Swiss albino mice of either sex were divided into six groups and treated for 5 d. Group I and II served as normal and toxic control, Group III were treated with Silymarin as a standard drug (50 mg/kg), and Group IV to VI was treated with 2-3,6 trimethyloct-6-enal at the dose of 50 mg/kg, 150 mg/kg and 250 mg/kg b.w. p.o. respectively. The liver protective activity of the compound was measured on biochemical parameters such as aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), triglycerides (TGL), total cholesterol (TC) and protein. Further antioxidant activity of the compound was also measured on antioxidant enzymatic and non-enzymatic levels such as reduced glutathione (GSH), lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

Results: The study revealed that the compound has protective activity at the dose of 50, 150 and 250 mg/kg b.w. p.o. against paracetamol induced toxicity. In some biochemical parameters such as aspartate amino transferase and bilirubin, the compound has showed better result at a dose of 150 mg/kg compared to standard drug silymarin (value of aspartate amino transferase [compound] = 71.9±0.12, (toxic) = 173.4±2.1, (silymarin) = 79.86±0.02 and total bilirubin [compound] = 1.04±0.11, (toxic) = 2.69±0.02, (silymarin) =1.11±0.01. The findings were also confirmed by histopathological observations.

Conclusion: 2,3,6 trimethyloct-6-enal from *Pajanelia longifolia* may be considered as a potent liver protective agent.

Keywords: 2,3,6 trimethyloct-6-enal, Liver protective activity, Paracetamol, Histopathological observations, *Pajanelia longifolia* (Willd.) K. Schuman

INTRODUCTION

Herbs play a significant role as health beneficial food and as source material for drug development to treat a wide variety of clinical diseases. Liver, the largest organ of the body, plays a key role in metabolism. It’s highly specialised tissues control high-volume biochemical reactions and continue the vital functions of the body [1-2]. Because of its diverse functional roles, the liver is a sensitive organ. Acute and chronic liver diseases interfere with the liver functions and substantially cause damage of it. Paracetamol is widely used as a hepatotoxin in hepatoprotective studies causing toxicity in an animal model. Herbal medicines have attracted attention to treat liver diseases because of its less side effects and cost efficiency. Silymarin is widely used as a standard hepatoprotective drug for the study of the hepatoprotective activity of natural product [3]. Numerous medicinal plants and their crude extracts have been reported for their hepatoprotective and antioxidant activity. In spite of great progress in modern medicine, there are hardly any suitable drug which has liver protective efficacy in all respect. To withstand this problem the potential hepatoprotective and antioxidant substance is tested in *in vivo* [4-6].

*Pajanelia longifolia* (Willd.) K. Schuman, belongs to the family Bignoniaceae. The plant is widely used in Southern Assam as a folkloric medicine for curing jaundice [7]. It is a terrestrial plant, medium in size, found both as wild and less cultivated. However, from the literature survey, it was found that there are less scientific studies have been carried out regarding the hepatoprotective activity of the plant. Therefore, it was our interest to investigate the hepatoprotective as well as the antioxidant activity of the isolated compound from the stem bark of mentioned plant against paracetamol induced hepatotoxicity in albino mice.

MATERIALS AND METHODS

Plant material

*Pajanelia longifolia* (Willd.) K. Schuman was collected from Cachar district of Southern Assam part of North East 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 gm) was defatted at room temperature with n-hexane followed by extraction with methanol (1500 ml). Qualitative phytochemical screening of the crude extracts was performed following the method suggested by Siddiqui and Ali [8].

Methodology for bioactivity analysis

Animal

Swiss albino mice (24-26 g) of either sex were obtained from animal house of Assam University. The animals were housed individually in acrylic cages and maintained under controlled conditions of temperature (24±2 °C) and relative humidity (30-70%) with a 12-12 light: dark cycle throughout the period of the experiment. They were provided with standard laboratory diet *ad libitum*. The animal studies were approved Institutional Animal Ethics Committee.

