Rheum palmatum root extract inhibits hepatocellular carcinoma in rats treated with diethylnitrosamine

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Keywords
diethylnitrosamine; DNA fragmentation; hepatocellular carcinoma; HPLC-MS/MS; Rheum palmatum

Abstract

Objectives The aim of this study was to investigate the potential anticancer properties of a methanol extract of Rheum palmatum roots against diethylnitrosamine (DENA)-induced hepatocellular carcinoma (HCC) in rats and to characterize its phytoconstituents.

Methods HPLC-PDA-MS/MS was used to profile the secondary metabolites in R. palmatum root extract. HCC was induced using diethylnitrosamine (DENA). The activity of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), alpha-fetoprotein (AFP), total proteins, serum albumin and serum globulin was determined. DNA fragmentation and histopathological examination and GST-P immunostaining were also studied.

Key findings LC-MS/MS analysis identified 16 compounds belonging to anthraquinones, flavonoids and tannins. The root extract significantly reduced the elevated liver enzymes ALT and AST and increased total proteins, albumin and globulin in HCC-rats. Also, the tumour markers AFP and GGT levels were significantly reduced in HCC-rats treated with the extract. In addition, the extract significantly reduced elevated DNA fragmentation and decreased the numbers and areas of GST-P positive putative foci in HCC-rats.

Conclusions Rheum palmatum is a potential candidate to be explored for the treatment of hepatocellular carcinoma.

Introduction

Cancer comes as a second cause of death worldwide after cardiovascular diseases. Among its different types, hepatocellular carcinoma (HCC) is considered to be the sixth most common cancer globally and is among the most predominant malignant tumours in adults. Furthermore, HCC represents the major complication of liver cirrhosis. Diabetes, virus infection (chronic hepatitis B or C), drugs and mycotoxins are the major promoters of HCC. In addition, obesity, tobacco, oral contraceptives and alcohol are included among the causative agents for HCC.[1,2]

Systematic therapeutic options for HCC include liver transplantation, chemotherapy, radiation therapy and percutaneous interventions. However, these treatments often fail.[3] There is evidence that medicinal plants could lower the risk of HCC and liver cirrhosis.[4–7]

Rheum palmatum, commonly known as Chinese rhubarb, belongs to the family Polygonaceae. Rheum palmatum is rich in anthraquinones and tannins, and its extracts exhibit a wide array of pharmacological properties including laxative, hepatoprotective, antiviral, antibacterial, antidiabetic and antimetastatic effects.[8–11]

Administration of nitrosamines in animals, among them diethylnitrosamine (DENA), induces severe hepatic
alterations, including HCC. Thus, application of DENA has become a robust experimental tool for HCC induction.\textsuperscript{[12]}

Although \textit{R. palmatum} was investigated for plethora of biological activity, its chemoprevention against hepatocarcinogenesis was not reported before. Therefore, the aim of our study was to investigate the chemical composition of a methanol extract of \textit{R. palmatum} roots using HPLC-PDA-MS/MS. Also, we determined its potential oncostatic effects against DENA-induced HCC in rats.

\section*{Materials and Methods}

\subsection*{Chemicals}

N-Nitrosodiethylamine (DENA) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and phenobarbital (PB) from ALEX PHARM Company, Egypt. Unless mentioned, all solvents were of analytical grade.

\subsection*{Extraction}

The root of \textit{R. palmatum} was collected from plants grown in a private garden, Cairo, Egypt. The plant was authenticated by Mrs. Therese Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and El-Orman Botanical Garden, Giza, Egypt. The root was dried at room temperature and then powdered in a grinder. To prepare a methanol extract, 100 g of the powder was soaked in methanol (1 l) for 48 h in a shaker. The residue was removed by filtration. The filtrate was evaporated until dryness at 40 °C under reduced pressure in a rotary evaporator giving a semisolid extract. The latter was frozen and lyophilized giving 12 g dry powder.

