Evaluation of Hepatoprotective Activity of the Ethanol Extract of *Limonia acidissima* against Paracetamol induced Hepatotoxicity in Experimental Rats

S. Kalpana*, S. M. Tasleem, P. G. Maheshwari, S. Nazeer, S. Rubeena, Y. Siva Kumar, S. Anu Malik

*Associate professor, Department of Pharmacology, P. Rami Reddy Memorial College of Pharmacy, Kadapa, Andhra Pradesh, India.

*Corresponding author’s E-mail: sana.kalpana35@gmail.com

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ABSTRACT

Plant is an important medicinal source, and plays a key role in world health. *Limonia acidissima* have been known to be an important potential source of therapeutics and curative aids. In previous studies it has been reported *Limonia acidissima* showed antibacterial activity, anti diarrhoeal activity, anticancer activity and wound healing potential. There are no reports on hepatoprotective activity of this plant. The present study was planned to scientifically investigate the hepatoprotective activity of ethanolic extract of *Limonia acidissima* against paracetamol (PCM) induced hepatotoxicity model. The animals were grouped into five groups of six animals each. Except the normal group all the other groups received PCM at a dose of 1.5 ml/kg b.w orally for 14 days. Normal groups received distilled water orally. The standard group received silymarin 100 mg/kg orally. Test groups received EELA 200 mg/kg and 400mg/kg b.w orally. On the 14th day, blood samples were collected and serum was separated which is in turn used to analyze liver function tests such as SGOT, SGPT, ALP, Total bilirubin, Total cholesterol, Total protein levels. The results thus obtained showed significant improvement in these parameters. Thus, concluding that the ethanolic extract of *Limonia acidissima* possesses hepatoprotective activity.

Keywords: *Limonia acidissima*, paracetamol, ethanolic extract, hepatoprotective activity.

INTRODUCTION

Liver is the most important organ, which plays a pivotal role in regulating various physiological processes in the body. It is involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences. 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 damages.

Drug-induced hepatotoxicity is a major cause of iatrogenic diseases, accounting for one in 600 to one in 3500 of all hospital admissions. Paracetamol being used as an analgesic and antipyretic it is highly used as OTC drug that has an adverse effect of hepatotoxicity on high usage and its over dose.

*Limonia acidissima* Linn syn Feronia limonia (Rutaceae) is a moderate sized deciduous tree grown throughout India. Its fruits are woody, rough and used as a substitute for bael in diarrhoea and dysentery while the bark and leaves are used for vitiated conditions of vata and pitta. The fruits are used for tumours, asthma, wounds, cardiac debility and hepatitis. It has been reported that this part of the plant contains flavonoids, glycosides, saponins, tannins, coumarins, and tyramine derivatives. Fruit shells of *L. acidissima* have been reported to have antifungal compounds namely psoralene, xanthotoxin, 2,6-dimethoxybenzoquinone and octenol. While the leaves have hepatoprotective activity. The stem bark of the plant contain (-) - (25) - 5',3' - dihydroxy 4'-methoxy-6',6'-dimethylchromeno-(7, 8, 2',3')-flavanone along with several known compounds including an alkaloid, five coumarins, a flavanone, a lignan, three sterols and a triterpene which were found to possess antimicrobial activity.

Yet, no systemic pharmacological studies were reported to support its use in hepatotoxicity. Hence present study attempts to study hepatoprotective potential of ethanolic extract of *Limonia acidissima* against paracetamol induced hepatotoxicity in rats.

MATERIALS AND METHODS

Collection and identification of Plant material

The stem bark of the plant *Limonia acidissima* was colleted from utukurkadapa district, Andhra Pradesh.

Preparation of extract

The dried stem bark of plant *Limonia acidissima* was taken, powdered in a grinder-mixer to obtain a coarse powder and then passed through 40 mesh sieve. About 200gms of powder was extracted by using methanol by maceration process up to 24hrs. The solution was filtered through Whatmann filter paper and the resultant filtrate was distilled under reduced pressure for recovery of solvent. The dried extract thus obtained was kept in desiccators and used for further experiments.
**Experimental animals**

Wistar albino rats of either sex (150-230gm) were used in the present study. The animals were housed in the clean propylene cages and maintained under standard conditions (25±2°C, relative humidity 44 - 56% and 12 hours light and dark cycles respectively) and fed with standard rat diet (Mysore feeds, Bangalore) and purified drinking water at libitum for 1 week before and during the experiments. Animals were handled with human care.

**Drugs & chemicals**

Silymarin (Sigma), paracetamol and all other reagents used were of analytical grade. Diagnostic kits used in this study were procured from Span Diagnostic Ltd., India and Excel diagnostic Ltd., India.

**Acute toxicity studies**

Acute oral toxicity study was performed as per OECD-423 guideline.

Wistar albino rats (n=6) of either sex selected by random sampling technique were used for acute toxicity study. The animals were kept fasting for an overnight provided with only water, after which the extract was administered orally at the dose level of 2000 mg/kg body weight by gastric intubation and observed for 24 hours. If mortality was ascertained in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher dose such as 4000 mg/kg of body weight.