Chemicals

Paracetamol (Apex Laboratories Limited). Silymarin received from Ranbaxy India Ltd. As gift sample. All other chemicals were of analytical grade and purchased from Merck India Ltd., Mumbai, India.

Paracetamol induced hepatotoxicity

The hepatic injury was created by inducing paracetamol (2 mg/kg body weight per orally) according to the literature [4] with slight...
modifications. On the basis of acute toxicity study the dose concentration of 2-3-6 trimethylol-6-enal were selected as 50 mg/kg, 150 mg/kg and 250 mg/kg b.w. p. o. Animals were divided into six groups with six animals each as follows, Group I: normal control, received liquid paraffin (1 ml/kb w. p. o.) for 5 d. Group II: negative control, received liquid paraffin (1 ml/kg b.w. p. o.) and the single dose of paracetamol (2 mg/kg) b.w. p. o. once daily for 5 d. Group III: positive control treated with Silymarin (50 mg/kg b.w. p. o.) and Group IV, VI treated with (50 mg/kg, 150 mg/kg and 250 mg/kg b.w. p. o) respectively for 5 d. Groups III-VI animals were administered simultaneously Paracetamol (2 mg/kg b.w. p. o.) with vehicles once daily for 5 d after 30 min. of administration of the doses. Animals were sacrificed 24 h after the last treatment i.e. on 6th-day blood samples were collected and the animals were sacrificed to assess the liver function and histological changes.

Biochemical assay

Measurement of biochemical parameters

Serum of Swiss albino mice was used for biochemical analysis, such as aspartate amino transferase (AST), alanine amino transferase (ALT), total cholesterol (TC) levels were assessed by kit methods as per instructions provided by the company Merck India Ltd. briefley, the tissue was homogenized in chilled 0.1M potassium phosphate solution pH 7.0, 1 mmol sodium azide, 0.2 mmol EDTA, 1 Unit/ml of GR and 1 mmol GSH. The reaction mixture was incubated at room temperature for 5 min before addition of 32 µl of NADPH, 1 Unit/ml of GR and 1 mmol GSH. The reaction mixture was incubated at room temperature for 5 min before addition of 32 µl of NADPH, 1 Unit/ml of GR and 1 mmol GSH. The reaction mixture was incubated at room temperature for 5 min before addition of 32 µl of NADPH, 1 Unit/ml of GR and 1 mmol GSH. The reaction mixture was incubated at room temperature for 5 min before addition of 32 µl of NADPH, 1 Unit/ml of GR and 1 mmol GSH. The reaction mixture was incubated at room temperature for 5 min before addition of 32 µl of NADPH, 1 Unit/ml of GR and 1 mmol GSH. The reaction mixture was incubated at room temperature for 5 min before addition of 32 µl of NADPH, 1 Unit/ml of GR and 1 mmol GSH. The reaction mixture was incubated at room temperature for 5 min before addition of 32 µl of NADPH, 1 Unit/ml of GR and 1 mmol GSH.

Preparation of liver homogenate

Liver samples from the sacrificed mice was quickly removed and Pursued with ice-cold saline. A portion of the liver was homogenized in chilled sodium phosphate buffer (0.1M, pH 7.4) using a Potter Eleven homogenizer. The homogenate obtained was centrifuged in a cooling centrifuge at 12,000xg for 30 min at 4 °C to separate the nuclear debris. The supernatant was collected and used for the subsequent assays [13].

Analysis of antioxidant activity by measuring antioxidant enzymatic and non-enzymatic levels

Catalase (CAT) assay

Catalase assay was performed following the method based on the disappearance of H2O2 at 25 °C catalase [14] 5 µl of diluted homogenate (1:40) was mixed with 720 µl of 30 mmol H2O2 in 10 mmol potassium phosphate solution and the reaction was followed at 240 nm. Decomposition of H2O2 by CAT contained follows a first order kinetics as K=2.3/1 log A0/A where K is the first order rate constant, t is the time for which the decrease of H2O2 due to CAT activity was measured for 15s and A0/A is the optical density at time 0 and at 15s respectively.