\subsection*{HPLC-PDA-MS/MS}

HPLC-PDA-MS/MS was employed to identify the chemical constituents in the methanol extract of \textit{R. palmatum}. The LC system was Thermofinnigan (ThermoElectron Corporation, Austin, TX) coupled with an LCQ-Duo ion trap mass spectrometer with an ESI source (ThermoQuest). A C18 reversed-phase column (Zorbax Eclipse XDB-C18, rapid resolution, 4.6 × 150 mm, 3.5 μm, Agilent, Santa Clara, CA) was employed.\textsuperscript{[13]} Water and acetonitrile (ACN) (0.1% formic acid each) were used from 5% to 30% ACN over 60 min and then increased to 90 in the following 60 min with flow rate 1 ml/min to elute the sample with a 1 : 1 split before the ESI source. The sample was injected automatically using autosampler surveyor ThermoQuest. The instrument was controlled by Xcalibur software (Xcalibur\textsuperscript{TM} 2.0.7, Thermo Scientific, Waltham, MA). The MS operated in the negative mode with a capillary voltage of −10 V, a source temperature of 200 °C and high purity nitrogen as a sheath and auxiliary gas at a flow rate of 80 and 40 (arbitrary units), respectively. Collision energy of 35% was used in MS/MS fragmentation.\textsuperscript{[14]} The ions were detected in a full scan mode and mass range of 50–2000 m/z.

\section*{Experimental animals}

This study was carried on healthy adult male Wistar rats (\textit{Rattus norvegicus}) of an average body weight about 160–200 g. They were purchased from Egyptian Vaccine and Antibody Company (VACSERA, Giza, Egypt). Animals were randomly selected and housed in designed cages with hardwood chips. They were kept in the laboratory under constant conditions of 25 °C and 12 h light/dark cycle for 2 weeks before experimentation. They were fed on a standard rodent pellet diet manufactured by the Egyptian Company for Oil and Soap. Also, animals were supplied with tap water during the period of experiment. The experiments were approved by the ethical committee of the University of Sadat City, Sadat City, Menoufia Province, Egypt.

\section*{Experimental design}

Animals were divided into four groups; each group contained eight rats. Group (A): Normal control that received drinking water over the experimental period. Group (B): HCC-rats that were injected intraperitoneally with a single dose of DENA (200 mg/kg of body weight) followed by administration of phenobarbital (PB) 0.05% for 12 weeks from the second week, phenobarbital (PB) acts as a tumour promoter.\textsuperscript{[15]} Group (C): The rats were also injected intraperitoneally with a single dose of DENA (200 mg/kg of body weight) followed by administration of PB 0.05% for 12 weeks from the second week. After 10 days of DENA injection; \textit{R. palmatum} extract (100 mg/kg body weight (b.w)) was given orally for 12 weeks. Group (D): The rats of this group obtained an oral dose of the \textit{R. palmatum} extract (100 mg/kg body weight) alone for 12 weeks.

\section*{Sampling and serum collection}

At the end of the treatment, the rats were sacrificed 24 h after the last dose under mild ether anaesthesia and both total body weight and liver weight were determined. Blood samples were collected by retro-orbital puncture in sterilized, heparinized tubes, which were then centrifuged using a microcentrifuge. The plasma was separated and used for the evaluation of serum biochemical parameters. After sacrificing the rats, their liver was dissected, drained off blood, washed with saline and stored in 10% formalin, for a histopathology study. A portion tissue was excised to prepare the homogenate for the assay of various parameters.
Blood samples were placed at room temperature for clotting and then centrifuged at 950g for 15 min. Sera were then separated and stored at −20°C in aliquots for individual biochemical investigations.