The mortality and morbidity was observed after 24 hours.

**Experimental design**

The experimental design used to carry out the hepatoprotective activity of ethanolic extract of *L. acidissima* against PCM induced hepatotoxicity.

The animals were grouped into five groups with six animals in each. Except the normal group all the other groups received paracetamol at a dose of 1.5 ml/kg b.w, orally for 14 days. Normal groups received distilled water orally. The standard group received silymarin 100 mg/kg b.w, orally. Test groups received EELA 200 mg/kg and 400mg/kg b.w orally. On the 14th day, blood was collected from each animal for serum analysis.

**Assessment of serum marker enzymes**

The blood samples were collected in EDTA-free vials on day 0, 7, 14, 21, 28. The collected blood samples were centrifuged under cooling condition at 4000 RPM for 10-15 minutes to separate plasma and serum. The separated serum was used for the estimation of biochemical parameters like SGOT, SGPT, ALP, total cholesterol, total protein and TB using commercial available diagnostic kit (Span Diagnostic, Ltd).

**Statistical analysis**

All parameters are expressed as a mean value ± SEM. Differences between the mean value of tests and control groups were evaluated statistically by using the one-way analysis of variance (ANOVA), Student’s t-test.

**RESULTS**

**Acute toxicity studies**

Acute oral toxicity study was performed as per OECD-423 guidelines. The ethanolic extract of *L. acidissima* was found to be safe since no animal died even at the maximum single dose of 4000 mg/kg when administered orally. The animal did not show any gross behavioral changes.

**Table 1: Effect of *Limonia acidissima* on serum biochemical parameters**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>SGOT (IU/L)</th>
<th>SGPT (IU/L)</th>
<th>Alk.P (IU/L)</th>
<th>TB (mg/dl)</th>
<th>TP (g/dl)</th>
<th>TC (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>18.83±8.035</td>
<td>23.17±10.76</td>
<td>53.67±14.72</td>
<td>6.55±0.187</td>
<td>5.65±0.7714</td>
<td>156±34.92</td>
</tr>
<tr>
<td>II</td>
<td>PCM treated</td>
<td>42.17±7.46##</td>
<td>48.33±8.31###</td>
<td>109.5±11.95##</td>
<td>2.067±0.5203###</td>
<td>8.383±0.9326###</td>
<td>274.3±36.09###</td>
</tr>
<tr>
<td>III</td>
<td>Silymarin</td>
<td>20.32±7.062**</td>
<td>18.61±8.021***</td>
<td>68±21.74*</td>
<td>0.483±0.2483***</td>
<td>5.317±1.196***</td>
<td>184±45.14*</td>
</tr>
<tr>
<td>IV</td>
<td>Test-1 (200 mg/kg PO)</td>
<td>28.97±7.674*</td>
<td>25.7±7.027**</td>
<td>76.8±21.55*</td>
<td>0.7167±0.2317***</td>
<td>6.883±0.8353*</td>
<td>196±39.52*</td>
</tr>
<tr>
<td>V</td>
<td>Test-2 (400 mg/kg PO)</td>
<td>22.75±8.219**</td>
<td>22.42±9.067**</td>
<td>65±22.56</td>
<td>0.6±0.2366***</td>
<td>6.267±0.6593***</td>
<td>193.7±40.54*</td>
</tr>
</tbody>
</table>

All values were shown as mean ± SEM and n=6, ### indicates p < 0.001 when compared to normal group, *** indicates p < 0.001 when compared to control group.
Hepatoprotective activity

Administration of PCM induced a significant increase (p<0.05) in serum SGOT, SGPT, ALP, Total bilirubin, total cholesterol levels on 28th day when compared to normal group. While a significant (p<0.05) reduction was observed in serum levels of above parameters on 14th day in rats treated with standard drug silymarin when compared to control group. The trend was same with that of both the groups (IV & V) receiving ethanolic extract of *L.acidissima* (200mg/kg & 400mg/kg) witnessing a significant fall in above biochemical parameters on 28th day when compared to control group as shown in Table 1.

The serum levels of total protein and albumin were significantly decreased in rats up on administration of PCM when compared to normal. Silymarin treated groups showed significant rise (p<0.05) in serum levels of total protein and albumin compared to control group. The EELA (200mg/kg & 400mg/kg) treated groups also showed significant increase (p<0.05) in total protein and albumin levels compared to control group as shown in Table 1.

