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Original Research Article

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, Antihepatotoxicity*

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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 *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

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 *R. cyprius*

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 µ/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 *R. cyprius*

The process applied in this study was cold extraction using *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 *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)

(flavonoids); chloroform-methanol-formic acid (97:3:0.2, v/v) (phenolic acids and anthraquinones).

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 CCl_4-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 CCl_4 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 bilirubin (TB), total cholesterol (TC) and triglycerides (TG). The mice were sacrificed to collect the liver, which were then cleaned and homogenized in PBS (ice-cold) to give 10 % w/v homogenates. Homogenates were stored at -80 °C until needed.

Biochemical analyses

Mindray BS-120 Clinical Chemistry Auto-analyzer (Shenzhen Mindray Bio-medical Electronics Co. Ltd, Shenzhen, China) was used to measure the levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), triglycerides (TG), and total bilirubin (TB). Elisa Kits (Biodiagnostics, Cairo, Egypt) were used for measuring the levels of glutathione (GSH), malondialdhyde (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 anthraquinons. showed the ESI (+)-MS representative of the ethyl acetate and methanol extracts of the same organs. In Figures 3-5, the compounds spectra, relative intensity and characteristic ions were noticed in the *m/z* range from 335 to 611, and that may be representative of the different mono- and di- glycosides. 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

No	Compound	Leaf			Fruit			Stem			CE	MW	Fragmentation pathway [m/z], ESI (+)
		C	EA	M	C	EA	M	C	EA	M			
1.	Benzoic acid	121.9	-	-	-	-	-	-	-	-	35	122	103, 106, 95, 92, 77, 51
2.	Pyrogallol	126.7	-	-	126.8	-	-	126.9		-	15	126	119, 109, 101,99, 85, 81, 78, 68.8
3.	Maleic acid	134.1	-	-	-	-	-	-	-	-	12	134	111, 114.8
4.	cinnamic acid	148.9	-	-	148.7	-	-	148.9		-	25	148	131.6, 103.6
5.	Salicylic acid	139.0	-	-	138.7	-	-	139.0		-	12	138	121, 95, 93
6.	Gallic acid	170.9	170.9	-	170. 9	-	-	170.7		-	25	170	152, 127.9
7.	Ferulic acid	194.8	194.7	-	194.8	-	-	195.1	-	-	35	194	177, 152, 162, 134
8.	Chrysophanol	254.9	-	-	254.9	-	-	254.9	-	-	12	254	237.19, 226.98, 207.25
9.	Emodin	270.9	-	-	270.6	-	-	270.6	-	-	30	270	253, 245, 224.4, 180
10.	Aloe emodin	271.3	-	-	271.2	-	-	271.3	-	-	30	270	252.2, 224.3, 212.2, 196.2
11.	Physcion	285.4	-	-	285.3	-	-	285.0	-	-	10	284	271.5, 267, 253, 239.6, 224
12.	luteolin	286.7	-	-	-	-	-	-	-	-	10	286	269, 246.8, 241, 153
13.	Kampferol	287.4	-	-	-	-	-	287.4	-	-	35	286	268.5, 242, 248
14.	Quercetin	303.3	-	-	302.9	-	-	-	-	-	15	302	283,275.6, 222.6, 204.7
15.	Ellagic acid	-	-	-	303.4	-	-	303.4	-	-	25	302	285, 257, 229, 183
16.	isorhamnetin	316.7	-	-	317.2	-	-	317.2	-	-	7	316	272, 255.7, 152.7
17.	p-coumaric acid hexoside	-	-	-	-	326.3	-	-	326.3	-	15	326	187,163, 143
18.	Ferulic acid derivative	-	335.9	335.8	-	335.9	-	-	335.9	-	35	336	195
19.	Vitexin	-	432.6	432.9	-	433.2	433.3	-	433.4	432.5	40	432	344, 312.9
20.	Isorhamnetin derivative	-	-	-	-	-	422.8	-	-	-	25	442	315
21.	Luteolin-7- *O*-glucoside	-	448.1	448.1	-	448.1	-	-	-	-	12	432	286, 246, 241.67, 153
22.	Orientin	-	448.5	-	-	448.5	-	-	448.5	448.5	40	448	327.9, 347.8, 429
23.	Isorhamnetin-7-*O*- pentosyl methyl pentosyl	-	-	593.3	-	593.3	592.9	-	-	592.9	8	592	592, 447.5, 316
24.	Luteolin-*O*- dihexoside	-	-	-	-	609.3	-	-	-	609.4	8	608	286, 447, 267.41, 252.79, 239.81
25.	Rutin	-	610.5	610.5	-	610.9	610.8	-	610.8	610.5	25	610	303

