POSSIBLE MECHANISM OF PLUMBAGO ZEYLANICA IN PREVENTION OF HEPATIC DAMMAGE IN WISTAR RAT

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ABSTRACT

Plumbago zeylanica root has been used traditionally to treat various body ailments including liver diseases. The present study was designed to investigate the hepatoprotective effects of standardized methanolic extract of Plumbago zeylanica root on paracetamol, CCl₄ and alcohol induced hepatic injuries in rats and its possible mechanism(s) of hepatoprotection. Administration of hepatotoxicants like paracetamol (3 g kg⁻¹, p.o.) or CCl₄ (1 mL kg⁻¹, s.c.) or alcohol (15 g kg⁻¹, p.o.) to Wistar albino rats (180-240 g of either sex, n = 8) caused significant increase in serum ALT, AST, ALP and bilirubin; tissue lipid peroxidation, nitrite/nitrate, collagen; and decrease in tissue GSH and SOD levels. The liver histopathology revealed central vein dilation, infiltration and fatty degeneration in hepatotoxicant control groups. Pretreatment with methanolic extract of Plumbago zeylanica (100, 200 and 400 mg kg⁻¹) and silymarin (50 mg kg⁻¹) as standard significantly reversed these toxic changes dose dependently, as compared to hepatotoxicant control. The results in present study suggested that Plumbago zeylanica root possesses marked hepatoprotective potential through its oxidative, inflammatory and fibrotic effects against experimentally induced liver toxicity. These findings support and extend the rational basis for the use of this plant as hepatoprotective in traditional and folk-lore medicine.

Keywords: Hepatoprotective, Plumbago Zeylanica, Anti-Oxidative, Anti-Inflammatory, Anti-Fibrotic

1. INTRODUCTION

Liver is the vital organ involved in major metabolic and secretory functions in the body and appears to be a sensitive target for exogenous and endogenous challenges, resulting liver damage (Zimmerman, 1976). The occurrence of hepatic damage is generally due to indiscriminate consumption of NSAIDS, alcoholics, antitubercular, anticancer and anticonvulsant drugs. It results clinically as jaundice, cirrhosis, hepatitis, steatosis, fibrosis, liver cancer and ultimately liver failure (Sherlock and Dooley, 2002). In spite of tremendous strides in modern medicine, there are hardly any drugs that stimulate liver function, offer protection to the liver from damage and help regeneration of hepatic cells. There is however, a number of plant drugs employed in traditional system of medicine for liver affections but the management of liver disorders by a simple and precise herbal drug is still an unmet medical need.
The literature revealed that there is an extended use of roots of PZ in traditional system of medicine to treat liver disorders, but very less scientifically validated reports are available. Although, an earlier report suggested the beneficial effect of roots of PZ in liver diseases (Kanchana and Sadiq, 2011) but the study was done with single dose using only one experimental model. Keeping this in view, the present study was designed to investigate the pharmacological potential of PZ root against paracetamol, CCl4 and alcohol induced liver toxicity in rats at various doses and to elucidate its possible mechanism of hepatoprotection.

2. MATERIALS AND METHODS

2.1. Collection, Extraction and Phytochemical Screening of Plant Material-PZ

Root was collected from a Khambadia village near Jamnagar, Gujarat, India. Plant parts were authenticated by Prof. A.K. Rawat, National Botanical Research Institute (NBRI), Lucknow, India and the voucher specimen was kept in institutional herbarium (NBRI-SOP-202). The plant material was dried under shade (≤40°C), pulverized and coarsely powdered. Extraction was done with 95% methanol using a soxhlet extractor. The methanolic extract was dried using rota-evap apparatus (yield 13.9% w/w). The qualitative phytochemical screenings of plant extract was also carried out to investigate the presence of class of constituents e.g., alkaloids, flavonoids, glycosides, tannins, phenols and steroids (Cannell, 1998).

2.2. High Performance Liquid Chromatography (HPLC) Study

The methanolic extract of PZ was standardized by the presence of plumbagin through HPLC. The isocratic elution was carried out by using Waters Spherisorb RP-18 (4.6 mm ID×250 mm; 5 mm) with mobile phase methanol: water (70:30) at flow rate: 1 mL/min. Photodiode array detector was used for data acquisition and the calculation was performed at 265 nm to quantify the plumbagin content using PZ extract and plumbagin standard.

