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An effective and facile approach for the determination of bioactive components of *Rheum ribes* in the Kurdish state of Iraq and Siirt region in Turkey

İBRAHIM TEGIN[1]*, BAKHTIYAR MAHMOOD FATTAH FATTAH[1], MEHMET FIDAN[2], ORHAN ACAR[3] and ERDAL YABALAK[4]**

[1]Siirt University, Faculty of Arts and Science, Department of Chemistry, Siirt, Turkey, [2]Siirt University, Faculty of Arts and Science, Department of Biology, Siirt, Turkey, [3]Gazi University, Science Faculty, Chemistry Department, Ankara, Turkiye and [4]Mersin University, Department of Nanotechnology and Advanced Materials, Mersin, Turkey

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Abstract: Developing a streamlined and accessible method for identifying the bioactive components of *Rheum ribes* (rhubarb) holds significant promise in unlocking its therapeutic potential and advancing research in natural medicine. In this study, the bioactive components of rhubarb such as total phenolics and flavonoids as well as the antioxidant activity of its methanolic extract were determined. Total phenolic content was between 84.02 and 387.53 mg/L gallic acid equivalent (GAE) in extracts. Total flavonoid contents determined by the aluminium chloride colorimetric method ranged from 69.98 to 935.75 mg L^{-1} of routine equivalents (RE) in the extracts. The antioxidant activities were determined using ferric reducing the antioxidant potential (FRAP) and 1,1-diphenyl--2-picrylhydrazyl (DPPH) methods. In the FRAP assay, the highest antioxidant activity (IC_{50}) was found as 25.18±0.04 mg L^{-1} extract. In the DPPH method, the maximum percentage inhibition was found as 88.11 %. Iron chelating activities of the samples were above 70 %. The chemical compound contents of the extracts were determined by LC–MS/MS. In this step, a total of 25 phenolic and flavonoid compounds in extracts were analysed qualitatively and quantitatively. Malic acid (15.72±0.53 mg kg^{-1}) and rutin (76.93±0.03 mg kg^{-1}) in the extract were identified as the major phytochemicals compounds. The results of the study confirm that rhubarb have potential biological activities and can be introduced as an important sources of natural antioxidants.

Keywords: rhubarb; phenolic compounds; antioxidant activities; flavonoids.

*,** Corresponding authors. E-mail: (*)ibrahim.tegin@gmail.com, (**)yabalakerdal@gmail.com
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INTRODUCTION

Rhubarb, comprising approximately sixteen plants within the Polygonaceae family,[1] is harvested in Turkey and Iraq and serves both as a culinary delight and a natural remedy.[2] It mostly grows in the spring season under the snow of the high mountains in the north and centre of Asia.

Not only are rhubarb samples consumed as food, but they are also valued for their medicinal properties, making them a versatile resource for human use. In addition to its medical importance, rhubarb is used in cuisine in desserts such as rhubarb crumble, as well as in jams, jellies and sauces, since its tart flavour became famous. Rhubarb is basically a vegetable but it is often thought to be a fruit.[3] Some of their leaves are toxic, but the stalks are used in foods for their tart flavour. Rhubarb stalks are either cooked or eaten raw, and raw stalks can also be dipped in sugar to eliminate sourness.[4]

They provide rich sources of natural antioxidants. The components of rhubarb are separated and predicted to have antiulcer, antioxidant, antidiabetic, antimicrobial, wound healing, nephroprotective, anticancer and hepatic protective activities.[5] They also include tocopherols, vitamin C, carotenoids and phenolic compounds. Since phenolic compounds oxidize and combine with proteins and other components, they can protect plants against tissue injuries. Phenolic compounds in plants may serve as defence systems against herbivores. Products of photosynthesis may also produce high levels of oxygen, free radicals and reactive oxygen species (ROS). The rhubarb plants have a myriad of antioxidant compounds to survive.[6]

Many of these compounds have basic similar molecular and have at least one aromatic ring and a hydroxyl group. They also include phenolic acids, flavonoids and gallate esters (hydrolysable tannins). Flavonoids are also recognized as a cause of wound healing potential of rhubarb since it is identified to decrease per oxidation of lipids not merely via averting or decelerating the beginning of cell necrosis but also via secularity improvement. Because of flavonoids and tannins' astringent and antimicrobial properties, which seem accountable for the contraction wound and epithelialization escalation rate, tannins and flavonoids promote the process of wound healing.[7]

