Protective effects of Parinari curatellifolia flavonoids against acetaminophen-induced hepatic necrosis in rats

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Abstract In the present study, we investigated the hepatoprotective potential of Parinari curatellifolia Planck (Chrysobalanaceae) in experimental rats in order to ascertain the validity of folkloric claims of its effectiveness in the treatment of hepatic-related disorders. Flavonoid extract of P. curatellifolia seed, PCF (10-, 20- or 30 mg/kg body weight) or silymarin (25 mg/kg), dissolved in corn oil, was administered by gavage to experimental animals once daily for 14 consecutive days before liver damage was chemically induced through the administration of acetaminophen (2 g/kg p.o.) on the 14th day. Hepatoprotection was assessed by analyzing liver homogenate and serum for markers of hepatotoxicity – alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) activities as well as prothrombin time (PT). Evaluation of biochemical indices of oxidative stress – level of lipid peroxides (LPO), activities of superoxide dismutase (SOD) and catalase, along with histological assessment of hepatic tissue sections were also carried out. Results revealed that all doses of PCF significantly (P < 0.001) and dose dependently prevented acetaminophen-induced increase in serum activities of hepatic enzymes (ALT, AST, GGT, LDH) and PT. Furthermore, PCF (10- and 20 mg/kg) significantly (P < 0.001) reduced lipid peroxidation in liver tissue and restored the activities of the antioxidant enzymes SOD and catalase toward normal levels. Histopathology of the liver tissue showed that PCF mitigated the toxicant-induced hepatocellular necrosis, reduced inflammatory cell infiltration and enhanced hepatocyte regeneration. The results indicated that P. curatellifolia flavonoids demonstrated remarkable hepatoprotective activity in acute liver injury caused by acetaminophen.

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in use have side effects. This contributes to hepatotoxicity which is becoming a serious health challenge since the liver is the major site of drug metabolism. Acetaminophen (paracetamol, N-acetyl-p-aminophenol) is a widely used over-the-counter analgesic and antipyretic drug with only weak anti-inflammatory property (Hinson et al., 2010). Acetaminophen could cause potentially fatal centriflobular hepatic necrosis. This occurs at high doses after its conversion by drug metabolizing enzymes to a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) that covalently binds to proteins. At toxic doses, the metabolite suppresses the antioxidant defense system and the amount of covalent binding correlates with the relative hepatotoxicity (Jollow, 1973). Acetaminophen-induced liver injury is now a viable model of hepatotoxicity popularly employed by clinical researchers to investigate the beneficial effects of drugs (Olaleye et al., 2010a, 2010b; Pattanayak et al., 2011).

Finding sustainable therapeutic approach to minimizing liver damage is important owing to the prevalence of this condition. Medicinal plants used in the folkloric management of liver-related diseases or diseases related to other organs could be considered an alternative therapeutic approach (Salama et al., 2013). The use of herbal and dietary supplements in disease management dates back to thousands of years (Schuppan et al., 1999). The therapeutic value of medicinal plants is largely dependent on the constituent antioxidant phytochemicals, notably the phenolics. This stems from the role of oxidative stress in the etiology or progression of various diseases including hepatic-related ones. Flavonoids constitute a large subset and one of the most ubiquitous groups of plant phenolics (De Groot and Raven, 1998). They are the major active nutraceutical ingredients in plants (Tapas et al., 2008). Apart from their antioxidant property, flavonoids have been recognized to possess antiinflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (Tapas et al., 2008). Silymarin, a standardized flavonoid extract from the seed of Silybum marianum, has been found to be highly effective as a hepatoprotective agent and is now widely used as a reference drug in hepatoprotective assessments (Gupta et al., 2011; Salama et al., 2013).