Superoxide dismutase (SOD) activity

The reaction mixture consisted of 1.5 ml phospho buffer (0.1 M, pH 7.4) 0.1 ml NBT (2.25 mmol, 0.1 ml tissue homogenate, 0.1 ml sodium carbonate (1.5 M), 0.2 ml methionine (200 mm), 0.1 ml EDTA (3 mmol) 1 ml distilled water and 0.1 riboavin (60 mmol)) in the total volume of 3 ml. It was incubated in light for 60 min at room temperature. The rate of reaction was measured by recording changes in absorbance at 560 nm due to the formation of formazone, a reaction product of NBT [15].

Glutathione peroxidase (GPx) assay

100 µl of the tissue homogenate diluted in 1:100 was added to 800 µl of a reaction mixture containing 50 mmol potassium phosphate solution pH 7.0, 1 mmol EDTA, 1 mmol sodium azide, 0.2 mmol NADPH, 1 Unit/ml of GR and 1 mmol GSH. The reaction mixture was incubated at room temperature for 5 min before addition of 32 µl of 2.5 mmol H2O2solution for the initiation of the reaction and finally, absorbance at 340 nm was recorded for 3 min and the activity was calculated focussing on millimolar absorption coefficient for NADPH being 6.22. In the case of blank reactions homogenates were replaced by distilled water which was subtracted from each assay [14].

Reduced glutathione (GSH) content

0.2 ml of tissue homogenate was mixed with 1.8 ml of EDTA solution. To this 3.0 ml of precipitating reagent (1.67 g of mg, phosphoric acid, 0.2g of EDTA disodium salt, 30g sodium chloride in 1 L of distilled water) was added mixed thoroughly and kept for 5 min before centrifugation. 2.0 ml of the filtrate, 4.0 ml of 0.3 M disodium hydrogen phosphate solution and 1.0 ml of DTNB (5,5-dithiobis-2-nitrobenzoic acid) reagent were added and absorbance was read at 412 nm [16].

Lipid peroxidation (LPO) assay

Determination of Hepatic TBARS which signifies the rate of lipid peroxidation was carried out by following the standard method [17]. Briefly, the tissue was homogenized in chilled 0.1M potassium chloride solution. The assay mixture contained 0.5 ml of liver homogenate, 3 ml of 1% HClO4 and 1 ml of 0.6% TBA. The mixture was heated at 100 °C for 45 min, the reaction mixture was then allowed to cool at room temperature and 3 ml of n-butanol was added to it and shaken vigorously so as to separate the butanolic phase, it was then subjected to centrifugation at 4000xg for 10 min. and absorbance was determined at 535 nm.

Histopathological observation

Liver tissues were fixed in 10% formalin and were graded with ethanol. The paraffin embedded liver tissue was cut into thin sections, stained with Haematoxylin-Eosin dye and observed under a microscope (BX41, OLYMPUS) to note the changes in the liver tissue [18].

Statistical analysis

The data presented here were expressed as means±SE. The results were analysed 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 acetone extract revealed the presence of secondary metabolites like-alkaloids, steroids, tannin, reducing sugar and flavonoids.

The probable structure of the compound

Considering spectral data the structure elucidation of the compound has reported in our previous study Datta and Choudhury (19).

Bioactivity of 2-3-6 trimethylol-6-enal

Measurement of biochemical parameters

Hepatotoxicity of mice with paracetamol induction significantly (P<0.001) altered the biochemical parameters when compared with control mice. Treatment with 2-3-6 trimethylol-6-enal at 50 mg/kg, 150 mg/kg and 250 mg/kg showed significant protective effect by decreasing the paracetamol induced elevated levels of serum biochemical parameters (P<0.001,P<0.01,P<0.05) and by increasing the decreased levels of Protein and triglycerides (P<0.001,P<0.01 and P<0.05). The protective efficacy showed by the compound was similar to that of the Standard drug, Silymarin. In some biochemical parameters such as aspartate amino transferase (AST) and bilirubin (TB), it was found that the compound at a dose of 150 mg/kg has showed protective efficacy which is better than the protective efficacy showed by standard drug silymarin (value of aspartate