Some rhubarb samples have large somewhat triangular-shaped leaves with long fresh petioles. Through its medical advantages can be used for the treatment of acidity in the stomach and to address constipation.[8,9] Additionally, it also kills intestinal worms and helps to purify the human liver.[8] They are used as a purgative, anti-inflammatory agent and herbal medicines for the treatment of constipation and cancer.[10] Some beneficial effects of rhubarb are also on Parkinson's disease, immune system and acute respiratory syndrome. *Rheum ribes* has been clinically used as a laxative agent,[11] antibacterial, isolation and identification of about 200 chemical compounds for at least 2000 years. It is identified that environmental and

genetic factors and their interactions involve the pharmaceutically important secondary metabolites in medical plants.[12] Besides, Rhubarb is one of the most well--known and widely used traditional Chinese medicines for the treatment of constipation, inflammation and cancer. It derives from the roots and rhizomes of *Rheum* officinal recorded in the Chinese Pharmacopoeia.[13]

Phenolic compounds in plants are essential for human diets and are of considerable interest due to their antioxidant properties. These compounds have an aromatic ring bearing one or more hydroxyl groups and their structures may range from a simple phenolic molecule to a complex high-molecular-weight polymer. The antioxidant activity of phenolic compounds depends on the structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings. The major effective constituents of rhubarb samples are phenolic compounds, sennosides and anthraquinone glycosides. Rhubarbs can lower sugar and lipid levels in human blood and could be used to treat hyper lipids, obesity and diabetes.[14]

Antioxidants may protect the cells against the effects of free radicals and molecules produced when the body is exposed to tobacco smoke and radiation. Free radicals may play a role in heart disease, cancer and other diseases. Antioxidants come up frequently in discussions about good health and preventing diseases. These powerful antioxidants mostly come from fresh fruits and vegetables. Aqueous and methanol extracts of the roots of Rhubarb emodin have been indicated to have anticancer and antioxidant potential.[15] One of the primary factors for the development and progression of many life-threatening diseases and disorders like cancer, atherosclerosis and diabetes is oxidative stress. Presently, in modern pharmacopoeia around 25 % of drugs originate from plants (phytomedicines) and several others are synthetic analogues built on the prototype compounds separated from plants. In Chinese folk medicine, *R. emodin* is used in the treatment of cancer, ulcers and liver treatments.[16] The rhubarb's anticancer influence is accredited to the aloe-emodin which not merely subdued the spread but also encouraged apoptosis of two human cancer cell lines.[17]

The aim of this study is to determine the antioxidant activity (DPPH % inhibition), the total phenolic content and total flavonoid concentrations, FRAP antioxidant capacities and iron chelating capacity of extracts of rhubarb samples. Besides, a comprehensive method was developed, optimised and validated to determine 37 phytochemicals in rhubarb samples using liquid chromatography––mass spectrometry/mass spectrometry (LC–MS/MS).

These include coumarin, hesperidin, *p*-coumaric acid, gallic acid, caffeic acid, vanillic acid, salicylic acid, quinic acid, *p*-hydroxybenzoic acid, ferulic acid, chlorogenic acid, rosmarinic acid, protocatechuic acid, cinnamic acid, sinapinic acid, fumaric acid, malic acid, syringic acid, naringenin, rutin, quercetin, quercitrin, isoquercitrin and nicotiflorin.[18,19] The developed method went thorough

validation, encompassing assessments of linearity, accuracy (recovery), inter-day and intra-day precision (repeatability), as well as determination of limits of detection and quantification (*LOD*, *LOQ*). The evaluation of measurement uncertainty (U % at 95 % confidence level with $k = 2$) was also conducted as part of the validation process. Furthermore, the developed and validated LC–MS/MS method was employed for phytochemical screening of methanol-chloroform extracts from the samples. Also, the presence of specific phenolic acids, including organic acids such as quinic, malic, fumaric, chlorogenic and vanillic acids, alongside flavonoids such as rutin, hesperidin and isoquercitrin, the total phenolic and flavonoid contents, as well as the antioxidant potential were analysed in rhubarb samples.