Physical investigation

Both of total body weight and liver weight were determined at the end of study for every rat from each group. The relative liver weights can be calculated from the following equation: Relative liver weight = Absolute liver weight (g)/Body weight of rat on sacrifice day (g) × 100.\(^{16}\)

Biochemical analysis

The activity of serum alanine aminotransferase (ALT) was measured colourimetrically according to the method described before.\(^{17}\) Aspartate aminotransferase (AST) activity was estimated by enzymatic rate method.\(^{18}\) Alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) activity was estimated by established methods.\(^{19}\) Alpha-fetoprotein (AFP) activity estimation was carried out by a commercial diagnostic centre. Total proteins were estimated according to Burtis.\(^{20}\) Thus, the intensity of the colour formed is proportional to the total protein concentration in the sample. The serum albumin concentration was estimated by bromocresol green colourimetric reaction, according to the method as described by Doumas.\(^{21}\) The serum globulin concentration was calculated by subtracting the value of serum albumin from the total serum protein (globulin conc. (g/dl) = Total protein conc. (g/dl) – albumin conc. (g/dl)).

DNA Fragmentation assay

DNA fragmentation assay was determined by the method described by Khelifa et al.\(^{22}\) Total genomic DNA was isolated from the rat livers belonging to different groups using a DNA extraction kit (TIANamp Genomic DNA Kit) and analysed by electrophoresis on 1.5% agarose gel containing 0.1 mg/ml ethidium bromide and visualized under an UV illuminator.

Histopathological examination

Liver tissue was fixed in 10% neutral buffered formalin solution for 24 h at room temperature at 37°C, following dehydration in ascending series of ethanol (70%, 80%, 90% and 100%). Tissue samples were processed with paraffin wax. Sections (5-μm) were stained with haematoxylin and eosin (H & E) and were examined under a light microscope. The sections were viewed and photographed.\(^{23}\) Immunostaining of GST-P was carried out using a polyclonal rabbit antirat GST-P antibody (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan; 1:500).\(^{24,25}\)

Statistical analysis

The results were expressed as mean ± SD of different groups. The differences between the mean values were evaluated by one-way analysis of variance ANOVA followed by Tukey-Kramer multiple comparison test using GraphPad Prism software (version 5, GraphPad Software, Inc., San Diego, CA, USA). P values <0.05 were considered to be statistically significant.

Results and Discussion

Chemical profiling of Rheum palmatum

The secondary metabolites were profiled using LC-MS/MS and yielded 16 compounds. The anthraquinone emodin and its glucoside dominated the extract along with the other anthraquinones, flavonoids and tannins (Table 1 and Figure 1). Among the detected constituents, the anthraquinone glucoside chrysophanol glycoside showed [M-H]− at m/z 415 and a main daughter ion at 253 (Figure 2a).

Another anthraquinone, rhein, gave [M-H]− at m/z 283 and two fragments ions at 257 and 239 as described before (Figure 2b).\(^{26}\) A sulphated anthraquinone, emodin sulphate, exhibited a molecular ion peak at [M-H] m/z 349 and a main fragment at 269 [M-H – 80] along with the anthraquinone emodin (the major compound) (Figures 1 and 2c,d, respectively).\(^{27}\) Several flavonoids, stilbenoids, tannins and phenolic acids were also recorded in the extract. A similar pattern had been reported for Chinese R. palmatum.\(^{9,26,28}\) Representative structures from some compounds are shown in Figure S1, and MS/MS fragmentation of some identified compounds is presented in Figures S2–S8.

Biological activity

Physical results

Body weight and liver weight are sensitive indicators for HCC development. Following DENA injection, the liver weight was significantly elevated in HCC-rats (group B) compared with the normal control group (P < 0.001) and the body weight in HCC-rats (group B) was significantly reduced compared with the normal control group (P < 0.05), indicative for a HCC induction. In extract-treated rats (group C) (a dose of 100 mg/kg b.w), a significant decline was observed compared with the HCC-rats (group B) (P < 0.001). As for the relative liver weight, a significant elevation was observed in HCC-rats (group B) compared...
with the normal control group ($P < 0.001$). The extract restored the relative liver value in the HCC-rats (group C), similar to the control group. On the other hand, the liver weight and the relative liver weight were not significantly altered in the \textit{R. palmatum} extract group (D) (Table 2).

