Hepatoprotective studies of floral extracts of *Gomphrena serrata* L. and piperic acid on CCl₄ induced hepatotoxicity

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Received 03 November 2018; Revised 29 December 2019

The present investigation aims to isolate, characterise and evaluate the phytoconstituents of *Gomphrena serrata* L. responsible for hepatoprotective activity in carbon tetrachloride-induced hepatotoxicity models both *in vitro* and *in vivo*. The plant species has not been explored for various therapeutic activities. HPLC analysis of subfraction of plant extract showed the presence of piperine, which was isolated and further hydrolysed to piperic acid. The results of the study indicate that the plant hydroalcoholic, acetone extracts at 500 mg/kg and compound piperic acid at 0.5 mg/kg exhibited better results in the regeneration of damaged hepatocytes and reduction of biochemical marker enzymes. The hepatoprotective activity might be due to inhibition of cytochrome P450 2E induced ER and oxidative stress. The present study reveals that the hepatoprotective activity of floral extracts might be due to *in situ* conversion of piperine into piperic acid. As piperic acid showed the equipotent potential to standard drug silymarin, it can be further developed as a hepatoprotective drug.

**Keywords:** *Gomphrena serrata* L., *G. serrata* extracts, Hepatoprotective, Piperic acid.

**IPC code:** Int. cl. (2015.01)- A61K 36/00, A61K 36/21, A61K 133/00, A61P 1/00, A61P 1/16

**Introduction**

The plant kingdom is an enormous resource of therapeutic entities. Therapeutic search for novel molecules always drives researchers to herbs. Damage to liver and liver diseases has become a common problem worldwide¹. Alcohol abuse and nonalcoholic fatty liver disease (NAFLD), a metabolic disorder, which promote oxidative stress and inflammation, are the most common causes of hepatic damage². Cirrhosis, jaundice and fatty liver include the more prominent liver diseases³. Current pharmacotherapy of liver disorders uses a limited number of drugs with profound side effects. Herbal medicines are used in the treatment of hepatic problems⁴. The therapeutic area of hepatic problems requires novel hepatoprotective agents with different modes of action. The drugs which activate endoplasmic reticulum (ER) stress response evolve as better therapeutic agents for hepatic problems⁵.

The genus *Gomphrena* is cosmopolitan with 140 species occurring in different temperate, and subtropical regions of the world. Many plants of the family Amaranthaceae are employed in folk medicine for their nutritive assets and treatment of several diseases. The plant *G. serrata* L. Amaranthaceae is an ornamental, edible, roadside plant grown in the regions of America, Antarctica, and Indo-Malaysia. The plant species has not been explored scientifically much for phytochemical and pharmacological studies. The closest species *G. celoisoides* is often confused with *G. serrata*⁶ and hence selected plant species (*G. serrata*) has been neglected. The genus *Gomphrena* is being used for the treatment of jaundice, high cholesterol and urinary problems in Latin America and Caribbean⁷,⁸. The folklore people of different regions use *G. Serrata* leaf extracts as natural blood coagulators, and the whole plant extracts in cardiovascular and diabetic disorders⁹-¹¹. The whole plant alcoholic, aqueous and *n*-hexane extracts were studied for antibacterial properties against *Bacillus cereus* and *Escherichia coli*¹². Oleuropein was isolated from the plant extracts¹³. The
plant acetone and hydroalcoholic floral extracts have been found to exhibit antiasthmatic activity by antihistaminic and anticholinergic models\textsuperscript{14}. The chloroform fraction of acetone floral extract of the plant species was subjected to gas chromatography-mass spectrometry (GC-MS) analysis\textsuperscript{15}. In order to explore the therapeutic principles of \textit{G. serrata}, an attempt has been made to isolate, characterize, and evaluate its hepatoprotective potential.