Fig. 1: Structure of 2-3-6 trimethylol-6-enal

Bioactivity of 2-3-6 trimethylol-6-enal

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amino transferase (compound) =71.10±0.12, (toxic) = 173.43±1.21, (silymarin) =79.86±0.02 and total bilirubin (compound) = 1.04±0.11), (toxic) = 2.69±0.02, (silymarin) = 1.11±0.01). The findings were also confirmed by histo-pathological observations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
<th>ALP (U/l)</th>
<th>TB (mg/dl)</th>
<th>TGL (mg/dl)</th>
<th>TC (g/dl)</th>
<th>Protein (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>59.19±0.02</td>
<td>52.36±0.21</td>
<td>126.17±1.11</td>
<td>0.53±0.09</td>
<td>91.32±1.01</td>
<td>65.03±0.12</td>
<td>68.32±0.12</td>
</tr>
<tr>
<td>Group II</td>
<td>173.43±1.21</td>
<td>106.03±1.11</td>
<td>24.75±1.22</td>
<td>2.69±0.02</td>
<td>38.26±1.34</td>
<td>194.97±1.72</td>
<td>1.63±1.12</td>
</tr>
<tr>
<td>Group III</td>
<td>70.86±0.02</td>
<td>67.73±1.24</td>
<td>170.39±0.35</td>
<td>1.11±0.01</td>
<td>54.15±1.09</td>
<td>92.32±0.12</td>
<td>51.84±1.19</td>
</tr>
<tr>
<td>Group IV</td>
<td>79.18±0.01</td>
<td>72.17±1.42</td>
<td>221.10±1.04</td>
<td>1.37±0.07</td>
<td>62.47±0.32</td>
<td>92.81±1.23</td>
<td>51.79±1.12</td>
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<tr>
<td>Group V</td>
<td>71.10±0.12</td>
<td>79.21±1.05</td>
<td>202.19±1.20</td>
<td>1.04±0.11</td>
<td>52.56±1.32</td>
<td>80.95±1.42</td>
<td>52.72±1.32</td>
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<tr>
<td>Group VI</td>
<td>101.46±1.12</td>
<td>85.46±1.02</td>
<td>197.22±1.49</td>
<td>0.90±0.12</td>
<td>52.66±1.32</td>
<td>81.66±0.45</td>
<td>58.07±1.17</td>
</tr>
</tbody>
</table>

Measurement of antioxidant enzymatic and non-enzymatic levels

Mice treated with a single dose of Paracetamol (2 mg/kg body weight per orally) developed significant hepatic damage as observed from elevated levels of LPO (4.02±0.01) and decreased levels of SOD (0.29±0.02), CAT (0.11±0.01), GPx (19.88±1.01), and GSH (13.84±0.51) in hepatic cells. Pretreatment with 2, 3,6 trimethyloct-6-enal at dose concentration of 50 mg/kg, 150 mg/kg and 250 mg/kg b.w. p. o. conferred significant (P<0.001 and P<0.01) protection in antioxidant enzymatic and non-enzymatic levels of Swiss albino mice by lowering the elevated levels of LPO and by increasing the decreased levels of SOD, CAT, GPx, and GSH. 150 mg/kg b.w. p. o. dose of 2,3,6 trimethyloct-6-enal exhibited a maximum level (P<0.001 compared to toxic) of antioxidant activity [LPO (0.92±0.01) and decreased levels of SOD (0.79±0.01), CAT (0.42±0.01), GPx (46.38±0.90), and GSH (37.60±1.32)] (fig. 2). The protective efficacy of 150 mg/kg dose of 2,3,6 trimethyloct-6-enal was similar to that of protective efficacy exhibited by standard drug Silymarin (50 mg/kg b.w. p. o.). The result was also supported by histopathological observations (fig. 3).