### Biochemical results

Liver enzymes, among them ALT, AST and ALP, are reliable markers to determine liver activity.\cite{29} When the liver is damaged or injured, a breakdown in the cell membrane architecture is likely to happen which leads to the release of these enzymes into the serum.\cite{30}

Injection of DENA-induced HCC, manifested by a significant elevation in the liver enzyme activity ALT, AST and ALP compared with the control group (Table 3). A significant decline was observed in ALT and AST in the treated group (C) compared with HCC-rats (group B; $P < 0.01$; Table 3).

Again, DENA injection significantly reduced total protein, albumin and globulin levels in HCC-rats (group B) compared with the control group (A) (Table 4). The root

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**Table 1** Chemical composition of the methanol extract from \textit{Rheum palmatum}

<table>
<thead>
<tr>
<th>No.</th>
<th>Rt</th>
<th>M-H</th>
<th>MS/MS</th>
<th>Tentative identification</th>
<th>Relative abundance (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.93</td>
<td>331</td>
<td>169, 125</td>
<td>Gallic acid glucoside</td>
<td>1.38</td>
<td>[9]</td>
</tr>
<tr>
<td>2</td>
<td>4.23</td>
<td>169</td>
<td>125</td>
<td>Gallic acid</td>
<td>1.25</td>
<td>[26]</td>
</tr>
<tr>
<td>3</td>
<td>15.92</td>
<td>451</td>
<td>289</td>
<td>Epicatechin glucoside</td>
<td>0.13</td>
<td>[9]</td>
</tr>
<tr>
<td>4</td>
<td>27.37</td>
<td>389</td>
<td>227</td>
<td>Resveratrol glucoside</td>
<td>1.20</td>
<td>[34]</td>
</tr>
<tr>
<td>5</td>
<td>31.46</td>
<td>419</td>
<td>257</td>
<td>Gentisin glucoside</td>
<td>4.42</td>
<td>[35]</td>
</tr>
<tr>
<td>6</td>
<td>33.31</td>
<td>541</td>
<td>389, 227</td>
<td>Resveratrol galloyl-glucoside</td>
<td>5.18</td>
<td>[34]</td>
</tr>
<tr>
<td>7</td>
<td>33.63</td>
<td>447</td>
<td>285</td>
<td>Kaempferol glucoside</td>
<td>4.56</td>
<td>[13]</td>
</tr>
<tr>
<td>8</td>
<td>39.63</td>
<td>431</td>
<td>269</td>
<td>Apigenin glucoside</td>
<td>3.43</td>
<td>[36]</td>
</tr>
<tr>
<td>9</td>
<td>45.50</td>
<td>583</td>
<td>431, 269</td>
<td>Apigenin galloyl-glucoside</td>
<td>3.46</td>
<td>[36]</td>
</tr>
<tr>
<td>10</td>
<td>50.22</td>
<td>431</td>
<td>269</td>
<td>Aloe-emodin glucoside</td>
<td>7.01</td>
<td>[9]</td>
</tr>
<tr>
<td>11</td>
<td>50.35</td>
<td>415</td>
<td>253</td>
<td>Chrysophanol glucoside</td>
<td>0.58</td>
<td>[28]</td>
</tr>
<tr>
<td>12</td>
<td>57.71</td>
<td>285</td>
<td>285, 179</td>
<td>Kaempferol</td>
<td>5.28</td>
<td>[13]</td>
</tr>
<tr>
<td>13</td>
<td>66.26</td>
<td>269</td>
<td>269</td>
<td>Aloe-emodin</td>
<td>1.39</td>
<td>[26]</td>
</tr>
<tr>
<td>14</td>
<td>68.68</td>
<td>283</td>
<td>257, 239</td>
<td>Rhein</td>
<td>7.01</td>
<td>[26]</td>
</tr>
<tr>
<td>15</td>
<td>77.29</td>
<td>349</td>
<td>269</td>
<td>Emodin sulphate</td>
<td>3.12</td>
<td>[27]</td>
</tr>
<tr>
<td>16</td>
<td>79.59</td>
<td>269</td>
<td>269</td>
<td>Emodin</td>
<td>48.15</td>
<td>[9]</td>
</tr>
</tbody>
</table>