EXPERIMENTAL

Collection and preparation of samples

The *Rheum ribes* samples were collected from seven different locations in Siirt (Pervari and Şirvan) and Northern Iraq (Gara, Korajar, Sor, Rash and Qalandar). The samples taken were air-dried completely at laboratory temperature in the shaded area and then powdered until dust particles were obtained with a mixer. Powdered plant samples were placed in glass jars and stored at laboratory temperature. The extracts were prepared for total phenolic matter content, DPPH radical scavenging activity analysis, total flavonoid content and FRAP analysis of *R. ribes* samples using the amounts specified in the literature.[20-22] For the analysis of phenolic compounds by LC–MS/MS, *R. ribes* samples were subjected to the Soxhlet extraction method proposed by Teğin *et al.*[23] For this purpose, 10 g of dried and powdered *R. ribes* samples were Soxhlet extracted with 160 mL 80:20 methanol–water mixture. After extraction, the solvents obtained were evaporated under vacuum at 35 °C by a rotary evaporator until dry extracts were obtained. Dry filtrates were diluted to 1000 mg/L and filtrated with a 0.2 µm syringe filter before LC–MS/MS analysis.[19,23] To determine the phenolic content composition by LC–MS/MS, the method given by Yılmaz *et al.*[18]

LC–MS/MS device and chromatographic conditions

Qualitative and quantitative analysis of phytochemicals were performed by using an LC––MS/MS system equipped with a Shimadzu – Nexera ultrahigh performance liquid chromategraphy (UHPLC) device and Shimadzu LCMS 8040 triple quadruple mass spectrometer (MS). The liquid chromatography system used consists of the LC-30 AD gradient pump, the DGU--20A3R degasser, the CTO-10ASvp column oven and the SIL-30AC autosampler. Chromatographic separation was performed on an RP-C18 Inertsil ODS-4 (100 mm×2.1 mm, 2 µm) analytical column. During the analysis, the column furnace temperature was set at 35 °C. In the elution gradient, ultrapure water for mobile phase A and acetonitrile for mobile phase B were used. In addition, 10 mmol/L ammonium formate and 0.1 % formic acid were added to the water phase A to facilitate better chromatographic separation and ionization. After several attempts to achieve optimal separation of analyses, the most suitable UHPLC gradient profile was obtained with a gradient profile of 5–20 % B (0–10 min), 20 % B (10–22 min), 20–50 % B (22–36 min) 95 % B (36–40 min) and 5 % B (40–50 min). The mobile phase flow rate was set at 0.25 mL/min and the injection volume was about 4 µL.[19]

The triple quadrupole mass spectrometer is equipped with an electrospray ionization (ESI) source operating in both a negative and a positive mode. The LC–ESI-MS/MS data were collected and processed by the registered Lab Solutions software (Shimadzu, Kyoto, Japan). The

quantitative analysis of the compounds was carried out using multiple reaction monitoring (MRM) mode and the parent ions were combined with one or two product ions (the first one was used for qualitative purposes and the second one for quantitative purposes). Other parameters optimized in the mass spectrometer were: interface temperature, 350 °C; DL temperature, 250 °C; heat block temperature, 400 °C; nebulizer gas flow (N_2), 3 L/min; drying gas flow (N_2) 15 L/min.[24]

LC–MS/MS method validation studies

In the presented study, a comprehensive LC–MS/MS method was optimized and validated for the qualification and quantification of 37 phytochemicals in Rhubarb samples. The performance characteristics of the method were determined by using standard solutions and wavelengths recommended by the manufacturer for detection. Furthermore, to increase the credibility of the results by compensating for the matrix effects and analyte losses during the sample preparation and analyses, quercetin D3, rutin D3 and ferulic acid D3 were used as the deuterated internal standards for flavonoid compounds, respectively. Within this context, the developed method was fully validated in terms of linearity, accuracy (recovery), inter-day and intra-day precision (repeatability), limits of detection and quantification (*LOD*/*LOQ*) and relative standard uncertainty (U % at 95 % confidence level ($k = 2$)). Parameters related to the LC–MS/MS method validation studies are given in Table S-I and Fig S-1 of the Supplementary material to this paper.[24,25]

Linearity

The linearity was evaluated using an external standard calibration curve with eight concentration levels for each analyte was analyzed in triplicate. The calibration curves were constructed as a plot of the ratio of the concentration of the analyte to the concentration of the internal standard (IS, x) *versus* the ratio of the area of the analyte to IS (y).