2.3. Animals

Wistar albino rats of either sex (180-240 g) were procured from Animal house, ISF College of Pharmacy, Moga and Punjab. Animals were maintained under controlled conditions of light/dark cycle (12/12 h), temperature (25±2°C) and humidity (45-50%) and provided with food pellets (Ashirwad Industries, Kharar, Punjab, India) and water ad libitum. The animals were acclimatized for at least 5 days to the laboratory conditions before doing experiments. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) and was performed under Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines.

2.4. Drugs and Chemicals

Paracetamol (Glaxo SmithKline Pharma, Bangalore), silymarin (Micro Labs Limited, Baddi), carbon tetrachloride (Merck Specialities Pvt. Ltd., Mumbai), alcohol (Rankem, Mumbai) and plumbagin (Sigma-aldrich, USA) were purchased. All other chemicals and biochemical reagents of analytical grade were used.

2.5. Acute Toxicity Study

Acute oral toxicity study was performed as per the OECD 423 guidelines (Acute toxic class method) (Botham, 2004). Wistar albino rats (n = 3) were selected by random sampling technique and fasted for 4 h with free access to water. The PZ: Methanolic extract (suspended in 1% CMC) was administered orally at dose of 5 mg kg−1 and observed for 14 days. If mortality was not observed, the procedure was repeated with doses 50, 300 and 2000 mg kg−1. The animals were observed for behavioral or toxic symptoms.

2.6. Paracetamol (PCM) Induced Hepatotoxicity

In this model, rats were divided into different groups, each comprising eight animals (n = 8): Saline Control (SC), PCM control, PZ Extract: 100 (PZE100), 200 (PZE200) and 400 (PZE400) mg kg−1 and silymarin 50 mg kg−1 (SILY50). The plant extracts and silymarin were given orally for 7 days. Paracetamol 3 g kg−1, p.o. was given on third and fifth days to all groups except saline control (Gutierrez and Solis, 2009).

2.7. Carbon Tetra Chloride (CCl4) Induced Hepatotoxicity

CCl4 was administered at the dose of 1 ml kg−1 (diluted in olive oil 1:1) to rats subcutaneously on fourth and fifth days to induce acute hepatotoxic damage served as CCl4 control. The schedule for administration of three doses of PZ extract and silymarin to different groups was...
similar as mentioned in PCM induced hepatotoxicity model, following CCl₄ intoxication (n = 8) (Porchezhian et al., 1981).

2.8. Alcohol Induced Hepatotoxicity

Ethanol (28%) at the dose 15 g kg⁻¹ was given to rats daily for 4 weeks to induce chronic hepatic injury served as alcohol control. PZ extract 100, 200 and 400 mg kg⁻¹ and silymarin 50 mg kg⁻¹, p.o. were given to different groups for 4 weeks, following alcohol intoxication (n = 8) (Tsukamoto, 1998).

After completion of each experimental protocol, blood samples were collected and serum separated for biochemical estimations. The animals were sacrificed, liver dissected out, washed in cold saline, blotted dry and weighed. The 10% liver homogenate was prepared in 0.15 M Tris HCl buffer or 0.01 M phosphate buffer (for glutathione only) for tissue biochemical estimations.

2.9. Serum Biochemical Estimations-Serum Biochemical Estimations

Alanine Transaminase (ALT), Aspartate Transaminase (AST), Alkaline Phosphatase (ALP) and bilirubin were estimated spectrophotometrically using UV-1700 Pharmaspec Shimatzu spectrophotometer and enzymatic biochemical kits.

2.10. Tissue Biochemical Estimations

Liver tissue biochemicals like lipid peroxidation in the form of Thiobarbituric Acid Reactive Substances (TBARS) by Wills (1966), Reduced Glutathione (GSH) by Ellman (1959), Superoxide Dismutase activity (SOD) by Kakkar et al. (1984), nitrite/nitrate level by Green et al. (1982) and collagen content by estimating hydroxyproline level by modified method of Jamall et al. (1981) were assessed.

2.11. Histopathological Study

The excised livers were fixed in formalin (10%), embedded in paraffin block, serially sectioned (5 μm size) and microscopically examined after staining with hematoxylin and eosin.

2.12. Statistical Analysis

The results expressed as mean ± Standard Deviation (SD) and were analyzed using one way ANOVA followed by Bonferroni’s multiple comparison tests as post-hoc analysis. p<0.05 was considered to be statistically significant.

3. RESULTS

In acute toxicity study, the methanolic extract of P. zeylanica caused no significant toxic symptom and mortality even at highest dose 2000 mg kg⁻¹ employed. Moreover, three doses: 100, 200 and 400 mg kg⁻¹ of the plant extract were selected for further pharmacological studies.