**Materials and Methods**

**Plant material**

The plant material (Fig. 1,2) was collected from local grounds of Prasadampadu and Enikepadu coordinates 16°32′45″N 80°34′12″E of Vijayawada rural region, Krishna district, Andhra Pradesh, India. Dr. P. Satya Narayana Raju, Plant Taxonomist, Department of Botany & Microbiology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India, identified and authenticated the plant specimen. A voucher specimen (001/VIPW) was deposited in the Department of Pharmacognosy, Vijaya Institute of Pharmaceutical Sciences for Women, Enikepadu, Vijayawada, for future reference.

**General chemicals**

Azocarmine, aniline blue, carnoy, and silymarin were purchased from Sigma-Aldrich Chemical Co., biochemical kits were purchased from Span Diagnostic Limited, Surat, India. All other chemicals used were of analytical grade and commercially available.

**Animals**

Wistar rats (100–150 g) of either sex were purchased from Mahaveer enterprises, Hyderabad, Telangana, India, housed in standard conditions of temperature (22±2 °C), relative humidity (55±5 %), and light (12 h light/dark cycles). They were fed with standard pellet diet and water \textit{ad libitum}. The experimental protocol was approved (No 012/IAEC/NCPA/PhD/2016-17) by the Institutional Animal Ethical Committee of Nirmala College of Pharmacy, Atmakur, Mangalagiri, Guntur district, Andhra Pradesh, India, nominated by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Department of Animal husbandry, Ministry of Environment and Forests, Government of India.

**Isolation of phytoconstituents**

Various methods of extraction are used for isolation of different alkaloids from plant material. In the extraction and isolation of alkaloids, one has to consider that alkaloids usually occur in plants as salts of organic or inorganic acids. The procedure of extraction depends on the class of alkaloids present\textsuperscript{16}. The study has been planned to isolate alkaloids. Plant material (1 kg) was initially extracted with light petroleum ether two times (2 L×2) to remove fatty materials, moistened with the dilute ammonia solution (1 L) and extracted with chloroform (2 L) by Soxhlet apparatus for 24 hours. The obtained extract was dried using a rotary vacuum evaporator. The dried chloroform extract (12.0 g) was subjected to column chromatography (Silica gel 60 for the column, 60-120 mesh, Merck, India) in chloroform. The chloroform fractions (F1-F5), 25 mL each was collected at a flow rate of 4-5 mL for every 2 minutes. The collected fractions were subjected to TLC studies with various solvent systems (Table 1,2) to separate different groups of alkaloids present. The pH of fractions was
adjusted with 25% ammonia. All the TLC plates were treated with Dragendorff's reagent and iodine vapour separately for the development of spots. Rf values were calculated for the well-developed chromatograms and compared with corresponding literature reports. The fractions (F1-F5) were subjected to column chromatography and run using TLC solvent systems (Table 2) to collect subfractions (Fa-Fe). The subfractions (Fa-Fe) were subjected to TLC with appropriate solvent systems (Table 2). The subfraction Fc that showed yellow spot was studied by HPLC analysis using standard drug piperine. Further, the phytochemical piperine belonging to a pyridine-piperidine group was isolated and hydrolysed to piperic acid.

**Instrument and chromatographic conditions for HPLC analysis of piperine**

HPLC analysis was performed on the Shimadzu LC-20 system (Shimadzu, Kyoto, Japan) equipped with a pump (LC-20AD), autosampler (SIL-20A), and column oven and diode array detector (SPD-M20A). The output signal of the detector was recorded using LC Solution software at 343 nm. The separation was executed on a YMC-Pack ODS-A C18 (250 mm×4.6 mm, 5 μm).

**Preparation of mobile phase for quantification of piperine**

The mobile phase composed of acetonitrile:water:acetic acid (60:39.5:0.5 v/v) at a flow rate of 1.0 mL/min. The injection volume was 20 μL (Table 1; Fig. 3,4).

**Preparation of standard solution for quantification of piperine**

The standard stock solution was prepared by taking 5 mg of piperine, dissolved in 1 mL of the mobile phase. The standard working solution was prepared by taking 0.2 mL of standard stock solution and made up to 1 mL by mobile phase to get 1000 μg/mL. The sample was sonicated for 10 min and filtered before injection and quantified (Table 1; Fig. 3,4).