The seeds of Parinari curatellifolia, commonly called Mobola Plum, are widely employed in traditional medicine for the management of various diseases including hypertension (Olaleye et al., 2010a, 2010b), diabetes and liver-related illnesses (Ogbonnia et al., 2011). Phytochemicals in the seed include polyphenols, glycosides, alkaloids and anthraquinones (Ogbonnia et al., 2008) but none of these has been specifically correlated with hepatoprotection. Bearing in mind the role of oxidative stress in both hepatic and non-hepatic diseases, we presumed that the therapeutic efficacy of P. curatellifolia could be related to its antioxidant components. Since there is paucity of information in this regard, the present study was aimed at determining the possible effect of the flavonoid extract of P. curatellifolia seeds on acetaminophen-induced liver injury.

2. Materials and methods

2.1. Chemicals

Thiobarbituric acid (TBA), malonaldehyde bis-(dimethyl acetal) (MDA), Epinephrine, 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) and hydrogen peroxide were purchased from Sigma Chem., Co. (London, UK). γ-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase and total protein kits were obtained from Randox Laboratories, UK. All other chemicals were of analytical grade and were obtained from British Drug Houses, (Poole, UK). The water used was glass distilled.

2.2. Plant material and preparation of crude extract

The seeds of P. curatellifolia were purchased at Oja-Oba in Akure, Nigeria, in the month of February, 2013. Botanical identification and authentication were carried out at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria. The seeds were air dried, pulverized and stored in air-tight containers. The crude extraction of the seeds was carried out with 80% methanol (Komolafe et al., 2013) using a Soxhlet apparatus. The solvent was evaporated to dryness and the dried methanolic extract (ME) was stored in an airtight bottle at 4 °C until needed.

2.3. Preparation of flavonoid extract

A portion (3 g) of ME was dissolved in 20 ml of 1% H2SO4 in a standard flask and was hydrolyzed by heating on a water bath for 30 min. The resulting mixture was placed on ice for 15 min to allow for the precipitation of the flavonoid aglycone. The cooled solution was filtered and the solid on the filter (flavonoid aglycone mixture) was dissolved in 50 ml of warm 95% ethanol at 50 °C. The resulting solution was re-filtered into a 100 ml volumetric flask which was made up to mark with 95% ethanol. The filtrate finally collected was concentrated to dryness using a rotary evaporator (El-Olemy et al., 1994). A calculated mass of the dried extract was dissolved in an appropriate volume of corn oil to prepare various concentrations which were administered to experimental animals by oral gavage.

2.4. Animals

Adult male rats (Wistar strain) weighing 180–220 g, obtained from a private breeder and housed in the primate colony of the Department of Biochemistry, Federal University of Technology, Akure, Nigeria were used for this study. The animals were kept in wire mesh cages under controlled light cycle (12 h light/12 h dark), fed with commercial rat chow (Vital Feeds Nigeria Limited) ad libitum, and liberally supplied with water. All animal experiments were conducted according to the guidelines of National Institute of Health (NIH publication 85-23, 1985) for laboratory animal care and use.

2.5. Experimental design

Age-matched rats were divided into nine groups (n = 6) and treated as follows:

- **Group I (Control)** Corn oil (1 ml/kg).
- **Group II** Corn oil (1 ml/kg) + 2 g/kg Acetaminophen (ACE).
- **Group III** 10 mg/kg P. curatellifolia flavonoids (PCF) + 2 g/kg ACE.
• Group IV 20 mg/kg PCF + 2 g/kg ACE.
• Group V 30 mg/kg PCF + 2 g/kg ACE.
• Group VI 25 mg/kg silymarin (SIL) + 2 g/kg ACE.
• Group VII 10 mg/kg PCF only.
• Group VIII 20 mg/kg PCF only.
• Group IX 30 mg/kg PCF only.

Corn oil (1 ml/kg), silymarin (25 mg/kg) or PCF (10-, 20- or 30 mg/kg) were administered by gavage to healthy experimental rats once daily for 14 consecutive days and thereafter, rats were challenged with a single oral dose of acetaminophen (2 g/kg) on the 14th day. Animals were sacrificed under mild ether anesthesia 24 h after ACE administration. Blood was collected by cardiac puncture and the liver tissues dissected out for biochemical and histological evaluation.