In HPLC study, The PZ extract was standardized by estimating plumbagin content and was found to be 0.367 % w/w (Fig. 1 and 2). Moreover, the phytochemical screening of the PZ extract showed the presence of chemical constituents like flavonoids, phenolics, steroids and glycosides.

Hepatotoxication with PCM, CCl₄ and alcohol caused significant (p<0.05) increase in serum transaminases: ALT, AST, ALP and bilirubin levels in comparison to saline control rats. Pretreatment with PZE100, PZE200, PZE400 and silymarin 50 mg kg⁻¹ significantly (p<0.05) attenuated the increase in these serum markers in a dose dependent manner. The effects of PZE400 (except in bilirubin level and silymarin in attenuating the each hepatotoxicant induced increase in serum markers, were not significantly (p>0.05) different from that of saline treated control rats (p>0.05). The decrease in these serum markers, produced by the highest dose (400 mg kg⁻¹) of PZE was not significantly (p>0.05) different from that produced by silymarin against PCM (for AST and ALP levels), CCl₄ (for ALT and ALP levels) and alcohol-induced hepatotoxicities in rat (Table 1).

All the hepatotoxicant control groups showed significant (p<0.05) increase in lipid peroxidation products (TBARS) as compared to SC. Pretreatment with PZE100, 200 and 400 and SILY50 produced significant (p<0.05) decrease in TBARS level in a dose-dependent manner as compared to hepatotoxictants. The effect of SILY50 was not significantly (p>0.05) different from that of saline treated control rats. PZE400 did not caused significant decrease (p>0.05) in TBARS level as compared to SILY50 against CCl₄ induced hepatotoxicity (Table 2).

PCM or CCl₄ or alcohol intoxications caused significant (p<0.05) decrease in tissue GSH and SOD levels as compared to SC. Pretreatment with PZE100, 200 and 400 and SILY50 significantly (p<0.05) restored the levels of GSH and SOD in a dose-dependent manner. The increase in GSH level on treatment with PZE400 and SILY50 against alcohol intoxication, produced was not significantly (p>0.05) different from that of SC (Table 2).

Hepatointoxications caused marked elevation (p<0.05) in tissue nitrite/nitrate level as compared to SC. On treatment with extract of PZE100, 200 and 400 mg kg⁻¹, significant (p<0.05) decrease in nitrite/nitrate levels were observed.
SILY50 and PZE400 showed prominent decrease up to the normal level against PCM and CCl₄ induced hepatic damage. The decrease in nitrite/nitrate level with PZE400 against PCM and CCl₄ and with SILY50 against all the hepatotoxicants, were not significantly (p>0.05) different from that of SC (Table 2).

In present study, PCM or CCl₄ or alcohol intoxication caused significant (p<0.05) increase in tissue collagen content in hepatotoxicant control groups in comparison to SC. Pretreatment with PZE100, 200 and 400 significantly decreased (p<0.05) the elevated collagen level in a dose dependent manner as compared to hepatotoxicant control group. The decrease in hydroxyproline level on treatment with PZE400 against PCM and CCl₄ and with SILY50 against all the hepatotoxicants were not significantly (p>0.05) different from that of SC (Table 2).

In histological study, liver sections from saline control group showed normal lobular architecture and hepatic cells (A).
Fig. 3. Effect of *P. zeylanica* extract (100, 200 and 400 mg kg\(^{-1}\), p.o.) on histopathological characteristics: A: Saline Control (SC); B: PCM control; C: PZE100+PCM; D: PZE200+PCM; E: PZE400+PCM; F: SILY50+PCM; G: CCl\(_4\) control; H: PZE100+CCl\(_4\); I: PZE200+CCl\(_4\); SILY50+CCl\(_4\); L: Alcohol control; M: PZE100+Alc.; N: PZE200+Alc.; O: PZE400+Alc.; and P: SILY50+Alc; (10x). (SC: Saline control, PZE 100, 200 and 400: *Plumbago zeylanica* 100, 200 and 400 mg kg\(^{-1}\), SILY50: silymarin 50 mg kg\(^{-1}\))

Table 1. Effect of *P. zeylanica* extract (100, 200 and 400 mg kg\(^{-1}\)) on serum biochemical estimations