**Table 2 — TLC profile of column fractions**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Type of alkaloid</th>
<th>pH</th>
<th>TLC Mobile phase</th>
<th>Sub Fr. Fa-Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phenanthrene</td>
<td>9.0</td>
<td>Fr. F1-F5</td>
<td>Fa</td>
</tr>
<tr>
<td>2.</td>
<td>Quinoline</td>
<td>10.5</td>
<td>Benzene:ethanol (17:1)</td>
<td>Ethylacetate:methanol:water (8:1:1)</td>
</tr>
<tr>
<td>3.</td>
<td>Pyridazine</td>
<td>10.0</td>
<td>Chloroform:methanol (85:15)</td>
<td>Methanol:n-hexane (7:3)</td>
</tr>
<tr>
<td>4.</td>
<td>Indole</td>
<td>8.5</td>
<td>Dichloromethane:methanol (83:17)</td>
<td>Toluene:ethylacetate (7:3)</td>
</tr>
<tr>
<td>5.</td>
<td>Pyrrolizidine</td>
<td>8</td>
<td>Chloroform:ethanol (9:1)</td>
<td>Methanol:chloroform (8:2)</td>
</tr>
</tbody>
</table>

**Table 1 — Quantification of piperine in the standard and test samples by HPLC**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mg/mL)</th>
<th>Rt (min)</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Piperine</td>
<td>1</td>
<td>5.67</td>
<td>916100</td>
</tr>
<tr>
<td>Test sub fraction Fc</td>
<td>1</td>
<td>5.61</td>
<td>37085</td>
</tr>
</tbody>
</table>

**Fig. 3** — HPLC chromatogram of standard piperine

**Fig. 4** — HPLC chromatogram of piperine from test subfraction Fc
Preparation of sample solution for quantification of piperine

The test sample stock solution was prepared using 5 mg of the dried sample of subfraction Fe dissolved in 1 mL of the mobile phase. The test working solution was prepared by taking 0.2 mL of test stock solution and made up to 1 mL by mobile phase to get 1000 μg/mL. The sample was sonicated for 10 min, filtered before injection, and quantified (Table 1; Fig. 3,4).

Isolation of piperine

The powder of the plant material was refluxed with 95% ethanol for six hours. The solution was concentrated to required volume using a rotary evaporator and warm ethanolic potassium hydroxide was added to it. It was shaken well and filtered. The filtrate was added with cold water to develop turbidity. It was kept overnight for the formation of the yellow precipitate of piperine. Further, it was recrystallised using hot acetone-hexane at 3:2 ratios and kept on ice to promote the formation of yellow needle-like crystals of piperine (Fig. 5a).

Hydrolysis/degradation of piperine to piperic acid

The obtained product was analyzed by physico-chemical studies and structural characterization by UV, IR, Mass, and NMR spectroscopy. The UV absorbance of 0.01% w/v solution of the drug was determined between 200-400 nm. State: Yellow coloured compound. UV absorbance: 344 nm UV-visible double beam spectrophotometer U-2900/U-2910 (Lab India). IR997.37 (aromatic C-H str); 3068.22 (O-H str); 2948.81 (C-H str); 1672.94 (C=O str); 1598.90 (C=C str); 1310.45 (C-O str); 1257.30 (C-O str). MS (APCI-MS)(m/z (%)): 522 (2M+H+; 100), 217.0 (M+H+; 35) (KBr disc method, Bruker optics-IFS 66v/s vacuum FT-IR). 1H NMR (in ppm); δ=7.75 (dd, J=15.2,11.8Hz, 1H,H-6); δ=7.39 (dd, J=9.1,1.3 Hz, 1H, H-7); δ=7.10 (d, J=1.5 Hz,1H,H-4); δ=5.97 (d, J=8.1,1.6Hz,1H, H-4); δ=5.97 (d, J=5.0 Hz, 2H, H 2); δ=6.79 (m, 3H, H-1, H-2, H-3).13CNMR (CDCl3) (in ppm); δ= 140.24 (C=O, C-5); δ= 137.22 (C-H, C-3); δ= 132.44 (C-H, C-2); δ= 122.92 (C-H, C-5); δ= 105.52 (C-H,C-7); δ= 101.32 (C-H,C-2); DEPT 13CNMR (in ppm); δ= 140.24; 137.22; 125.78; 123.44; 22.92; 108.01; 105.52; 101.32 for C-5, C-3, C-1, C-6, C-2, C-5, C-4 and C-2(Oxford 60 and 90 MH z NMR spectrometer, deuterated chloroform (CDCl3) as solvent.