Precision (repeatability) and accuracy (recovery)

Precision and accuracy studies for the developed method were carried out by standard addition to a selected extract of a species. Intra-day (within-day) precision (repeatability) was evaluated by analysing six replicates of fortified samples within a single day. On the other hand, to conduct an inter-day precision assay six replicates of fortified samples were examined per day for three consecutive days. As a consequence of the intra-day and inter-day studies, percent relative standard deviation (*RSD* / %) and recovery values were used to assess the precision and accuracy (Table S-I). Eq.(1) was used to calculate the recovery:

$$\text{Recovery (\%)} = 100(DC - OC)/SC \quad (1)$$

where *DC*, *OC* and *SC* signify detected, original and spiked concentrations, respectively.

*Limit of detection (*LOD*) and limit of quantification (*LOQ*)*

The limits of detection (*LOD*) and quantification (*LOQ*) data for each phytochemical analyte used were determined by serial dilution of standard solutions and analysing them under the described LC–MS/MS conditions until the detection of the lowest concentration signalled by the standards (*S*/*N* ratio 3:1). After determining the lowest detectable concentrations for each analyte, ten internal standard solution mixtures (ISs included) at these concentrations were prepared and injected to the LC–MS/MS system. Calculation of *LOD* and *LOQ* data were carried out using Eqs. (2) and (3), Table S-I:

$$LOD = \text{Mean} + 3SD \quad (2)$$

$$LOQ = \text{Mean} + 10SD \quad (3)$$

where *SD* indicates the standard deviation.

Preparation of plant samples for extraction

After the samples collected were dried at the laboratory environment temperature in the shaded area, they were pulverized. Powdered samples were placed in glass jars and stored at laboratory temperature. A 4 g of the powdered sample was placed in a beaker and 40 mL of 80 vol. % methanol was added. After the mixture was sonicated for 2 min on the Wiggin Hauser homogenizer and 5 min on the Sonopuls HD 2070, it was left overnight on the incubated shaker Standard & Cooled – MD13. The extract was then filtered through ordinary filter paper. After the extracts were dried at 38 °C in an oven, the stock concentrations were formed by adding 80 vol. % methanol so that the concentration of the solid part remaining in the bottom of the tube was 10 mg/mL.

Determination of phenolic content

The total phenolic contents of samples were determined according to the Folin–Ciocalteu reactivity and Gallic acid standard.[27,28] From the extract solution, 0.1 mL of Folin–Ciocalteu reactant was added to the flask and then thoroughly shaken. After 3 min, 1 mL of 6 % Na_2CO_3 solution was added and the mixture was allowed to stand for 1 h with intermittent agitation. Absorbance was measured at 760 nm with a Uvmini-1240 spectrophotometer. The same procedure was repeated in the gallic acid solutions. The calibration graphs of the analytes obtained are given in Fig. S-2 of the Supplementary material.

Determination of total flavonoid contents in samples

Total flavonoid contents in samples were determined using the aluminium chloride colorimetric method.[30,31] The 0.5 mL of the extract solution was mixed with 2 mL of distilled water and 150 μL of 5 % sodium nitrate. After 6 min, 150 μL of 10 % aluminium chloride and 2 mL of 1 mol/L sodium hydroxide were added and left at room temperature for 15 min. The absorbance values of the sample solutions were measured at 510 nm with a Shimadzu UV–Vis Uvmini-1240 spectrophotometer. The routine was used as a standard. The calibration graphs of the analytes obtained are given in Fig. S-3 of the Supplementary material.

Preparation of plant samples for DPPH analysis

For DPPH analysis,[32] 4 mL of 0.01 mM DPPH solution prepared in 80 % methanol was added to 1 mL of extract and the mixture was absorbed at 517 nm wavelength with Shimadzu Uv–Vis Uvmini-1240 spectrophotometer after waiting 15 min in the dark. The control solution was 1 mL of solvent and 4 mL of DPPH mixture.[18,33] DPPH activities were calculated using Eq. (4):

$$\text{DPPH activity (\% incubation)} = 100\frac{A_C - A_1}{A_C} \quad (4)$$

where A_C is the control absorbance and A_1 is the sample absorbance.