2.6. Assessment of hepatoprotective activity

The collected blood was allowed to clot and serum was obtained after centrifugation at 3000 rpm for 15 min. The activities of AST, ALT, LDH and GGT were estimated in serum using assay kits obtained from Randox Laboratories Ltd., UK according to the instructions of the manufacturer.

2.6.1. Prothrombin time test

The prothrombin time test is based on the time required for a fibrin clot to form after the addition of Tissue Factor (factor VIIa), phospholipid and calcium to decalcified, platelet poor plasma. The assay was carried out using the assay kit provided by Quinica Clinica Aplicada, S.A., Spain according to the manufacturer’s instruction (Hills and Ingram, 1973).

2.7. Assessment of hepatic antioxidant status

Excised livers were rinsed in 1.15% KCl and homogenized in aqueous Tris–HCl buffer (50 mM, pH 7.4). Homogenates were centrifuged at 10,000 g for 20 min at 4 °C to obtain the supernatant fraction which were used for analyses.

2.7.1. Assessment of hepatic lipid peroxidation

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979).

2.7.2. Determination of superoxide dismutase (SOD) activity

The method of Misra and Fridovich (1972) was employed in determining the activity profile of SOD in the homogenates. Briefly, an aliquot of the diluted sample was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer. The reaction was started by the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml of buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of water. The increase in absorbance at 480 nm was monitored every 30 s for 150 s. 1 unit of SOD activity was defined as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 min.

2.7.3. Determination of catalase activity

Catalase activity was determined by following the decomposition of H₂O₂ according to the method of Sinha (1971). An aliquot (1 ml) of properly diluted enzyme preparation was rapidly mixed with an assay mixture containing 4 ml of H₂O₂ (800 μmol) solution and 5 ml of 0.1 M phosphate buffer (pH 7.0) in a conical flask by a gentle swirling motion. The reaction was run at room temperature. A 1 ml portion of the reaction mixture was withdrawn and blown into 2 ml dichromate/acetic acid reagent at 60 s intervals. The H₂O₂ content of the withdrawn sample was determined by taking the absorbance at 570 nm. Catalase activity was expressed as μmol H₂O₂ consumed/min/mg protein.

2.8. Histopathology

Representative liver tissue of each group was excised, trimmed of fat and other connective tissues and prepared for histological studies. The tissues were fixed in 10% phosphate buffered neutral formalin, dehydrated in graded (50–100%) alcohol and embedded in paraffin. Thin sections (4–5 μm) were cut and stained with hematoxylin and eosin (H & E) stain for photomicroscopic assessment.

2.9. Statistical analysis

All values are expressed as mean ± SD of six animals. Statistical evaluation was done using One Way Analysis of Variance (ANOVA) followed by the Newman–Keuls comparison of means. The significance level was set at p < 0.05.

3. Results

3.1. Serum markers of hepatotoxicity

Toxicity arising from acetaminophen-intoxication was manifested in rats as observed in the significant elevation (P < 0.001) in serum activities of GGT (195%), ALT (139%), AST (107%) and LDH (141%) compared to the control (Table 1). Hepatoprotection by PCF (10-, 20- and 30 mg/kg) demonstrated the best protection were almost comparable to those of the standard hepatoprotective drug, silymarin. PCF alone produced a significant decrease in ALT (10, 20, 30 mg/kg; P < 0.001) in serum activities of GGT (45%, 50% and 58%); ALT (47%, 56% and 52%); AST (37%, 43% and 49%); LDH (27%, 38% and 40%) compared to the ACE-induced group (P < 0.001). The observed effects of PCF (30 mg/kg) which demonstrated the best protection were almost comparable to those of the standard hepatoprotective drug, silymarin. PCF alone produced a significant decrease in ALT (10, 20, 30 mg/kg; P < 0.001) and LDH (20, 30 mg/kg; P < 0.01) but caused no change in other markers when compared to the control.

