Electrospray ionization-mass spectrometry of different extracts of the organs of *Rumex cyprius* and their antihepatotoxic effect

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Abstract

**Purpose:** Phytochemical and biological investigations of the valuable plant, *Rumex cyprius*, family Polygonaceae, wildly grown in Saudi Arabia.

**Methods:** Chloroform, ethyl acetate and methanol extracts were prepared from the different organs of *R. cyprius*. The extracts were analyzed by electrospray ionization source coupled to a mass spectrometer (ESI-MS) and tandem mass spectrometer (ESI-MS/MS) at different collision energies. The plant organs (leaf, fruit and stem) were standardized on the bases of quercetin by HPLC, and determined for their hepatoprotection in tetrachloride-induced acute liver toxicity using a mouse model.

**Results:** Twenty-five phenolic compounds distributed between the leaf, fruit and stem of *R. cyprius* were identified. They were related to classes of anthraquinones, phenolic acids, flavonoid aglycones, glycosides and polyphenols. Twenty-two compounds in total were found and identified, and for the hepatoprotective effects, the leaf exhibited the best activity.

**Conclusion:** *R. cyprius* is a source of potentially active phytoconstituents and a good natural hepatoprotective drug. This study is being documented for the first time.

**Keywords:** *Rumex cyprius*, Mass spectrometry, HPLC, Antipathotoxicity

INTRODUCTION

Genus *Rumex* is made up of about 150 species, belongs to the family: polygonaceae, and is distributed in various regions around the world. Several species of this genus have numerous therapeutic potentials, and there are reports in some literature about the evaluation of a few species of *Rumex* for its extensive medicinal potential.

*Rumex cyprius* is native to the Arabian Peninsula. It is an annual herb with an ascending erect stem, and it branches from the base. Leaves are petiolate, ovate-triangular or oblong in shape, acute to acuminate apices, entire margins. Its inflorescences are elongated, and its flowers are 2-3 mm long with 1-2 on each pedicel. It is also membranous and cordate in shape, with distinct veins and spiny margins. The fruit it produces is achene [1]. *Rumex cyprius* Murb. is safely used...
as an antifungal [2], antibacterial [3] and antioxidant [4]. Phytochemically, it has been analyzed for the presence of emodin [2,5], flavonoids and phenolic acids [6,7].

The objective of this study was to characterize the dried extracts prepared from different organs of \textit{R. cyprius} by ESI-MS/MS and HPLC in order to determine the major constituents of these extracts, and also to evaluate the extracts for their hepatoprotective activity.

**EXPERIMENTAL**

**Plant material**

\textit{R. cyprius} L. herb was harvested during the rainy season in February/March 2018 from Wadi Dahakan Mountains in Deba City, Kingdom of Saudi Arabia (KSA, Figure 1). The plant was authenticated and identified by comparing it with the previously identified samples in the Dept. of Natural Products, Faculty of Pharmacy, King Abdulaziz University (KAU). Samples of fresh leaves, fruits and stems were separated and dried at a temperature of 25 ± 2 °C for 15 days. The dried tissues were then pulverized and kept in a dark glass container in a freezer for phytochemical and biochemical experiments.

![Figure 1: Source of collection of \textit{R. cyprius}](image)

**Chemicals, standards and reagents**

All solvents bought from Sigma Chemical Co., St Louis, MO, USA: Progallol, cinnamic acid, salicylic acid, gallic acid, ferulic acid, physcion, chrysophanol, emodin, aloe-emodin, luteolin, quercetin, apigenin, kampferol, isorhamnetin, orientin, vitexin, ellagic acid, luteolin7-O-glucoside and rutin standards were supplied by NODCAR, Giza, Egypt. Thin layer chromatography (TLC) was performed on pre-coated sheets using silica gel F254 (Fluka, Sigma-Aldrich, Germany).