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Group</th>
<th>ALT</th>
<th>AST</th>
<th>ALP</th>
<th>Bilirubin</th>
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<tbody>
<tr>
<td>1</td>
<td>SC</td>
<td>51.02 ± 9.19</td>
<td>67.14 ± 8.82</td>
<td>161.38 ± 26.76</td>
<td>0.26 ± 0.06</td>
</tr>
<tr>
<td>2</td>
<td>PCM control</td>
<td>112.91±13.68*</td>
<td>127.45±24.39*</td>
<td>239.89±67.94*</td>
<td>0.742±0.12*</td>
</tr>
<tr>
<td>3</td>
<td>PZE100 + PCM</td>
<td>102.51±4.94</td>
<td>92.53±13.16a</td>
<td>173.28±31.79a</td>
<td>0.586±0.061a</td>
</tr>
<tr>
<td>4</td>
<td>PZE200 + PCM</td>
<td>80.51±15.20a</td>
<td>86.91±17.85a</td>
<td>157.4±30.68a</td>
<td>0.586±0.114a</td>
</tr>
<tr>
<td>5</td>
<td>PZE400 + PCM</td>
<td>67.87±14.07ad</td>
<td>78.88±10.06de</td>
<td>119.9±25.93ad</td>
<td>0.458±0.134a</td>
</tr>
<tr>
<td>6</td>
<td>SILY50 + PCM</td>
<td>52.89±21.11d</td>
<td>78.31±7.7b</td>
<td>181.49±29.91d</td>
<td>0.032±0.15d</td>
</tr>
<tr>
<td>7</td>
<td>CCl(_4) control</td>
<td>146.28±40.99*</td>
<td>185.96±53.93*</td>
<td>288.58±26.76*</td>
<td>0.758±0.136*</td>
</tr>
<tr>
<td>8</td>
<td>PZE100 + CCl(_4)</td>
<td>106.42±12.8b</td>
<td>129.65±24.04b</td>
<td>185.06±31.29b</td>
<td>0.579±0.079b</td>
</tr>
<tr>
<td>9</td>
<td>PZE200 + CCl(_4)</td>
<td>117.55±47.24b</td>
<td>155.5±55.8b</td>
<td>161.6±21.98b</td>
<td>0.591±0.094b</td>
</tr>
<tr>
<td>10</td>
<td>PZE400 + CCl(_4)</td>
<td>91.83±24.58bde</td>
<td>118.26±30.76bde</td>
<td>126.2±28.083bde</td>
<td>0.498±0.114bde</td>
</tr>
<tr>
<td>11</td>
<td>SILY50 + CCl(_4)</td>
<td>64.28±12.29bd</td>
<td>70.76±14.8bd</td>
<td>102.68±32.26bd</td>
<td>0.456±0.104bd</td>
</tr>
</tbody>
</table>

Results: mean ± SD; * = p<0.05 Vs SC; a = p<0.05 Vs PCM control; b = p<0.05 Vs CCl\(_4\) control; c = p<0.05 Vs Alc. control; d = p>0.05 Vs SC; e = p>0.05 Vs PCM control; e’ = p>0.05 Vs CCl\(_4\) control; f = p>0.05 Vs Bilirubin control; f’ = p>0.05 Vs PCM hepatotoxicity; f” = p>0.05 Vs CCl\(_4\) hepatotoxicity; g = p<0.05 Vs SILY50 (against PCM hepatotoxicity); h = p<0.05 Vs SILY50 (against CCl\(_4\) hepatotoxicity); i = p<0.05 Vs SILY50 (against alcohol hepatotoxicity). (SC: Saline control, PZE 100, 200 and 400: *Plumbago zeylanica* 100, 200 and 400 mg kg\(^{-1}\), SILY50: silymarin 50 mg kg\(^{-1}\)).
The present study provides the evidences for the pharmacological potential of PZ roots against three experimental models, paracetamol, CCl4 and alcohol-induced hepatocellular damage in Wistar rat.

PCP or CCl4 or alcohol induced liver injuries are the models for experimental liver damage. PCP overdosage (Porchezian and Ansari, 2005), CCl4 intoxication (Gutierrez and Solis, 2009) and chronic alcohol administration (Tsukamoto, 1998) are well reported experimental models for the evaluation of hepatoprotective agents. PCP is converted to its toxic reactive electrophile: N-Acetyl-P-Benzoquinone-Imin (NAPQI) (Jollow et al., 1974). CCl4 is reduced to a highly reactive trichloromethyl free radical catalyzed by cytochrome P450 2E1 (Wong et al., 1997). The acetaldehyde and acetate, byproducts formed due to chronic ethanol intake, are primarily responsible for free radicals production and oxidative damage in hepatocytes (Hoek and Pastorino, 2002). The reactive metabolites and free radicals are capable of binding to proteins and lipids and thereby initiating lipid peroxidation, rupture the membrane, impairment of cell functions, cell shrinkage, cell necrosis and finally liver damage (Vermeulen et al., 1992). The extent of hepatic cell damage raises the serum levels of cytoplasmic hepatic enzymes: ALT, AST, ALP and bilirubin which have been used as indices of hepatocellular injury (Zimmerman, 1976).