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*

Organ	Leaf			Fruit			Stem		
Fractions of each organ	***C***	***EA***	***M***	***C***	***EA***	***M***	***C***	***EA***	***M***
No. of identified compounds in each extract	15	7	5	12	8	4	12	5	5
Total no. of identified compounds in each organ		27			24			22	

C, chloroform extract; EA, ethyl acetate extract; M, methanol extract

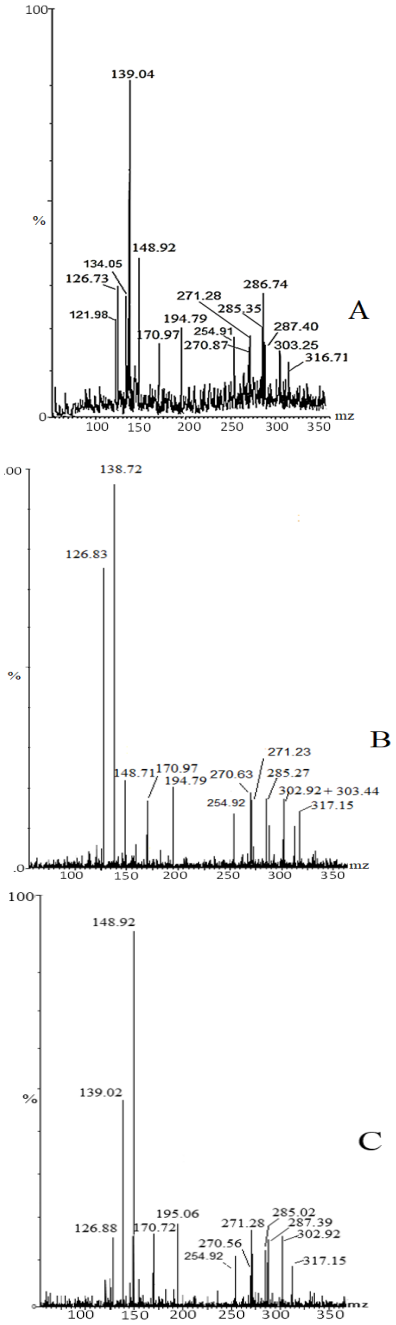

Figure 2: ESI (-)-MS of non-polar(chloroform) extracts of the leaf (A), fruit(B) and stem (C) of *R. cyprius*

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

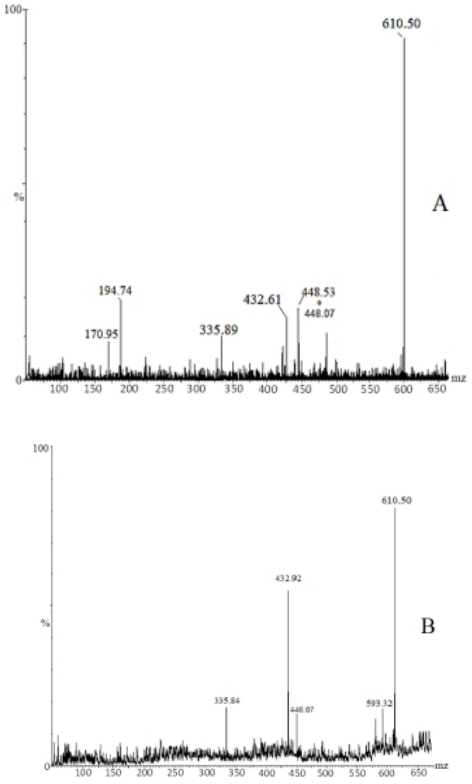

Figure 3: ESI (-)-MS of ethyl acetate (A) and methanol(B) extracts of the leaf of *R. cyprius*