FRAP analysis

FRAP analysis was performed by adding 3 mL of FRAP reagent into 100 μL of sample diluted appropriately.[34] The incubation was allowed to proceed for 6 min at room temperature in the dark and then the absorbance was measured at 593 nm with a Shimadzu UV–Vis Uvmini--1240 spectrophotometer. FRAP solution was 10 mL acetate buffer + 10 mL $FeCl_3 \cdot 6H_2O$ + 1 mL TPTZ solution. Acetate buffer (300 mmol/L at pH 3.6) solution was prepared using 3.1 g of CH_3COONa in 16 mL of acetic acid/L distilled water. HCl (40 mmol/L) solution was prepared by 400 μL of HCl (32 %)/100 mL of purified water. TPTZ (10 mmol) solution was pre-

pared by dissolving 31.2 mg TPTZ in 10 mL of 40 mmol/L HC1. $FeCl_3$ solution was prepared by dissolving 54.1 mg of $FeCl_3 \cdot 6H_2O$ in 10 mL of pure water.

Preparation of plant samples for DPPH analysis

For DPPH analysis,[32] 4 mL of 0.01 mM DPPH solution in 80 % methanol was added to 1 mL of extract and the mixture was absorbed at 517 nm wavelength with Shimadzu Uv–Vis Uvmini-1240 spectrophotometer after waiting 15 min in the dark. The control solution was 1 mL of solvent and 4 mL of DPPH mixture.[18,33] DPPH activities were calculated using Eq. (4).

RESULTS AND DISCUSSION

Determination of phenolic content

As shown in Fig. S-2 of the Supplementary material, the linear relation between the concentration of phenolic compound and the absorbance ($R^2 = 0.9996$) was illustrated.[29] The mean and standard deviation values of phenolic acids and flavonoids found in samples taken from seven regions are given in Table I. The major phenolic is gallic acid. Mean values of it were found as 835, 1079, 719, 796, 410, 2026 and 973 mg/kg in Pervari, Shirvan, Sor, Rash, Korajar, Gara and Qalandar, respectively. In addition, the mean values of quinic acids were found as 881, 1362, 1842, 542, 1487, 363 and 968 mg/kg in Pervari, Shirvan, Sor, Rash, Korajar, Gara and Qalandar, respectively.

TABLE I. Determinations of phenolic acids and flavonoid compounds (mean ± *s*, μg kg^{-1}); nd.: not detected

Analyte	Pervari	Şirvan	Sor	Rash
Coumarin	8.330±0.002	5.530±0.001	9.890±0.002	7.750±0.002
Hesperidin	24.510±0.006	2.660±0.001	23.640±0.006	6.070±0.002
p-Coumaric acid	2.660±0.001	1.760±0.001	4.040±0.002	7.660±0.004
Gallic acid	834.74±0.24	1079±0.3	719.24±0.20	796.100±0.225
Caffeic acid	0.900±0.001	0.14±0.00	0.13±0.00	0.14±0.00
Vanillic acid	8.850±0.004	12.200±0.006	6.500±0.003	10.800±0.005
Salicylic acid	nd.	nd.	1.43±0.00	0.23±0.00
Quinic acid	881.30±0.07	1361.86±0.11	1841.99±0.15	541.46±0.04
p-Hydroxybenzoic acid	4.600±0.001	1.250±0.000	1.400±0.000	nd.
trans-Ferulic acid	1.160±0.001	2.920±0.001	0.950±0.001	1.06±0.00
Chlorogenic acid	0.020±0.000	nd.	0.020±0.003	nd.
Rosmarinic acid	73.59±0.05	nd.	2.38±0.05	nd.
Protocatechuic acid	2.700±0.001	3.530±0.001	3.290±0.001	3.580±0.001
Cinnamic acid	71.84±0.01	54.27±0.01	118.75±0.01	125.48±0.02
Sinapinic acid	nd.	nd.	nd.	nd.
Fumaric acid	3.50±0.00	2.21±0.00	2.64±0.00	1.82±0.00
Malic acid	3311.7 ±3.7	5438.7±0.4	8495.8±0.6	3882±1
Syringic acid	nd.	nd.	5.86± 0.14	3.780±0.001
Naringenin	2.480±0.001	nd.	nd.	1.120± 0.001
Rutin	240.65±0.04	380.15±0.04	677.46±0.06	926.10±0.11
Quercetin	45.03±0.02	47.31±0.02	87.08±0.02	145.45±0.04
Quercitrin	nd.	nd.	nd.	nd.