3.2. Prothrombin time and hepatic antioxidant status

Serum prothrombin formation time and extent of hepatic membrane lipid peroxidation are shown in Fig. 1. Hepatic injury induced by ACE caused significant increases (P < 0.001) in both the time for the formation of the clotting factors in the blood and in the extent of lipid peroxidation in rat hepatocytes. Significant inhibition of these effects was observed in PCF-treated rats. A >200% increase in MDA level, an index of membrane lipid peroxidation, was recorded in the ACE group. This was reduced in PCF (10- and 20 mg/kg) groups by 32% and 37% respectively (P < 0.001). ACE (2 g/kg) significantly reduced hepatic activities of SOD (69%,
and CAT (64%, \( P < 0.001 \)) when compared with the control (Fig. 2). The protective effect of PCF on antioxidant enzymes was reflected in the significant increases, at all doses employed, in the activities of SOD and CAT compared with values obtained in the induced, untreated animals. The observed effects of PCF were dose-dependent and the highest effective concentration (30 mg/kg) was almost equipotent with the reference drug, silymarin with regard to % increase in SOD (185, \( P < 0.001 \) vs. 205, \( P < 0.001 \)) and CAT (72, \( P < 0.01 \) vs. 83, \( P < 0.001 \)) respectively.

### 3.3. Histopathology

The hepatoprotective effect of PCF was confirmed by histological studies of liver sections (Fig. 3) which support the results obtained from serum enzyme assays. Normal control rats (A) showed normal hepatic architecture with no evidence of pathological changes whereas liver sections of the ACE-induced group (B) showed total loss of hepatic architecture with patterns of cell necrosis, inflammatory collections and accumulation of fatty lobules providing histopathological evidence of tissue injury in the ACE-treated group. The pretreated animals showed noticeable protection from acetaminophen as evident from the improved histologic indices. The histological architecture of liver sections of PCF (10 mg/kg)-treated rats (C) showed a more or less normal lobular pattern with a mild edematous change in the hepatic tissue. The 20 mg/kg PCF group (D) showed patterns of regeneration of hepatocytes around the central vein while the group treated with 30 mg/kg PCF extract (E) only showed mild hepatocyte degeneration. The liver architecture of E was comparable to the group pretreated with the standard drug, silymarin (F) which showed less disarrangement of hepatocytes, indicating marked regeneration activity with an almost near normal liver architecture. The liver of non-induced animals treated with PCF (10, 20 and 30 mg/kg) only (G–I) showed normal histological features.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of <em>Parinari curatellifolia</em> flavonoids (PCF) on serum markers of acetaminophen-intoxicated rats.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>GGT (U/I)</td>
</tr>
<tr>
<td>Control</td>
<td>3.26 ± 0.2</td>
</tr>
<tr>
<td>ACE</td>
<td>9.61 ± 0.6***</td>
</tr>
<tr>
<td>ACE + PCF (10 mg/kg)</td>
<td>5.33 ± 0.7a</td>
</tr>
<tr>
<td>ACE + PCF (20 mg/kg)</td>
<td>4.89 ± 0.6a</td>
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<tr>
<td>ACE + PCF (30 mg/kg)</td>
<td>4.06 ± 0.5a</td>
</tr>
<tr>
<td>ACE + SIL (25 mg/kg)</td>
<td>2.22 ± 0.4a</td>
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<tr>
<td>PCF (10 mg/kg)</td>
<td>3.08 ± 0.5a</td>
</tr>
<tr>
<td>PCF (20 mg/kg)</td>
<td>2.92 ± 0.4a</td>
</tr>
<tr>
<td>PCF (30 mg/kg)</td>
<td>2.35 ± 0.8a</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (\( n = 6 \)). PCF, *P. curatellifolia* flavonoids; GGT, gamma glutamyl transpeptidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase. \( P \) values: **<0.01, ***<0.001 compared with the normal control group I; \( b <0.01, c <0.001 \) compared with the ACt-induced group II.