**Electron spray ionization and direct mass spectrometry**

Analysis was performed on an XEVO-TQD (Waters, Milford, MA, USA) using electrospray ionization operated in positive ionization mode over a mass range of 100 to 2000 m/z. Capillary, cone and RF voltages were set at 3.47 kV, 59 V, 2.50 V, respectively. Source temperature was 150 °C, and desolvation temperature was 200 °C. Desolvation gas flow was 350 L/hr, collision gas flow was 0.21 mL/min, LM 1 resolution 15.00 and HM 1 resolution 15.00. Samples were dissolved in pure methanol (10 µl/ml) and directly injected in the apparatus. For the MS/MS analysis, collision energies were set at 10, 12, 15, 25, 30, 35 and 40 eV.

**Preparation of extracts of different organs of \textit{R. cyprius}**

The process applied in this study was cold extraction using \textit{n}-hexane, chloroform, ethyl acetate and methanol. The air-dried and weighed powders of the leaf, fruit and stem were placed in a flat bottom conical stoppered flask, and a suitable amount of the solvent was added and allowed to stand for 24 h with agitation at intervals. The extract was decanted through a piece of cotton, and the process was repeated for three more times. After the fourth extraction, the damp marc was pressed, allowed to dry, and the process was repeated using the next solvent. The collective extracts of each solvent were filtered, concentrated to dryness and weighed.

**Chromatographic determination of the different organs of \textit{R. cyprius}**

The instrument used by the HPLC system was Agilent 1260, and it included a quaternary pump with solvent delivery module, column compartment, autosampler and diode array detector (Agilent Technologies, Germany). ChemStation (Rev. B.01.03 SR2 (204)) was used for controlling the HPLC instrument and data analysis. Kromasil 100 C18, 5 µ, 250 x 4.6 mm column (Teknokroma, S. Coop. C. Ltd., Barcelona, Spain), was used for separation and was kept at 25 ±2 °C. Acetonitrile-water with 0.1 % acetic acid (45: 55, v/v) was used for the mobile phase. The flow rate was 0.8 mL/min and the injection volume was 10 µL. The LC system was isocratic at a maximum wavelength of 360 nm. Elution systems for TLC were ethyl acetate-formic acid-acetic acid-water (100:11:11:25, v/v)
Calibration curve of standard quercetin

Quercetin (10 mg) was placed in a 10 mL volumetric flask, and methanol (10 mL) was used for dissolution at concentrations of 2.5, 5, 10, 25, 50, 100 and 200 ng/μL. Calibration levels were analyzed in triplicates. A 50 ng/μL was injected 7 repetitive times, and all separation parameters were calculated. The RSD values were also calculated.

Preparation of *Rumex cyprius* extract for biological study

The air-dried and powdered leaf, fruit, and stem of *R. cyprius* (100 g, each) were separately extracted using methanol via cold maceration. The dried and weighed residues were kept for biological analysis.

Experimental design

The mice were divided into 6 groups (8 animals per group). Group 1 (control) was administered water with an intraperitoneal (IP) injection of corn oil after 4 h; Group 2 mice were given an injection of 1 ml/kg of 50% mixture of CCl₄-corn oil. Group 3 mice were given silymarin (200 mg/kg b.wt., orally) pretreatment and served as a positive control. Mice in Groups 4 - 6 were given *R. cyprius* extracts of leaf, stem and fruit (200 mg/ kg b.wt., orally), pre-treatment respectively. Groups 3 - 6 mice were administered a single IP injection of the CCl₄ mixture after 4 h of the pre-treatment.

Tissue and serum preparation

Cardiac puncture was used for blood sample collection. The blood was left to clot and the serum was centrifuged for 10 min at 3000 rpm, and kept at -80 °C for analysis. Hepatotoxicity was determined by measuring hepatic markers in the serum; alanine transaminase (ALT), aspartate transaminase (AST), total cholesterol (TC), triglycerides (TG), and total bilirubin (TB). Elisa Kits (Biodiagnostics, Cairo, Egypt) were used for measuring the levels of glutathione (GSH), malondialdehyde (MDA) and the activity superoxide dismutase (SOD).