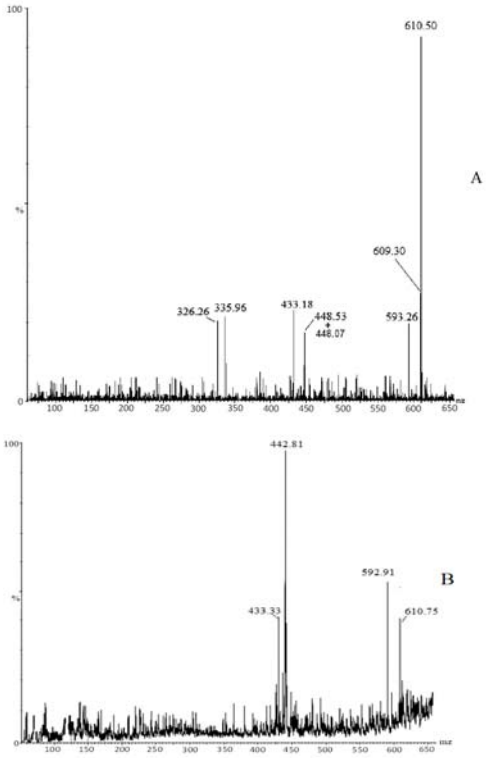

Figure 4: ESI (-)-MS of ethyl acetate (A) and methanol (B) extracts of the fruit of *R. cyprius*

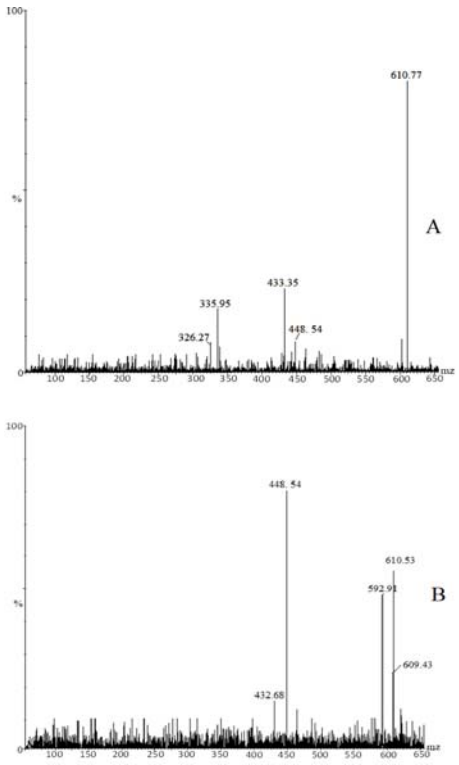

Figure 5: ESI (-)-MS of ethyl acetate (A) and methanol (B) extracts of the stem of *R. cyprius*

occurred as a result of their leakage during extraction.

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 isorhamentin-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 ellagitannin which was identified as ellagic acid [9].

Anthraquinones

Compounds **8-11** were the anthraquinones physcion, 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.

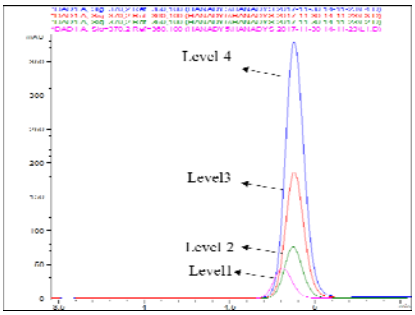

Figure 6: Overlaid HPLC-DAD chromatogram of calibration solutions of quercetin (2.5, 25, 50, 100 ng/µL)