The findings from present study confirmed the experimental hepatocellular damage due to exposure of hepatotoxicants: PCP, CCl4 and alcohol in wistar rats (Saraswat et al., 1999; Yanpallewar et al., 2002). However, pretreatment with PZ extract produced significant attenuation in toxic effects of experimental hepatic damage in a dose dependent manner and this

<table>
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<th>Table 2. Effect of P. zeylanica extract (100, 200 and 400 mg kg⁻¹) on tissue biochemical markers</th>
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<tr>
<td><strong>S. No.</strong></td>
</tr>
<tr>
<td>1</td>
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<tr>
<td><strong>PCP induced hepatotoxicity</strong></td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
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<tr>
<td>4</td>
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<td>5</td>
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<td>6</td>
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<tr>
<td><strong>CCl4 induced hepatotoxicity</strong></td>
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<td>7</td>
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<td>8</td>
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<td>9</td>
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<tr>
<td>10</td>
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<tr>
<td>11</td>
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<tr>
<td><strong>Alcohol induced hepatotoxicity</strong></td>
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<tr>
<td>12</td>
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<td>13</td>
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<td>14</td>
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<td>16</td>
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</table>

All the results are expressed as mean ± SD; a =p<0.05 Vs SC; b =p<0.05 Vs PCP control; c =p<0.05 Vs CCl4 control; d =p<0.05 Vs SILY50 (against PCP hepatotoxicity); e =p<0.05 Vs SILY50 (against CCl4 hepatotoxicity); f =p<0.05 Vs SILY50 (against alcohol hepatotoxicity). (SC: Saline Control, PZE 100, 200 and 400: Plumbago zeylanica 100, 200 and 400 mg kg⁻¹; SILY50: Silymarin 50)
effect may be due to its ability to act as a radical scavenger and thereby preventing cell injury. Moreover, the highest dose of PZ (400 mg kg\(^{-1}\)) was able to reduce markers of hepatic damage up to the normal level and was as effective a hepatoprotectant as silymarin (containing highly purified flavonoids).

PZ is reported to contain plumbagin as a chief constituent and which is having anti-oxidant ability (Liu et al., 2006; Tilak et al., 2004). The findings of HPLC study provide the evidences for the presence of plumbagin which standardized the methanolic extract of PZ.

The toxic reactive metabolites, free radicals and peroxidative products cause direct cell widespread damage of macromolecules leading to increased lipid peroxidation, damage to mitochondrial DNA (Hoek et al., 2002) and diminish or impair the antioxidant homeostasis (Hampel et al., 2004). Increased Nitric Oxide (NO) expression in hepatocytes, hepatic macrophages and kupffer cells after activation with various toxic stimuli (Guler et al., 2004), initiates the cascades of free radical mediated necrotic and apoptotic reactions (Jaeschke et al., 2002). The toxic effects of oxidative stimuli result increased cell damage characterized by decreased GSH and SOD levels (Genet et al., 2000). Glutathione and superoxide dismutase (Husain and Somani, 1997) play the important role in detoxification of toxic reactive metabolites (Pigeolet et al., 1990). It can be assumed that PZ attenuated hepatic damage in the present study by preventing lipid peroxidation reactions and restoring tissue antioxidants level. This contention is supported by the observation that PZ extract dose dependently reduced TBARS, NO and restored tissue GSH and SOD levels.

The production of collagen from hepatic stellate cells with induction of Tissue Growth Factor (TGF)-\(\beta\) represents a critical step in the progression of hepatic fibrosis (Bissell, 2003). In present study, an increase in collagen content has been noted upon exposure with hepatotoxins. Pretreatment with PZ extract dose dependently attenuated the progressive recruitment of collagenase in liver and thereby ameliorated the anti-fibrotic cellular effects. The protective effect of PZ extract was further confirmed by histopathological examination of the livers showing significant protection against experimentally induced liver tissue damage.

5. CONCLUSION

The findings of present investigations reported, for the first time, the evidences for the effectiveness and possible mechanism of PZ: Root in a dose-dependent manner against experimentally induced liver toxicity in rat. In this way, the present study added the knowledge about the use of roots of PZ in prevention of hepatic diseases and provided the pharmacological rationale for its extended folklore use in treatment of liver disorders.

6. ACKNOWLEDGMENT

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7. REFERENCES