Statistical analysis

Results are shown as mean ± standard deviation. One way analysis of variance (ANOVA) followed by Tukey’s test for post hoc analysis was used. A level of \( p < 0.05 \) was taken as significant. GraphPad InStat software, version 3.05 (GraphPad Software, Inc. La Jolla, CA, USA) was employed for statistical analysis. GraphPad Prism software, version 5.00 (GraphPad Software, Inc La Jolla, CA, USA) was used for figure preparation.

**RESULTS**

**Phytochemical profile**

The results of the ESI-MS and ESI-MS/MS revealed 25 phenolic compounds that were distributed between the chloroform, ethyl acetate and methanol extracts prepared from different organs of the herb (Table 1). The leaf, fruit and stem contained 27, 24 and 22 compounds, respectively (Table 2). ESI (+)-MS (Figure 2) showed the characteristic peaks with different intensities of the chloroform extracts of the leaf, fruit and stem, respectively. They were observed within the \( m/z \) range of 126 to 317, corresponding to protonated molecule ions of phenolic acids, aglycones of flavonoids and anthraquinones. The identification of the compounds was based on the available standards, NIST or other published literature.

**Phenolic acids**

Decarboxylation or water elimination identified the fragmentation patterns of most organic acids. Results indicated that compound 1 was benzoic acid [8], compound 3 was malic acid [9], compound 4 was cinnamic acid [10], compounds 5-7 were salicylic, gallic and ferulic acids [8], respectively.
Table 1: ESLCMS of the different fractions of Leaf, fruit and stem of *R. cyprius* and identification of their Molecular mass in the positive mode

<table>
<thead>
<tr>
<th>No</th>
<th>Compound</th>
<th>Leaf C</th>
<th>EA</th>
<th>M</th>
<th>C</th>
<th>EA</th>
<th>M</th>
<th>C</th>
<th>EA</th>
<th>M</th>
<th>CE</th>
<th>MW</th>
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<td>122</td>
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<td>12</td>
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<td>16</td>
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<td>610.5</td>
<td>25</td>
<td>610</td>
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C, chloroform fraction; EA, ethyl acetate fraction; M, methanol fraction; CE, collision energy; MW, molecular weight

Table 2: Number of identified compounds in different fractions of fruit and stem of *R. cyprius*

<table>
<thead>
<tr>
<th>Organ</th>
<th>Leaf</th>
<th>Fruit</th>
<th>Stem</th>
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<tr>
<td>Fractions of each organ</td>
<td>C</td>
<td>EA</td>
<td>M</td>
</tr>
<tr>
<td>No. of identified compounds in each extract</td>
<td>15</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Total no. of identified compounds in each organ</td>
<td>27</td>
<td>24</td>
<td>22</td>
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</table>

C, chloroform extract; EA, ethyl acetate extract; M, methanol extract
Figure 2: ESI (-)-MS of non-polar (chloroform) extracts of the leaf (A), fruit (B) and stem (C) of *R. cyprius*

Compound 17 revealed the presence of a molecular ion at m/z 356 and a major fragment at m/z 164 of the p-coumaric acid moiety after the loss of the hexose sugar. Most likely, compound 17 was p-coumaric acid hexoside [11]. Compound 18 showed a molecular ion peak at (M+H) + 335.9 and another peak at m/z 194. This indicated the presence of a ferulic acid derivative. Portions of gallic and ferulic acids were detected in chloroform and ethyl acetate extracts. This

Figure 3: ESI (-)-MS of ethyl acetate (A) and methanol (B) extracts of the leaf of *R. cyprius*

Figure 4: ESI (-)-MS of ethyl acetate (A) and methanol (B) extracts of the fruit of *R. cyprius*
Flavonoids