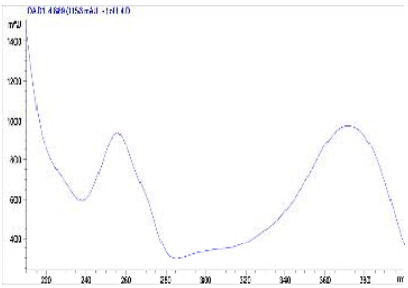

Figure 7: HPLC-DAD chromatogram of standard quercetin, 50 ng/µL

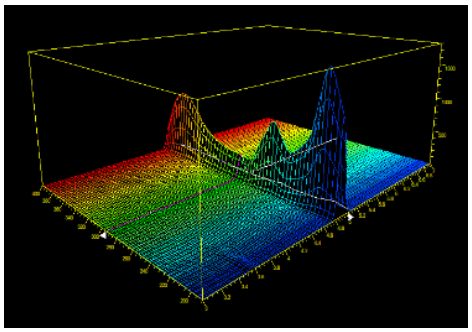

Figure 8: UV-VIS scan of peak corresponding to standard quercetin at 4.9 min (190 – 400 nm). Maximum absorption at λ max of 370 and 255 nm

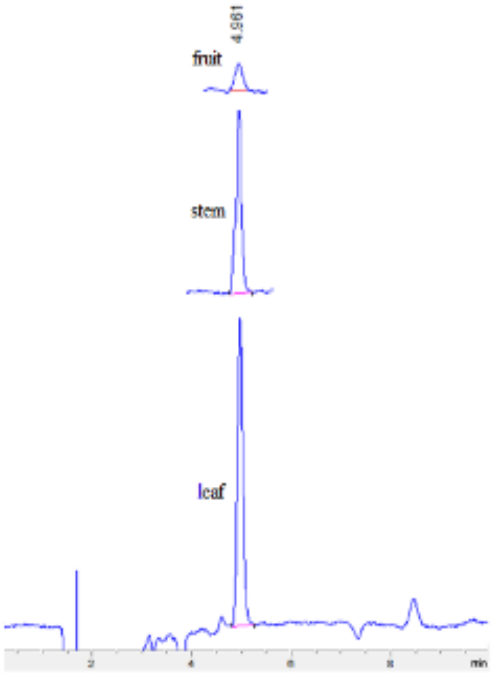

Figure 9: HPLC chromatogram of analyzed the methanol extract of different tissues of *R. cyprius* after hydrolysis

Hepatoprotective effect

After the carbon tetrachloride challenge on mice, 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 CCl_4 administration resulted in significantly increased TB, TC and TG. Pre-treatment 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 (CCl_4) 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 CCl_4-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 CCl_4 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 3: Effect of *R. cyprius* extracts pretreatment on hepatotoxicity markers in mouse model of acute CCl_4 intoxication; n = 6

Group	ALT (U/L)	AST (U/L)	TB (mg/dL)	TC (mg/dL)	TG (mg/dL)
Control	$15.4^{b} \pm 4.07$	$12.7^{b} \pm 1.90$	$0.22^{b} \pm 0.08$	$56.7^{b} \pm 2.90$	$70.3^{b} \pm 3.60$
CCl_4	$124.2^{a} \pm 16.4$	$91.8^{a} \pm 3.40$	$1.15^{a} \pm 0.23$	$121.6^{a} \pm 6.30$	$150.8^{a} \pm 7.80$
CCl_4 + Silymarin	$21.3^{b} \pm 5.03$	$18.7^{b} \pm 4.10$	$0.27^{b} \pm 0.09$	$61.8^{b} \pm 3.20$	$76.6^{b} \pm 3.90$
CCl_4 + leaf extract	$26.9^{a, b} \pm 0.48$	$28.02^{a, b} \pm 4.90$	$0.34^{b} \pm 0.12$	$66.7^{b} \pm 3.40$	$82.2^{b} \pm 4.30$
CCl_4 + Fruit extract	$31.7^{a, b} \pm 2.40$	$32.1^{a, b} \pm 3.20$	$0.33^{b} \pm 0.12$	$64.7^{b} \pm 3.30$	$80.3^{b} \pm 4.16$
CCl_4 + stem extract	$66.1^{a, b} \pm 4.90$	$60.2^{a, b} \pm 4.50$	$0.98^{a} \pm 0.35$	$113.2^{a} \pm 5.80$	$140.4^{a} \pm 7.2$