TABLE I. Continued

Analyte	Pervari	Şirvan	Sor	Rash
Isoquercitrin	3.100±0.000	5.96±0.00	8.130±0.001	22.060±0.001
Cosmosiin	1.780±0.001	N.D.	0.190±0.001	nd.
Nicotiflorin	8.780±0.002	15.270±0.002	21.330±0.004	nd.
	Karajar	Gara		Qalandar
Coumarin	6.030±0.001	4.950±0.001		9.310±0.002
Hesperidin	1.910±0.001	nd.		nd.
p-Coumaric acid	36.87±0.02	96.96±0.05		21.40±0.01
Gallic acid	410.480±0.116	2025.970±0.571		972.680±0.274
Caffeic acid	1.560±0.001	4.850±0.002		1.480±0.001
Vanillic acid	10.130±0.005	12.270±0.006		127.280±0.065
Salicylic acid	nd.	nd.		1.31±0.01
Quinic acid	1487.37±0.12	363.41±0.03		967.81±0.07
p-Hydroxynenzoic acid	nd.	1.07±0.00		nd.
trans-Ferulic acid	33.270±0.001	73.91±0.02		71.17±0.04
Chlorogenic acid	nd.	nd.		nd.
Rosmarinic acid	nd.	nd.		nd.
Protocatechuic acid	3.760±0.001	5.950±0.002		6.730±0.002
Cinnamic acid	65.66±0.02	66.80±0.01		90.40±0.01
Sinapinic acid	nd.	nd.		3.65±0.01
Fumaric acid	2.79±0.00	2.72±0.00		1.69±0.00
Malic acid	10552.5±0.4	4656.31±1.19		15721.9±0.5
Syringic acid	2.590±0.001	6.750±0.001		3.020±0.002
Naringenin	N.D.	96.72±2.00		2.06±0.05
Rutin	185.18±0.15	76927.45±0.03		545.45±12.23
Quercetin	nd.	nd.		nd.
Quercitrin	nd.	5.20± 0.01		nd.
Isoquercitrin	1.980±0.003	1886.910±0.001		6.360±0.251
Cosmosiin	nd.	nd.		nd.
Nicotiflorin	nd.	17.010± 1.004		nd.

DPPH and FRAP analyses

The DPPH activities are presented in Table II, along with the FRAP analysis results of samples collected from seven regions. Additionally, the percentage of iron chelating activity observed in the samples is also provided in Table II.

Analysis of method validation parameters

In the linearity analysis, the developed analytical method was found to be linear for all compounds between the ranges of tested concentrations with acceptable correlation coefficients ($R^2 > 0.90$). The calibration curve equations and the coefficients of determination (R^2) are presented in Table S-I.

Intraday and inter-day repeatabilities were lower than 2.13 and 2.51 %, respectively. On the other hand, intra-day and inter-day recoveries were between 99.5–100.8 and 99.2–100.7 %, respectively. The results of the study demonstrate

that the accuracy and precision evaluation of the LC–MS/MS method was quite satisfactory for routine monitoring purposes.[24] Relative standard uncertainties (U^{95}) of the analytes were also determined by the accuracy (recovery) and the precision (repeatability) studies according to Eurachem's guide.[26]

TABLE II. Antioxidant activity, total phenolic compounds as gallic acid equivalent, total flavonoid analysis, FRAP antioxidant analysis as $FeSO_4$ equivalent and iron chelating percentages

Sample location	DPPH inhibition, %	Total phenolic, mg gallic acid/L extract	Total flavonoid, mg routine/L extract	FRAP, mg $FeSO_4$/L extract	Iron chelating, %
Gara	75.83±0.18	84.02±0.00	69.98±0.01	3.12±0.01	95.18±0.00
Korajar	85.89±0.01	128.42±0.01	246.46±0.01	7.30±0.04	90.18±0.01
Sor	87.29±0.01	387.53±0.02	935.75±0.02	25.18±0.04	73.88±0.01
Rash	87.45±0.00	358.77±0.02	683.86±0.02	23.85±0.06	71.22±0.00
Qalandar	87.45±0.01	278.03±0.02	659.73±0.01	17.76±0.01	81.36±0.01
Pervari	70.27±0.01	85.28±0.00	110.71±0.01	3.14±0.00	97.34±0.00
Şirvan	88.11±0.01	134.73±0.01	333.94±0.06	8.55±0.02	91.85±0.01

The developed LC–MS/MS method for the quantification of 25 phytochemicals was fully validated. Five different linearity ranges (25–1000, 100–5000, 250––10000, 1000–20000 and 5000–20000 μg/L) were used for the studied analytes in the method. The inter-day and intra-day *RSD* values of the analytes were smaller than 0.032 and 0.026, respectively.