Acute toxicity testing

The animals were overnight fasted prior to the experiment. Different doses (50–3000 mg/kg, orally) of the hydroalcoholic and acetone extracts of the plant and 2-piperidone were administered to groups of rats. The animals were observed continuously for 1 hour, next half-hourly intervals for 4 hours for any gross changes in their behaviour and then up to 24 hours for any mortality and toxicity as per the Organization for Economic Cooperation and Development (OECD) guidelines 425.14,31, 32.

Hepatoprotective activity by carbon tetrachloride-induced liver toxicity in rats

Wistar rats (100-150 g), of either sex, 54 in number were equally divided into 9 groups containing 6 animals.

Fig. 5 — a) Structure of piperine and b) Hydrolysis of piperine to piperic acid

Structural characterization of piperic acid

The obtained product was analyzed by physico-chemical studies and structural characterization by UV, IR, Mass, and NMR spectroscopy. The UV absorbance of 0.01% w/v solution of the drug was determined between 200-400 nm. State: Yellow coloured compound. UV absorbance: 344 nm UV-visible double beam spectrophotometer U-2900/U-2910 (Lab India). IR997.37 (aromatic C-H str); 3068.22 (O-H str); 2948.81 (C-H str); 1672.94 (C=O str); 1598.90 (C=C str); 1310.45 (C-O str); 1257.30 (C-O str). MS (APCI-MS)(m/z (%)): 522 (2M+H+; 100), 217.0 (M+H+; 35) (KBr disc method, Bruker optics-IFS 66v/s vacuum FT-IR). 1H NMR (in ppm); δ=7.75 (dd, J=15.2,11.8Hz, 1H,H-6); δ=7.39 (dd, J=9.1,1.3 Hz, 1H, H-7); δ=7.10 (d, J=1.5 Hz,1H,H-4); δ=5.97 (d, J=8.1,1.6Hz,1H, H-4); δ=5.97 (d, J=5.0 Hz, 2H, H 2); δ=6.79 (m, 3H, H-1, H-2, H-3).13CNMR (CDCl3) (in ppm); δ= 140.24 (C=O, C-5); δ= 137.22 (C-H, C-3); δ= 132.44 (C-H, C-2); δ= 122.92 (C-H, C-5); δ= 105.52 (C-H,C-7); δ= 101.32 (C-H,C-2); DEPT 13CNMR (in ppm); δ= 140.24; 137.22; 125.78; 123.44; 22.92; 108.01; 105.52; 101.32 for C-5, C-3, C-1, C-6, C-2, C-5, C-4 and C-2(Oxford 60 and 90 MH z NMR spectrometer, deuterated chloroform (CDCl3) as solvent.

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Hepatoprotective activity by carbon tetrachloride-induced liver toxicity in rats