Compounds 12-14 and 16 were luteolin [12], kampferol, quercetin and isorhamnetin [8], respectively. The present study suggests that the A-ring of the flavone is potentially linked at the 7-OH to the sugar molecule and therefore, compound 21 is luteolin 7-O-glucoside (cynaroside) [12]. Compound 23 showed the molecular ion at m/z 593 with a prominent peak at m/z 165 A-ring of isorhamnetin. MS/MS yielded the base peak at m/z 315. The loss of 146 mu of methyl pentose allows a fragment at m/z 447, followed by the loss of 132 mu of a pentose results in an aglycone with 7-OH attachment pointing out that it might correspond to isorhammentin-7-O-pentosyl methyl pentoside structure [13]. Compound 20 showed a fragment at m/z 315, which may be identified as isorhamnetin. The compound was probably an isorhamnetin derivative. Compound 24 has a molecular ion peak at m/z 609, in addition to the fragments at [M+H−162]⁺ and [M+H−324]⁺ (formed by two consecutive losses of sugar residues) to give luteolin that was most probably for the two hexose sugars, attached together with an O link and identified as luteolin-O-dihexoside. Compound 25 is rutin [8] Compounds 19 and 22 and were also identified as the C-glycosides vitexin and orientin, respectively [14].

Regarding polyphenols, compound 2 was pyrogallol [15] and compound 15 was an ellagittannin which was identified as ellagic acid [9].

Anthraquinones

Compounds 8-11 were the anthraquinones physiocrin, emodin, chrysophanol and aloe emodin respectively [16].

Chromatographic profile of the different organs of R. cyprius

HPLC-DAD of the standard quercetin (Figure 6) revealed a peak at Rt 4.92 of the different concentrations, and has a UV-VIS scan detection at λ max of 370 and 255 nm (bands I and II of flavonols, Figure 7). This result is well presented by the 3-D figure of the flavonoid bands (Figure 8), and helped in the construction of a standard curve of the reference sample. The methanolic extract of leaves, fruit and stem of R. cyprius (Figure 9) was hydrolysed by an acid, followed by their HPLC–DAD. This revealed the presence of a total quercetin of 0.274, 0.145, 0.098 mg/g powder, respectively.
a notable leakage of the liver enzymes, ALT and AST was noticed from hepatic cells in the blood. This was evidenced by a marked rise in ALT and AST in the serum relative to controls (Table 3). Furthermore, acute CCl₄ administration resulted in significantly increased TB, TC and TG. Pretreatment with test samples resulted in much lower levels of ALT and AST. Interestingly, silymarin and leaf extract has shown better hepatoprotection than the other extracts.

Oxidative stress markers

Carbon tetrachloride (CCl₄) caused a marked depletion of Glutathione (GSH) and doubled malondialdehyde (MDA) levels, when compared to controls, as shown in Figure 10. Silymarin and extracts of leaf and fruit of R. cyprius pretreatment reversed these effects and significantly increased GSH concentrations while reducing MDA, relative to the CCl₄-treated mice. MDA levels were not altered after stem pretreatment. Pretreatment with silymarin and extracts of leaf and fruit caused reduced SOD (Figure 10).

In this experiment, there was a notable rise in MDA and a lowering of GSH after CCl₄ administration. After pre-treating with the leaf and fruit extract, MDA and GSH levels were similar to controls, which suggests that they possess antioxidant and free radical scavenging actions.

DISCUSSION

In this study, the low molecular weight of polar phytoconstituents in different extracts of R. cyprius were examined. This was done by a simple direct mass spectrometry without carrying out HPLC. ESI-MS and ESI-MS/MS include ionization, analysis of positive ions and fragmentation of the protonated molecules. Direct injection was used to ease the structural identification of the constituents through the application of the positive ion mod.