The average and the standard deviation (*STD*) of the antioxidant activity (DPPH inhibition, %), average and *STD* between gallic acid equivalent, total flavonoid analysis, average and *STD* of FRAP antioxidant analysis and iron chelating average and *STD* of samples were determined and given in Table II. As seen in the results of sample analysis in Table II, the DPPH fractions of 1 mL extract prepared with methanol were given. Accordingly, the highest inhibition was indicated by the sample gathered from the Şirvan region (88.11 %) and the lowest inhibition was in the Pervari sample (70.27 %). There is a slight difference in the antioxidant activity between all the samples. It is also evident that rhubarb samples have high antioxidant activity.[25] In a study conducted by Öztürk *et al.*, the antioxidant activity of chloroform and methanol extracts of rhubarb roots and stems was investigated. The roots exhibited activity with 93.1 and 84.1 % inhibition of chloroform and methanol extracts, while the stem extracts showed 82.2 and 82.0 % inhibition, respectively.[35]

Following the outcomes of the analysis, the total phenolic concentration in 1 mL extract was given in Table II. Total phenolic concentrations were given as gallic acid equivalents. Gallic acid was calculated according to the standard regression curve of the gallic acid equivalent (Fig. S-2). According to these results, the highest value showed the sample collected from the Sor region (387.6 mg/L extract) and the lowest value collected from the Gara region (84.02 mg/L extract).

This difference may be due to the growth of samples in different regions. The concentration results of the samples are plotted and given in Fig. 1. In a study conducted by Öztürk *et al.*, the highest phenolic amounts were found in the chloroform extract of the rhubarb root and the methanol extract of the stem, with 48 and 35 µg PEs/mg extract, respectively. The phenolic compounds are known as powerful chain-breaking antioxidants. In the study conducted by Eman *et al.*, the total phenolic content in the ethanol extract and water extract of Rhubarb roots was found to be 655.47 and 1115.04 mg/100g d.w.[29]

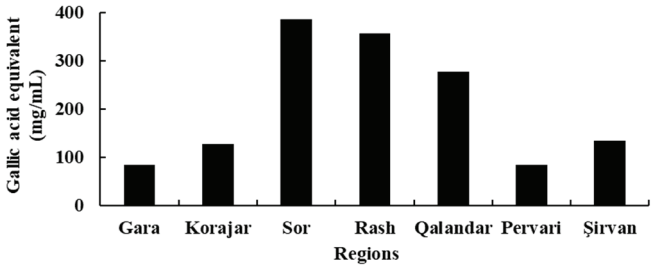

Fig. 1. Total phenolic concentrations as gallic acid equivalent.

The results of the total flavonoid concentrations found in 1 mL extract are given in Table II. Total flavonoid concentrations are given as routine equivalents. The routine equivalence calculation is based on the routine standard regression curve given. According to these results, the highest value showed the sample collected from the Sor region (935.8 mg/L extract) and the lowest value collected from the Gara region (69.98 µg/mL extract). The results of the concentration of the samples are plotted and given in Fig. 2. In a study conducted by Öztürk *et al.*, the total amounts of flavonoids in the chloroform extract of rhubarb roots and stems were found to be highest in chloroform extracts, with 145 and 20 µg QEs/mg extract, respectively.[35]

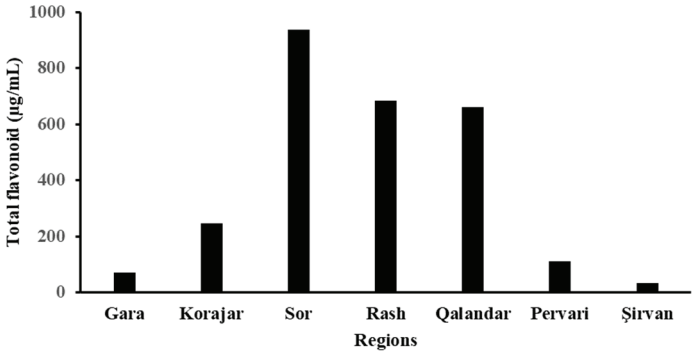

Fig. 2. Total flavonoid concentrations.