Wistar rats (100-150 g), of either sex, 54 in number were equally divided into 9 groups containing 6 animals.
in each to assess the hepatoprotective potential of plant extracts and isolated phytoconstituents. The animals from Group I served as the control, received the vehicle (olive oil) p.o. at a dose of 1 mL/kg body weight. Group II served as the positive control, received the carbon tetrachloride in olive oil (1:1) 2 mL/kg body weight orally once a day for 2 successive days a week. Group III received standard drug silymarin at a dose of 17.5 mg/kg p.o. twice (9:00 a.m. and 4:00 p.m.) at 6 hours interval a day at the volume of 7.2 mL/kg. Silymarin was suspended in 0.5% sodium carboxymethyl cellulose in distilled water. Test groups IV and V received G. serrata hydroalcoholic extract (GSHA) at a dose of 250 mg/kg at the volume of 7.2 mL/kg and 500 mg/kg at the volume of 7.5 mL/kg. Test groups VI and VII received acetone extract (GSAE) at a dose of 250 mg/kg at the volume of 7.5 mL/kg and 500 mg/kg at the volume of 7.8 mL/kg. Test groups VIII and IX received isolated compound (GSC) at a dose of 0.25 mg/kg at the volume of 6.6 mL/kg and 0.5 mg/kg at the volume of 7.5 mL/kg by gavage twice daily33,34. The stock solution of GSC was prepared in distilled water. The study duration was 28 days for all the treated groups. On termination, all animals were anaesthetized by injecting ketamine (100 mg/kg I.M.) in the thigh muscle35 and blood samples were collected separately by carotid bleeding into a sterilized dry centrifuge tube and allowed to coagulate at 37 °C for 30 minutes. The clear serum was separated and investigated for biochemical marker enzyme levels such as alanine aminotransferase (ALT/ SGPT) or serum glutamic pyruvic transaminase, aspartate aminotransferase (AST/ SGOT) or serum glutamic oxaloacetic transaminase and alkaline phosphatase (ALP) to assess the liver function (Table 3)32,36,37.

### Histopathology studies

Livers were carefully collected, examined, rinsed with a solution of 10% NaCl, weighed and preserved in 10% formalin. Liver pieces (3-5) weighing about 1 g were collected, fixed in formalin and carnoy solution. About 3-5 sections of each liver were embedded, cut, stained with azocarmine blue (AZAN), and observed under a trinocular microscope (Esaw) (Plate 1a-i)32,36-38.

### Statistical analysis

The results were expressed as mean±SEM (Standard error mean) of 6 animals from each group. The data were evaluated by One-way ANOVA (Analysis of variance), followed by Dunnett’s multiple comparison tests. Differences among groups were significant at *P <0.05, **P <0.01, and ***P <0.001 respectively (Table 3).

### Results and Discussion

The basified chloroform fractions (F1-F5) obtained by column chromatography were subjected to TLC to separate various types of alkaloids using appropriate solvent systems (Table 2). The TLC chromatograms developed reddish-brown spots after spraying with dragendorff’s reagent and brown spots after treatment with iodine vapour separately. The TLC profile of fractions (F1-F5) showed multiple spots. Further, the fractions (F1-F5) were subjected to column chromatography using the same solvent system (Table 2) to get subfractions (Fa-Fe). The Fa, Fb, Fd and Fe showed multiple spots whereas Fc showed a single yellow spot in TLC studies (Table 2). All the subfractions were kept in the refrigerator for future study except Fc. The subfraction Fc was studied to detect the presence of pyridazine alkaloids as it showed a single prominent yellow spot. In TLC study the Rf value (0.63) obtained was comparable to the literature.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SGPT/ ALT (Mean) U/L</th>
<th>SGOT/ AST (Mean) U/L</th>
<th>ALP (Mean) U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control/ Normal</strong></td>
<td>127.7±0.57</td>
<td>102.1±0.40</td>
<td>199.25±1.20</td>
</tr>
<tr>
<td>CCl4 in olive oil (1:1), 2 mL/Kg</td>
<td>279.2±0.90</td>
<td>275.23±0.70</td>
<td>315.11±2.10</td>
</tr>
<tr>
<td>Silymarin 17.5 mg/kg</td>
<td>131.0±0.80***</td>
<td>106±0.60***</td>
<td>207±0.50***</td>
</tr>
<tr>
<td>GSHA 250 mg/kg</td>
<td>181.2±6.68*</td>
<td>154.5±6.10***</td>
<td>229.8±4.31**</td>
</tr>
<tr>
<td>GSHA 500 mg/kg</td>
<td>147.5±2.77***</td>
<td>144±4.38***</td>
<td>208±0.77***</td>
</tr>
<tr>
<td>GSAE 250 mg/kg</td>
<td>192.2±4.68*</td>
<td>145.5±7.10***</td>
<td>238.8±1.31***</td>
</tr>
<tr>
<td>GSAE 500 mg/kg</td>
<td>147.5±6.77***</td>
<td>134±4.58***</td>
<td>217±0.32***</td>
</tr>
<tr>
<td>GSC 0.25 mg/kg</td>
<td>160±0.59***</td>
<td>127±1.24***</td>
<td>236±1.03***</td>
</tr>
<tr>
<td>GSC 0.5 mg/kg</td>
<td>140±0.21***</td>
<td>117±0.44***</td>
<td>219±0.12***</td>
</tr>
</tbody>
</table>