Fig. 2: Antioxidant efficacy offered by 2,3,6-trimethyloct-6-enal at different dose concentration manner on tissue enzymatic and non-enzymatic levels of Swiss albino mice against Paracetamol induced hepatic damage. (A) Changes in LPO level, (B) changes in GSH level, (C) changes in CAT level, (D) changes in SOD level, (E) changes in GPx level.

Histopathological studies of the liver in paracetamol induced hepatotoxicity

Group I: Section studied shows normal hepatocytes with normal parenchyma and sinusoids.

Group II: Section studied shows liver parenchyma with effaced hepatocytes with macrosteatosis, and also some degenerative changes.
Group III and V: Section studied shows liver parenchyma with the healing of necrosis. (table 2.)

Group IV and VI: Section studied shows liver parenchyma with partially healing of necrosis. (fig. 3)

Table 2: Histopathological changes in paracetamol induced liver injury in mice

<table>
<thead>
<tr>
<th>Microscopic Observation</th>
<th>Control</th>
<th>Paracetamol treated</th>
<th>Compound 50 mg/kg</th>
<th>Compound 150 mg/kg</th>
<th>Compound 250 mg/kg</th>
<th>Silymarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear disintegration</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytoplasmic vacuolation</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Necrobiosis</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Necrosis</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kupffer cell</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>Hypoplasia</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sinusoidal dilatation</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Central venous dilatation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 3: Histopathological studies of sections of mice liver on 6th day after treatment. (a) Control, (b) paracetamol (2 mg/kg b.w. p.o.), (c) Silymarin (50 mg/kg b.w. p.o.), (d) 50 mg/kg b.w. p.o. dose of 2-3-6 trimethyloct-6-enal, (e) 150 mg/kg b.w. p.o. dose of 2-3-6 trimethyloct-6-enal. In (b) arrow mark is the necrosis and in (c), (d), (e), (f) arrow mark is the healing of necrosis

DISCUSSION

Liver damage occurred mainly cellular necrosis which associated with an increase in tissue LPO and depletion in the tissue GSH levels. In addition, hepatocellular injury can also be measured by measuring the serum enzyme levels as because the hepatospecific enzymes are released in circulation and therefore it can be measured by measuring the serum enzyme levels. The hepatic cells consist of a higher concentration of AST and ALT in cytoplasm and AST particularly exists in mitochondria. 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 of ALP level in serum is due to increased synthesis in presence of increasing biliary pressure. A significant alteration in serum enzymes, bilirubin and tissue enzymatic, non-enzymatic levels were recorded after 6th day of paracetamol intoxication, indicating considerable hepatocellular injury.

N-acetyl p-benzoquinine amine (NAPQI) is a toxic electrophile which is activated by paracetamol induction and covalently interact with membrane molecule by losing one electron resulting membrane damage. This results into the leakage of biochemical markers into the serum [4]. Compound 2-3-6 trimethyloct-6-enal from the bark of the selected plant were exhibited significant activity in dose dependent manner. The dose was selected by acute toxicity study. In a previous study, it was found that the compound 2-3-6 trimethyloct-6-enal has some oxidative stress reducing activity against CCl₄ induced hepatotoxicity in mice. In this study, the compound exhibited subsequent recovery in the levels of tissue enzymatic, non-enzymatic and serum biochemical parameters towards normacy against paracetamol induced toxicity in mice. In histopathological observations, it was found that the compound exhibited similar and in some parameters better result than the protective activity exhibited by standard drug Silymarin.

CONCLUSION

It can be concluded that the compound 2-3-6 trimethyloct-6-enal from the methanol extract of *Pajanelia longifolia* (Willd.) K. Schuman has protective effects against paracetamol induced liver damage in mice as it ameliorates almost to normacy the damage caused by paracetamol. Hence, this study suggests that the compound 2-3-6 trimethyloct-6-enal at a dose of (i.e. 150 mg/kg) should be considered as an effective liver protective agent.

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CONFLICT OF INTERESTS

Declared none
REFERENCES


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