\( P < 0.001 \) and CAT (64%, \( P < 0.001 \)) when compared with the control (Fig. 2). The protective effect of PCF on antioxidant enzymes was reflected in the significant increases, at all doses employed, in the activities of SOD and CAT compared with values obtained in the induced, untreated animals. The observed effects of PCF were dose-dependent and the highest effective concentration (30 mg/kg) was almost equipotent with the reference drug, silymarin with regard to % increase in SOD (185, \( P < 0.001 \) vs. 205, \( P < 0.001 \)) and CAT (72, \( P < 0.01 \) vs. 83, \( P < 0.001 \)) respectively.

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4. Discussion

Plants have provided active ingredients of medicines for years and are still sources of lead compounds in the development of new therapeutics (Newman, 2008). Some naturally occurring substances have the potential to be hepatoprotective and therefore can be considered for use in the treatment of acute and chronic liver diseases. The therapeutic efficacy of flavonoids

Figure 2  Effects of *P. curatellifolia* flavonoids on superoxide dismutase (A) and catalase (B) activities in the hepatocytes of acetaminophen-intoxicated rats. Values are expressed as mean ± SD (*n* = 6). CrnO, corn oil; PCF, *P. curatellifolia* flavonoids; ACE, acetaminophen, SLM, silimarini. *P* values: **<0.01, ***<0.001 compared with the normal control group I; b< 0.01, c< 0.001 compared with the ACE-induced group II.

Figure 3  Photomicrographs of liver sections from rats treated with acetaminophen and the effect of PCF pretreatment. (A) Control (vehicle) (B) ACE (C) 10 mg/kg PCF + ACE (D) 20 mg/kg PCF + ACE (E) 30 mg/kg PCF + ACE (F) 25 mg/kg SLM + ACE (G) 10 mg/kg PCF alone (H) 20 mg/kg PCF alone (I) 30 mg/kg PCF.
against numerous disease and pathological conditions has been firmly established (Tapas et al., 2008). In the present study, the hepatoprotective effects of \textit{P. curatellifolia} flavonoids (PCF) on liver necrosis and damage induced by acute exposure to ACE (2 g/kg) were assessed using biochemical and histological parameters.

In line with previous findings on acetaminophen toxicity (Olaleye et al., 2010a, 2010b; Pattanayak et al., 2011), we observed significant increases in the activities of serum markers of hepatotoxicity; AST, ALT, GGT and LDH in acetaminophen-induced, untreated rats. Some of the most commonly used biochemical markers of hepatocellular necrosis are serum activity concentrations of the aminotransferases, AST and ALT which are localized in the hepatocytes (Friedman et al., 1996). Serum activities of these enzymes increase following cellular membrane damage and consequent leakage to the extra-cellular milieu. Highest levels occur in acute hepatocellular injuries, such as drug-induced necrosis or acute viral hepatitis leakage (Sturgill and Lambert, 1997). GGT, on the other hand, is a microsomal enzyme abundant in hepatocytes and biliary epithelial cells and involved in the transfer of γ-glutamyl groups from peptides to amino acids and the metabolism of glutathione conjugates. Abnormally elevated serum activity, as observed in the present study for ACE-induced animals, is mainly attributed to hepatobiliary dysfunction (Friedman et al., 1996). That levels of these markers tend toward normal in animals treated with the flavonoid extract suggest the hepatoprotective effects of the intervention. Parallel findings have been previously reported (Tapas et al., 2008; Kumar, 2012). In the same vein, elevation in serum LDH activity has been correlated with liver damage following administration of hepatotoxicant to rats (Ravikumar et al., 2010; Yakubu et al., 2013). The significantly decreased ($P < 0.001$) serum LDH activity in PCF-treated animals signifies improvement over acetaminophen-dependent damage to the liver. Prothrombin time (PT), an index of hepatic synthetic capacity, has prognostic value in both acute and chronic liver injuries. Worsening prolongation of the PT in the setting of acute hepatocellular necrosis is associated with an increased risk of fulminant injury (Clark et al., 1973; Sturgill and Lambert, 1997). It could be established from the present study that ACE-induced PT prolongation in rat serum was prevented by PCF treatment. The observation that PCF alone did not increase the serum levels of hepatic indices but instead caused significant reduction of serum ALT and AST, could suggest the non toxicity of the flavonoid extract by itself to the hepatocytes (Olaleye et al., 2010a, 2010b).