Values are Mean±S.E.M. (n= 6 rats per each group). *P <0.05, **P <0.01, ***P <0.001 significantly different from the group treated with CCl4, One-way ANOVA, Dunnett’s multiple comparison tests.

Table 3 — Hepatoprotective effects of G. serrata extracts and isolated compound by CCl4 induced hepatotoxicity method

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INdIAN J NAT PROD RESOUR, DECEMBER 2019

242
reports of one of the pyridine-piperidine alkaloids *i.e.*, piperine. Further, subfraction Fc was subjected to HPLC analysis to confirm the presence of piperine.

In HPLC analysis the subfraction Fc showed similar retention time (Rt- 5.61 min) to that of standard piperine (Rt- 5.67 min) (Fig. 3,4). The subfraction Fc was injected into the HPLC system in the concentration of 1 mg/mL and the amount of piperine present was found to be 3.92% (Table 1). The separation was monitored at 343 nm. The isolated compound (70.1 mg) was yellow crystalline in nature with an ammonical smell, pepper-like taste with a melting point at 126 °C. It was slightly soluble in water, soluble in alcohol, chloroform, and ether. It showed UV absorbance at 343 nm. The obtained values correlated with literature data. In the present study, piperine was isolated for the first time from this plant species (*G. serrata*). On treatment with
alcoholic basic hydrolysis, the piperine was converted into piperic acid (Fig. 5b). The obtained piperic acid was subjected to structure elucidation studies. The hydrolysis into piperic acid further proves the presence of piperine in the selected species.

The piperic acid obtained on hydrolysis was yellow in colour with a characteristic smell, m.p. 214 °C, insoluble in cold water and petroleum ether, soluble in boiling methanol, sparingly soluble in ether and benzene, which were comparable to literature data. UV absorbance was found to be 344 nm. The IR spectrum showed a characteristic band at 2917.22 cm\(^{-1}\) (O-H stretching) which indicates the presence of hydroxyl moiety. The other characteristic band at 1672.94 cm\(^{-1}\) indicates the presence of carbonyl (C=O, stretching) group. The band at 1310.45 and 1257.30 cm\(^{-1}\) correspond to (C-O stretching) (Fig. 6). The mass spectrum showed a molecular ion peak at m/z 217.0 which corresponds to the molecular weight and molecular formula (C\(_{12}\)H\(_{10}\)O\(_4\)) of piperic acid (Fig. 7). In proton NMR spectrum (Fig. 8a-c) the compound showed a doublet of doublet at \(\delta = 7.75\) ppm.
Fig. 8 — a) $^1$H NMR spectrum of piperic acid (0.5-7.5 ppm), b) $^1$H NMR spectrum of piperic acid expanded (0.0-6.5 ppm), and c) $^1$H NMR spectrum of piperic acid expanded (6.70-7.80 ppm)