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**Figure 1** LC-MS base peak in the negative ionization mode ESI of the methanol extract of \textit{Rheum palmatum} roots.
extract (a dose of 100 mg/kg b.w.) significantly improved the level of total proteins, albumin and globulin in group (C) to normal values as found in the control group (Table 4).

The liver enzyme (GGT) is a membrane-bound enzyme and is one of the specific indicators of liver injury. Various pathologic conditions may alter its activity, among them, development of carcinogenesis.[30,31] In HCC-rats (group C), DENA injection increased AFP and (GGT) levels compared with control rats (group A) (P < 0.001). HCC-rats, which were treated with R. palmatum extract (group C) (100 mg extract/kg b.w.), exhibited a significant reduction in AFP and GGT levels when compared with HCC-rats (group B; Table 5).

DNA fragmentation analysis in liver

DNA fragmentation analysis is a reliable test to determine the DNA quality. In this study group, HCC-rats (group B) showed a strong degree of hepatic DNA breaks, whereas

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**Table 2** Rheum palmatum root extract affects body weight, liver weight and relative liver weight

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (gm)</th>
<th>Liver weight (gm)</th>
<th>Relative liver weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group (A)</td>
<td>230.30 ± 27.77</td>
<td>6.50 ± 0.44</td>
<td>2.85 ± 0.35</td>
</tr>
<tr>
<td>HCC group (B)</td>
<td>193.60 ± 13.34</td>
<td>10.14 ± 1.84</td>
<td>5.23 ± 0.85</td>
</tr>
<tr>
<td>HCC-rats treated with root extract (C)</td>
<td>231.50 ± 29.73</td>
<td>7.13 ± 0.61</td>
<td>3.00 ± 0.35</td>
</tr>
<tr>
<td>Normal rats treated with root extract (D)</td>
<td>246.00 ± 14.55</td>
<td>6.78 ± 0.43</td>
<td>2.97 ± 0.23</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SD. *Significantly different from the control group (P < 0.05). **Significantly different from the control group (P < 0.001). †Significantly different from the HCC group (P < 0.001).

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**Table 3** Effect of Rheum palmatum root extract (a dose of 100 mg/kg b.w.) on the hematopoietic enzyme (ALT, AST, ALP) levels

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (U/l)</th>
<th>AST (U/l)</th>
<th>ALP (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group (A)</td>
<td>22.35 ± 1.05</td>
<td>43.55 ± 1.37</td>
<td>19.41 ± 4.87</td>
</tr>
<tr>
<td>HCC-rats group (B)</td>
<td>87.28 ± 2.92b</td>
<td>110.0 ± 9.81b</td>
<td>26.38 ± 3.22‡</td>
</tr>
<tr>
<td>HCC-rats treated with root extract (C)</td>
<td>24.05 ± 1.30c</td>
<td>47.95 ± 3.71c</td>
<td>22.74 ± 4.33</td>
</tr>
<tr>
<td>Normal rats treated with root extract (D)</td>
<td>23.20 ± 1.92c</td>
<td>44.91 ± 1.61c</td>
<td>19.65 ± 5.31†</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase. All data are expressed as mean ± SD. *Significantly different from the control group (P < 0.01). ‡Significantly different from the control group (P < 0.001). †Significantly different from the HCC group (P < 0.01).
DNA fragmentation was significantly elevated compared with the control group (A) \( (P < 0.001) \). *Rheum palmatum* extract exhibited promising antimutagenic activity; it significantly decreased the value of DNA fragmentation in group C by 72.12% compared with the HCC-rats (group B) \( (P < 0.001; \text{Figure 3}) \). Also, rats which were treated with 150 mg/kg b.w. (group D) did not show DNA fragmentation. This confirmed that the extract has no genotoxic activity. Similar results were reported from other extracts rich in anthraquinones.\[9\]