The hepatoprotective effect of PCF was further confirmed by findings from the histological studies of liver sections. The typical derangement in hepatic architecture observed in ACE-treated rats (Hewawasam et al., 2003; Pattanayak et al., 2011) was improved in animals pretreated with PCF in a similar manner to silymarin-treated rats. The observed close-to-normal lobular pattern with mild cellular necrosis and lymphocyte infiltration as well as patterns of hepatocyte regeneration in the liver sections of PCF-treated rats all point to the protective effect of the flavonoid extract. In the present study, PCF exhibited a dose-dependent activity with an overall optimum protective effect at the maximum dose of 30 mg/kg comparable with the control and standard (silymarin-treated) groups.

Evidences support the generation of reactive oxygen species during ACE hepatotoxicity and the fact that this plays a critical role in cell death (Jaeschke et al., 2003, 2010). Estimation of lipid peroxide levels gives quantitative analysis of cellular membrane damage resulting from oxidative stress. In the present study, the induction of oxidative stress and the overwhelming of the antioxidant defense by ACE in rat hepatocytes resulted in aggravated membrane lipid peroxidation as observed in the elevated TBARS levels in liver homogenates. Inferring from the reduced levels of TBARS in PCF-treated rats, it can be argued that the flavonoid extract promoted significant recovery of rat hepatocyte membrane integrity possibly through its antioxidant activity. The consequent oxidative stress arising from ACE toxicity could be responsible for the significant loss of SOD and catalase activities. These endogenous antioxidant enzymes are responsible for the dismutation of superoxide radicals to \(\text{H}_2\text{O}_2\) and detoxification of \(\text{H}_2\text{O}_2\) to water respectively thereby offering protection against tissue damage. For instance, administration of encapsulated SOD has been reported to decrease the toxicity of acetaminophen in rat (James et al., 2003). As observed in the present study and in line with previous reports (Donovan et al., 1999; Manach et al., 2005), orally administered \textit{P. curatellifolia} flavonoids were absorbed to biologically relevant concentrations in the hepatocytes of rats where they elicited the recovery of the antioxidant enzyme status. Apart from their direct antioxidant and free radical scavenging effects, flavonoids are capable of upregulating the synthesis of antioxidant enzymes (Li et al., 2006; Quinones et al., 2013). We can hypothesize that \textit{P. curatellifolia} flavonoids, through its antioxidant activity, prevented the overwhelming of the antioxidant defense and the covalent binding of reactive intermediates of ACE to critical proteins with the resultant reduction of acetaminophen-induced liver necrosis as evident in the biochemical and histological results (Hinson et al., 2010).

In vivo, some flavonoid metabolites exhibit prooxidative tendencies which proceed via concentration-dependent stimulation of hydrogen peroxide production (Metodiew et al., 1999; Procházková et al., 2011). Such prooxidative properties do not necessarily indicate toxicity in its entirety but could be associated with a cell signaling mechanism by which flavonoids contribute to the co-ordination of cell functions (Procházková et al., 2011). In some instances, these effects could be beneficial and capable of causing overall cytoprotection, since levels of some antioxidant defenses and biotransformation enzymes might be elevated due to the mild degree of oxidative stress ensuing (Halliwell, 2008). Going by the findings in the present study, the possibility of a prooxidative tendency of PCF by itself, at higher concentrations in rats, cannot be completely ignored. Such could account for the decreased catalase activity and the attendant membrane lipid peroxidation, especially at higher concentrations, in rats treated with the flavonoid extract alone. This observation, notwithstanding, does not put the hepatoprotective effect of PCF in doubt going by the array of results corroborating this property.

5. Conclusion

Going by the present study, it could be concluded that \textit{P. curatellifolia} flavonoids exhibited hepatoprotective activity.
Further research is ongoing in our laboratory to characterize the specific flavonoids involved as well as to ascertain their individual contributions and exact mechanism(s) of action.

References