(dd, 1H, $J = 15.2, 11.8$) which is assignable to the aromatic proton of C-6. The spectrum exhibited another doublet of doublet at $\delta = 7.39$ ppm (dd, 1H, $J = 9.1, 1.3$) indicating the single aromatic proton at C-7. Presence of doublet at $\delta = 7.10$ ppm (d, 1H, $J = 1.5$) and doublet of doublet at 6.98 (dd, 1H, $J = 8.1, 1.6$) indicates single aromatic protons of C4 and C4’, respectively. A doublet at $\delta = 5.97$ ppm (d, 2H, $J = 5.0$) was assignable to two protons of C-2. A multiplet at $\delta = 6.79$ indicates three protons of C-1’, C-2’, C-3’. The $^{13}$C NMR spectrum showed carbonyl carbon at $\delta = 140.24$ ppm, at $\delta$ 125.78, 123.44, 122.92, 108.01, 105.52 and 101.32 ppm showed methylene carbons at C-6, C-2’, C-5, C-4, C-7, and C-2 (Fig. 9). DEPT $^{13}$CNMR spectrum showed methylene groups at $\delta=140.24, 125.78, 123.44, 122.92, 108.01, 105.52, 101.32$ ppm for carbons at positions 5’, 3’, 1’, 6’, 2’, 5, 4, 7 and 2 (Fig. 10). The compound passes dragendorff’s test for carboxyl functional groups. The results of spectroscopy agree with spectral data reported for piperic acid$^{12}$, hence the obtained compound was confirmed to be piperic acid (Fig. 11). Acute toxicity studies revealed that both plant extracts and GSC (Piperic acid) did not produce any toxic symptoms when administered orally to rats at doses of 100-3000 mg/kg. The experiment recorded no toxic symptoms and no death of the animals during the study.

The animal group administered with CCl$_4$ in olive oil (1:1) showed significant increase in the levels of marker enzymes. Plant extracts and standard drug silymarin demonstrated ability to counteract the CCl$_4$ induced hepatotoxicity by decreasing the raised marker enzyme levels ALT, AST and ALP at $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels of significance compared to CCl$_4$ induced positive control group. GSC treated group at 0.5 mg/kg showed a better reduction in the raised levels of marker enzymes ALT, AST and ALP than CCl$_4$ (ALT 279 and AST 275 U/L) induced positive control group. GSAE treated group at 500 mg/kg prominently reduced the raised levels of ALP (217 U/L) than GSC (ALP 219 U/L) at 0.5 mg/kg. GSAE treated group at 500 mg/Kg b/w (ALT 147, AST 144 and ALP 208 U/L) and TPC treated group at 0.5 mg/kg b/w (ALT 140, AST 117, and ALP 219 U/L) have exhibited better reduction in the raised levels of enzymes than other groups when compared to CCl$_4$ induced positive.
Fig. 9 — $^{13}$CNMR spectrum of piperic acid

Fig. 10 — DEPT $^{13}$CNMR spectrum of piperic acid
control group at 17.5 mg/kg (ALT 279, AST 275, and ALP 315 U/L) \((P < 0.001)\) (Table 3).

It was observed from the liver histopathological study that the normal control group had normal hepatocellular degeneration such as necrosis and fatty changes. Prominent damage to central lobular region appeared in the liver cells \(^3\) (Plate 1b). The histopathological studies of the liver section in the standard group have shown the normal cellular architecture, cytoplasm and visible central veins \(^3\) (Plate 1c). GSHA treated group at 250 mg/kg and 500 mg/kg has shown moderate recovery and protection of hepatocytes degradation (Plate 1d,e). GSAE treated group at 250 mg/kg has shown moderate recovery (Plate 1f), but at the dose of 500 mg/kg offered normal hepatocytes recovery and protection of hepatocytes degradation (Plate 1g). GSC treated group at 0.25 and 0.5 mg/kg restored the structural damages (Plate 1h,i) induced by CCl\(_4\). Treatment with GSHA, GSAE, and GSC decreased the abnormal liver architecture induced by CCl\(_4\) (Plate 1g) and restored the altered histopathological changes. GSAE treated group at 500 mg/kg (Plate 1g) and GSC treated group at 0.5 mg/kg (Plate 1i) exhibited better regeneration of hepatocytes when compared to normal control. GSC treated group showed better recovery of hepatocytes than plant extracts compared to control. The results indicate that plant extracts exhibited hepatoprotective activity due to pipereric acid obtained by hydrolysis of piperine. Pipereric acid was studied for the first time for hepatoprotective activity. Therefore both plant extracts and pipereric acid can treat hepatic ailments.