<table>
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<th>Group</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>TB (mg/dL)</th>
<th>TC (mg/dL)</th>
<th>TG (mg/dL)</th>
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<tr>
<td>Control</td>
<td>15.4 ± 4.07</td>
<td>12.7 ± 1.9</td>
<td>0.22 ± 0.08</td>
<td>56.7 ± 2.9</td>
<td>70.3 ± 3.60</td>
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<td>CCl₄</td>
<td>124.2 ± 16.4</td>
<td>91.8 ± 3.4</td>
<td>1.15 ± 0.23</td>
<td>121.6 ± 6.30</td>
<td>150.8 ± 7.80</td>
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<td>CCl₄ + Silymarin</td>
<td>21.3 ± 5.03</td>
<td>18.7 ± 4.10</td>
<td>0.27 ± 0.09</td>
<td>61.8 ± 3.20</td>
<td>76.6 ± 3.90</td>
</tr>
<tr>
<td>CCl₄ + leaf extract</td>
<td>26.9 ± 0.48</td>
<td>28.02 ± 4.90</td>
<td>0.34 ± 0.12</td>
<td>67.7 ± 3.40</td>
<td>82.2 ± 4.30</td>
</tr>
<tr>
<td>CCl₄ + Fruit extract</td>
<td>31.7 ± 2.40</td>
<td>32.1 ± 3.20</td>
<td>0.33 ± 0.12</td>
<td>64.7 ± 3.30</td>
<td>80.3 ± 4.16</td>
</tr>
<tr>
<td>CCl₄ + stem extract</td>
<td>66.1 ± 4.90</td>
<td>60.2 ± 4.50</td>
<td>0.98 ± 0.35</td>
<td>113.2 ± 5.80</td>
<td>140.4 ± 7.2</td>
</tr>
</tbody>
</table>

Statistical analysis was carried out by one-way ANOVA followed by Tukey post-hoc test. *Statistically significant from the corresponding control at p < 0.05; **Statistically significant from CCl₄-treated group at p < 0.05
This study determined the hepatoprotective activity of *R. cyprius* in an acute CCl₄-induced liver toxicity model in mice. A single dose of CCl₄ causes steatosis and major hepatic necrosis, and it has been reported that this single day model is used since CCl₄ causes peak toxic effects one day after injection, after which values begin to normalize.

As presented in previous studies and furthermore in this study, CCl₄ resulted in raised serum levels of AST, ALT, TB, TC and TG. This could be explained by the hepatocellular effects it causes after being metabolized. MDA is a well-recognized oxidative stress marker, which raises levels indicating tissue injury and improved lipid peroxidation [17]. However, GSH is a non-enzymatic host antioxidant defense mechanism. The enzymatic parts are represented by SOD. GSH acts as a free radical scavenger and is oxidized to glutathione disulfide by GSP.

The glutathione disulfide can be converted back into GSH by GR reduction, a reaction that requires NADPH. Another action of GSH is that it interacts with metabolites, xenobiotics and electrophiles in order to give mercapturic acids. SOD is responsible for catalyzing the conversion of superoxide anion to O₂ and H₂O₂, which can be removed by CAT and GSP [18].

**CONCLUSION**

Twenty-five compounds, including four free anthraquinones, eight phenolic acids, four flavonoid aglycones, seven flavonoid glycosides and two polyphenols have been successfully identified from the Saudi-grown *Rumex cyprius*. The total quercetin content (combined and free) for leaf, fruit and stem of the plant as well as the hepatoprotective effects of the extracts have also been determined. The findings provide a basis for profiling the active components of the herb when formulated in a simple pharmaceutical preparation.

**DECLARATIONS**

**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

We declare that the present study was done by the authors named in the article. Nadia Sokkar designed and performed the phytochemical experiments, analyzed the data and wrote the manuscript. Basma Eid performed the biological
analysis. The two authors read and approved the final manuscript.

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REFERENCES