### Table 4
Effect of *Rheum palmatum* extract on the serum protein profile (total protein, albumin, globulin, albumin/globulin ratio).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total proteins</th>
<th>Albumin (g/dl)</th>
<th>Globulin (g/dl)</th>
<th>Albumin/Globulin ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group (A)</td>
<td>6.65 ± 1.69</td>
<td>4.09 ± 0.32</td>
<td>2.56 ± 0.71</td>
<td>1.45 ± 0.25</td>
</tr>
<tr>
<td>HCC group (B)</td>
<td>4.33 ± 0.41(^a)</td>
<td>2.96 ± 0.24(^a)</td>
<td>1.37 ± 0.24(^b)</td>
<td>1.48 ± 0.53</td>
</tr>
<tr>
<td>HCC-rats treated with root extract (C)</td>
<td>6.35 ± 1.28(^c)</td>
<td>3.84 ± 0.64(^c)</td>
<td>2.50 ± 0.19(^c)</td>
<td>1.97 ± 0.69</td>
</tr>
<tr>
<td>Normal rats treated with root extract (D)</td>
<td>6.528 ± 1.24(^c)</td>
<td>4.37 ± 0.63(^c)</td>
<td>2.16 ± 0.38(^c)</td>
<td>1.81 ± 1.22(^c)</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SD. \(^a\)Significantly different from the control group \( (P < 0.01) \). \(^b\)Significantly different from the control group \( (P < 0.001) \). \(^c\)Significantly different from the HCC group \( (P < 0.01) \).

### Table 5
Effect of *Rheum palmatum* extract on the tumour markers [alpha-fetoprotein (AFP) and gamma-glutamyl transferase (GGT)] levels.

<table>
<thead>
<tr>
<th>Groups</th>
<th>AFP (UI)</th>
<th>GGT (UI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group (A)</td>
<td>4.69 ± 0.66</td>
<td>11.71 ± 3.89</td>
</tr>
<tr>
<td>HCC group (B)</td>
<td>60.33 ± 3.02(^a)</td>
<td>25.49 ± 4.95(^a)</td>
</tr>
<tr>
<td>HCC-rats treated with root extract (C)</td>
<td>23.58 ± 0.66(^b)</td>
<td>15.80 ± 2.97(^b)</td>
</tr>
<tr>
<td>Normal rats treated with root extract (D)</td>
<td>5.60 ± 0.69(^b)</td>
<td>12.67 ± 3.84(^b)</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SD. \(^a\)Significantly different from the control group \( (P < 0.001) \). \(^b\)Significantly different from the HCC group \( (P < 0.01) \).

Figure 3 (I) Influence of *Rheum palmatum* root extract (a dose of 100 mg/kg b.w.) on DENA-induced hepatic DNA fragmentation. Group A (control lane): No DNA fragmentation in normal control. Group B (HCC lane): Strong DNA fragmentation was observed in HCC-rats. Group C (HCC-rats treated with the extract): Weak DNA fragmentation. Group D (*R. palmatum* lane): No DNA fragmentation in normal rats administered with *R. palmatum* root extract. (II) The effect of *R. palmatum* extract on the intensity of released DNA fragments of liver genomic DNA fragmentation; the intensity of released DNA fragments was measured by ImageJ software, as a mean of optical density values. \(^a\)Significantly different from the control group \( (P < 0.001) \). \(^b\)Significantly different from the HCC group \( (P < 0.01) \).