CCl\(_4\) induced hepatotoxicity causes peroxydative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes. Further, it causes liver steatosis, a process involving endoplasmic reticulum (ER) stress-induced cytochrome P450 2E1 activation and ROS (Reactive Oxygen Species) production\(^4\). Cytochrome P450 2E1 is a key enzyme in the metabolic activation of many low molecular weight toxicants, an important contributor to oxidative stress found predominantly in the ER of the liver. The changes associated with CCl\(_4\) are biotransformed by the cytochrome P-450 system to produce CCl\(_3\) a free radical, that binds to lipoprotein, leads to peroxydation of lipids of ER and finally result in cell death\(^4\). Cytosol releases a variety of enzymes into the bloodstream during liver cell plasma membrane damage. The increased levels of ALT, AST and ALP show cellular leakage and loss of functional integrity of the cell membrane as a result of hepatic damage\(^4\). Their estimation in the serum is a useful measure for determining the hepatocellular damage\(^4\).

All the treated animals showed significantly increased levels of the liver markers. The treatment with plant extracts and GSC decreased the raised levels of biochemical marker enzymes (Table 3).

A comparative histological examination of liver from the study groups further corroborated the hepatoprotective effect of \(G.\ serrata\) extracts and pipereric acid. Histological profile from the normal control group showed prominent central vein and normal arrangement of hepatic cells (Plate 1a). Histopathological examination of CCl\(_4\) induced liver section showed higher degrees of pathological changes centrilocular necrosis of hepatic cells, vacuolization and fatty regeneration (Plate 1b). The animals treated with plant extracts (GSHA 250 and 500 mg/kg) (Plate 1d,e) showed moderate recovery where GSAE at 500 mg/kg (Plate 1g) and pipereric acid (GSC) at 0.5 mg/kg (Plate 1i) showed more prominent recovery of hepatic cell damage. It was clear that necrosis was absent, normal hepatic cords and lesser fatty infiltration were present. The observations show that acetone extract exhibited hepatoprotective response due to piperidine moiety. HPLC analysis indicated piperine from chloroform fraction of acetone extract (Table 1; Fig. 3,4). The compound pipereric acid obtained on the degradation of piperine demonstrated more effective functional improvement of hepatocytes than plant extracts. The results of the histopathological study also support the results of biochemical trials.

\(G.\ serrata\) exhibited antidiabetic and antioxidant activity on streptozotocin-induced diabetic rats where the plant extracts inhibited ROS formation induced by streptozotocin\(^4\). Phytochemicals isolated from \(G.\ serrata\), kaempferol\(^4\) and oleuropein\(^4\) have exhibited hepatoprotective activity. Comparative results show that pipereric acid exhibited better activity than kaempferol and oleuropein. Further, the
hepatoprotective studies conducted on plant extracts and piperic acid were comparable to the earlier literature reports. Moreover, piperic acid inhibits lipooxygenase (LOX) activity. Piperic acid prevents experimentally induced ER stress in vitro. The anti hyperlipidemic activity was reported with respect to simvastatin, a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which regulates cholesterol biosynthesis, mainly in the liver. It also exhibited potent anti-inflammatory and free radical scavenging properties. Therefore, from the current research findings and in correlation to the available literature reports the possible mechanism of the current research findings and in correlation to the pharmacological studies.

**Conclusion**

In conclusion, the results of the study indicate that the compound piperic acid obtained by hydrolysis of piperine showed a significant decrease in the raised enzyme levels as well as regeneration of hepatocytes equipotent to standard drug silymarin against CCl4-induced hepatotoxicity. Further studies can be carried out to develop piperic acid as a potential hepatoprotective agent.

**Acknowledgement**

Authors thank Radiant Research Laboratories, Bangalore, Karnataka, India for providing facilities to carry out the phytochemical analysis. Authors thank Vijay Kumar N, Research scholar, CSIR, Jammu, India for help in interpretation studies. Authors also thank Dr. M. Narender, Associate Professor, Department of Pharmaceutical Chemistry, and Mr N. Kodanda Ram, Associate Professor, Department of Pharmacology, Vijaya Institute of Pharmaceutical Sciences for Women, Enikepadu, Vijayawada, for reviewing and giving suggestions in phytochemical and pharmacological studies.

**Conflict of interest**

The authors declare no conflict of interest.

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