Statistical analysis was carried out by one-way ANOVA followed by Tukey post-hoc test. [a]Statistically significant from the corresponding control at $p < 0.05$; [b]: Statistically significant from CCl_4-treated group at $p < 0.05$

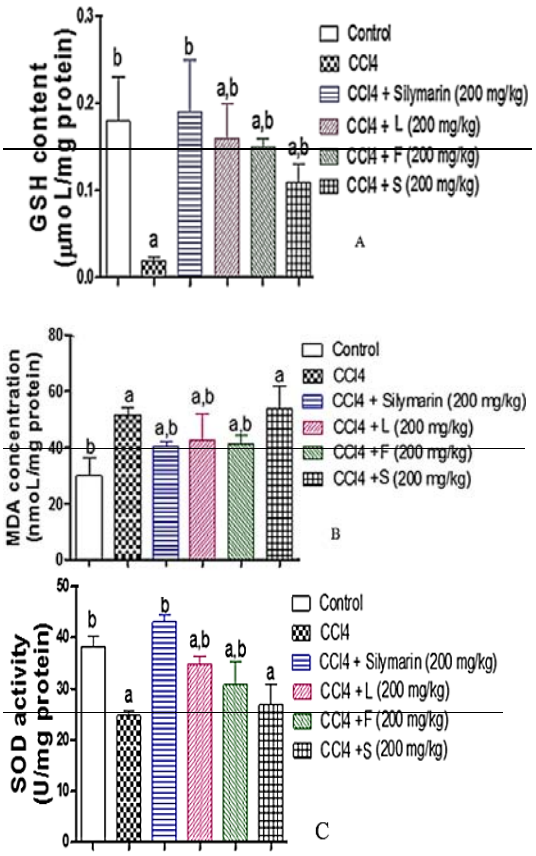

Figure 10: Effect of *R. cyprius* extracts pretreatment on: A, GSH hepatotoxicity model content; B, MDA consent mice ion; C, on SOD activity, in mice model of acute CCl4 intoxication. n=6. Statistical analysis was carried out by one-way ANOVA followed by Tukey post-hoc test. [a]Statistically significant from the corresponding control at $p < 0.05$; [b]Statistically significant from CCl4-treated group at $p < 0.05$. L= Leaves, F= Fruit and S= Stem

This study considered the exploration of 25 compounds of phenolic acids, flavonoids and antraquinones, and their distribution in the different extracts of the tissues of *R. cyprius*. The MS/MS experiments were performed at different values of CE. Even though the optimum CE might vary for different compounds. Data in low collision energy was used to illustrate the aglycone fragment (Y– 0) and distinguish their glycosides

Previous publications revealed the identification of isoorientin, vitexin, luteolin-7-*0*-glucoside (cynarosid) [6] and emodin [1,4] in *R.* cyprius. On the other hand, the results of this study concluded that 22 phenolic compounds were identified for the first time using an easy and fast method of analysis: mass spectrometry. The quantitative HPLC of the leaf, fruit, and stem proved that the leaf content of flavonoids is higher compared to the other two organs.

This study determined the hepatoprotective activity of *R. cyprius* in an acute CCl_4-induced liver toxicity model in mice. A single dose of CCl_4 causes steatosis and major hepatic necrosis, and it has been reported that this single day model is used since CCl_4 causes peak toxic effects one day after injection, after which values begin to normalize.

As presented in previous studies and furthermore in this study, CCl_4 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 mercaptumicees. SOD is responsible for catalyzing the conversion of superoxide anion to O_2 and H_2O_2, 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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