**Histopathological findings**

The livers of the control group showed normal hepatocytes, arranged normally in cords around the central vein (Figure 4a). The liver of HCC-rats revealed an intense alteration in hepatocytes morphology towards malignancy indicated by the presence of numerous neoplastic hepatic nodules, which showed great cellular hyperplasia, pleomorphism, atypia as well as an increase in both nucleo-cytoplasmic ratio and mitotic figures. Multiple altered foci including clear, eosinophilic and basophilic types in addition to malignant nodules were observed (Figure 4b). In group (C), the extract ameliorated the deleterious effect of DENA in the histological structure of the liver, which revealed by marked decrease tissue disarrangement, neoplastic nodules and altered foci within the hepatic tissues (Figure 4c). The livers of normal animals treated with *R. palmatum* root extract alone were mostly of normal morphology and arrangement (Figure 4d). Regarding GST-P expression, negative expression was noticed within the liver of control animals (Figure 4e). An increase in both numbers and areas of positive foci was detected in HCC-induced group (Figure 4f). Meanwhile, a significant decline
was observed in the foci numbers and areas in diseased animals treated with the *R. palmatum* extract (Figure 4g).

The liver of *R. palmatum* extract-treated animals did not reveal any positive immunostaining (Figure 4h). Statistical analysis of GST-P foci numbers and areas revealed a significant decrease in both parameters in HCC-induced animals treated with *R. palmatum* root extract in comparison with HCC-induced animals (*P* < 0.001; Figure 5).

Diethylnitrosamine induces a series of neoplastic alterations mostly within hepatocytes morphology is known as preneoplastic or putative foci, which further develop into HCC. These foci were expressed GST-P and could be efficiently used as an endpoint of hepatocarcinogenesis process.

Interestingly, *R. palmatum* root extract markedly decreased the numbers and areas of putative foci.
Altogether, similar protective properties were reported from the Chinese *R. palmatum* as well as its individual components emodin, rhein, physcion, aloe-emodin and chrysophanol.[9,26,30] Additionally, emodin, the dominant compound, along with other anthraquinones, is likely responsible for the potential anticancer properties of *R. palmatum*. Also, rhein and emodin, isolated from *R. palmatum*, impeded hepatic fibrosis through inhibition of TGF-β1 expression.[11] In vitro studies of chrysophanol, rhein and emodin revealed apoptosis and necrosis of hepatic cancer cells. The major compound, emodin, inhibited cell adhesion in a number of different cancer cells as well.[31-33]

**Conclusions**

In this study, LC-MS profiling of a methanol extract of *R. palmatum* revealed 16 compounds belonging to anthraquinones, flavonoids and tannins. The extract exhibited substantial anti-hepatocarcinogenic activity against DENA-induced HCC in rats. To conclude, the extract showed pronounced improvements in all studied parameters indicating its beneficial effect in the prevention of HCC, which needs to be studied in more detail in additional systems.

**References**

7. Liu SY et al. Anthraquinones in *Rheum palmatum* and *Rumex dentatus* (Polygonaceae), and phorbol esters in *Jatropha curcas* (Euphorbiaceae) with molluscsidal activity against the schistosome vector snails *Oncomelania*, *Biophalaria*, and *Bulinus*. *Trop Med Int Health* 1997; 2: 179–188.
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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Representative structures from some compounds identified in the methanol extract of *Rheum palmatum* roots.

**Figure S2.** MS/MS spectrum of gallic acid.

**Figure S3.** MS/MS spectrum of gallic acid glucoside.

**Figure S4.** MS/MS spectrum of epicatechin glucoside.

**Figure S5.** MS/MS spectrum of resveratrol glucoside.

**Figure S6.** MS/MS spectrum of kaempferol glucoside.

**Figure S7.** MS/MS spectrum of apigenin glucoside.

**Figure S8.** MS/MS spectrum of kaempferol.