**DISCUSSION**

The liver performs the normal metabolic homeostasis of the body as well as biotransformation, detoxification and excretion of many endogenous and exogenous compounds, including pharmaceutical and environmental chemicals. Hepatocytes are the main component that regulates various metabolic activities of liver. Distortion of this organ will result in disorder of body metabolism. An accidental over dosage administration of PCM as an antipyretic drug and over-the-counter analgesic can result in hepatic damage. N-acetyl-p-benzoquinoneimine (NAPQI), which is one of the metabolites of PCM after the latter undergoes metabolism in the liver via the action of cytochrome P450 (cyP450) monooxygenase, is highly responsible for the PCM toxic effect to the liver. Several CYP450 enzymes have been known to participate in the bioactivation of NAPQI. NAPQI is normally conjugated with glutathione (GSH) and excreted in urine. GSH has been highlighted to be responsible in the antioxidant defense of our body by scavenging the free radicals produced through the metabolism processes within the liver in order to prevent any subsequent cell damage. Overdosage of PCM will result in accumulation of NAPQI, which will bind to GSH to form conjugates that will lead to the oxidation and conversion of GSH to glutathione disulfide (GSSG) resulting in the reduced level of blood and liver GSH. Depletion of GSH level in blood and liver due to this process can result in mitochondrial dysfunction, increase of lipid peroxidation, and development of acute hepatic necrosis. Hepatocellular necrosis releases the enzymes such as AST and ALT into the circulation, and hence it can be measured in the serum. Hepatic parenchymal cells produce pool of ALT that is regarded as specific enzyme for detection of liver abnormalities.

Based on the role of PCM metabolite, NAPQI, as described previously, the development of PCM-induced hepatotoxicity seems to depend partly on the existence of free radicals and oxidative processes. For that reason, it is hypothesized that extracts/compounds possessing free radical scavenging and/or antioxidant activities could also demonstrate hepatoprotective activity against the PCM toxic effect. This is supported by claim that the combination of hepatoprotective effect and antioxidant activity synergistically prevents the process of initiation and progress of hepatocellular damage.

Interestingly, our findings did demonstrate the EELA to exert antioxidant activity due to presence of chemical constituents like coumarins, flavonoids, steroids and terpenoids. Moreover, the inflammatory processes activated by PCM or other toxic agents are intimately involved in the chemical-induced hepatotoxic processes. The inflammatory processes are thought to be responsible for producing various mediators, which are involved in the production of nitric oxide (NO) that can affect liver damage or repair. Therefore, it is also possible to postulate that extracts/compounds possessing anti-inflammatory activity might also exhibit hepatoprotective activity. It is again interesting to highlight that the stem bark of *L.acidissima* possess anti-inflammatory activity.

Phytochemical screening of EELA demonstrated the presence of flavonoids, saponins, condensed tannins, and steroids. Flavonoids have been reported to exhibit antioxidant, anti-inflammatory, and hepatoprotective activities. Furthermore, condensed tannins have been suggested to possess free radical scavenging and antioxidant, anti-inflammatory, and hepatoprotective activities, while saponins have been reported also to exhibit hepatoprotective activity via modulation of its antioxidant and anti-inflammatory activities. Taking all these reports into consideration, it is plausible to suggest that the hepatoprotective activity of EELA involved, partly, synergistic action of flavonoids, condensed tannins, and saponins.

The use of rat as experimental animals for hepatoprotective activity is mainly because of structural homology of rat CYP450 enzymes with that of humans.

The efficacy of any hepatoprotective drug id dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been disturbed by a hepatotoxin. Both low dose group and high dose group decreased PCM induced elevated enzymes levels, indicating the protection of structural integrity of hepatocytic cell membrane or the regeneration of damaged liver cell.

Both the test group for e.g., low dose and high dose treated groups shown dose dependent hepatoprotective activity. The test group containing the plant extract alone showed an improvement in liver activity. It clearly indicates that the plant *Limonia acidissima* has the hepatoprotective activity.
CONCLUSION

Hepatotoxicity may be defined as any damage or injury to the liver caused by a drug, chemical or other agent. The agents which protect and prevent liver damage are termed as hepatoprotective agent.

Liver diseases caused by the use of over the counter drug i.e.; paracetamol (PCM) as an analgesic and antipyretic is increasing continuously. So, our aim was to overcome this issue by the use of herbal extracts like Limonia acidissima and others.

As the literature survey reveals that works carried out for Limonia acidissima plant is having many pharmacological activities, in which many of the activities or not yet proved. So, this plant was taken for the thesis work. The stem bark of the plant was taken and it was extracted using ethanol as solvent and preliminary phytochemical studies were performed on the extract and the chemical test show the presence of flavonoids, phenolic compound, tannins along with other components.

The results obtained from the analysis of biochemical parameters conclude that the co-treatment of Limonia acidissima extract prevents paracetamol induced hepatotoxicity in rats. The high dose Limonia acidissima extract, showed better results as compared to low dose, and results were comparable with that of standard drug, silymarin. Purifying the extract and identifying the active principles like flavonoids, tannins and phenolic compounds may yield a good hepatoprotective drug.

This study showed that EELA has a significant protective action against the hepatotoxicity induced by the PCM. The hepatoprotective role of EELA might be due to its antioxidant potential mechanism suggesting that the extract of plant may be useful to prevent the PCM induced liver damage.

Thus, the present study indicates that the ethanolic extract of stem bark of Limonia acidissima may be used as an effective hepatoprotective agent. Further studies on isolation and structural determination of active principle might be worthy.

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REFERENCES


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