In various studies, the antioxidant activity of plant extracts (in the ethanolic extract) has been associated with their flavonoid content. In the study conducted by Eman *et al.*, the extracts richest in flavonoids were water and ethanol extracts.[29] The total amount of flavonoids in them was found to be 149.01 mg/100g d.w in the water extract and 687 mg/100g d.w in the ethanolic extract.[29]

The results of FRAP antioxidant capacities (total antioxidant amount) obtained in samples with 1 mL extract, prepared in methanol, were given in Table I. FRAP antioxidant capacities are given as $FeSO_4$ equivalents. According to these results, the highest antioxidant effect was obtained from the Sor region (25.18 mg/mL extract) and the lowest antioxidant effect from the Gara region (3.14 mg/mL extract). Calculation of the $FeSO_4$ equivalent is based on the FRAP standard regression curve given in Fig S-3 of the Supplementary material. The concentration results of FRAP antioxidant capacities obtained from seven regions are plotted and given in Table II.

Iron chelating capacities of samples found in extracts prepared with methanol are given in Table I. According to the results, the highest iron chelating capacity (97.34 %) was collected from the Pervari region and the lowest iron chelating capacity (71.22 %) was collected from the Rash region.

Quantitative determinations of phytochemicals in samples by LC–MS/MS

The developed, optimized and validated LC–MS/MS method (Table S-I and Fig. S-1) was applied for the simultaneous determination of 37 phytochemicals in methanol extracts of rhubarb samples. Codes from 1 to 37 were used for the studied species. LC–MS/MS-total ion chromatogram (TIC) chromatograms of the analysed extracts for compounds were given in the supplementary file (Figs. S-4––S-10). The quantitative analysis data of sample extracts related to LC–MS/MS data are given in Table II. According to LC–MS/MS results, extracts were found to be rich in phenolic acid and flavonoid content.

Generally, according to the LC–MS/MS results, the phenolic acid contents of the studied extracts were richer than flavonoid contents. Specifically, quinic, malic, fumaric, chlorogenic, vanillic and caffeic acid amounts were noteworthy in the overall assessment of the quantification results. Additionally, several phenolic compounds such as vanillic acid, syringic acid and sinapic acid were detected to be in low amounts in some of the extracts of the studied species. Rutin, hesperidin, isoquercitrin and quercetin were the most abundant flavonoids in the studied extracts. On the other hand, contents of gallic, *p*-hydroxybenzoic, *p*-coumaric, cinnamic, sinapinic and rosmarinic acids, coumarin, quercitrin, quercetin were determined. Quercitrin was reported to have benefits in delaying skin aging in humans.[36] Although rutin (a flavonoid) detected at an extract concentration of 2404 μg/g has been reported to have potential bioactivities such as cardioprotective, neuroprotective and antioxidant activities, its potential for use as a thera-

peutic agent is limited due to its low bioavailability.[37] Nicotiflorin (a flavonoid) has been reported to have several bioactivities such as α-glucosidase inhibitory (19.36±2.43 μM for KR),[38] *in vitro* antiglycation and hepatoprotective activities.[39] Flavonoid glycoside hesperidin (more than 2000 mg kg^{-1}) was reported to exhibit various pharmacological actions such as cardioprotective, antihyperlipidemic, antidiabetic and antihypertensive activities.[40] The phytochemical screening of hesperidin was carried out by LC–MS/MS method and several different species contained various amounts of hesperidin. Naringenin, a highly bioactive flavonoid aglycone, was reported to have several pharmacological effects such as antibacterial, antioxidant, anticancer and cardioprotective activities.[41,42] Quinic acid (minimum 541 μg kg^{-1} and maximum 1841 μg kg^{-1}) was found predominantly in all extracts of the studied samples. It was one of the most abundant phytochemicals in the studied plant species. The malic acid has the highest quantity in all extracts of samples. As for fumaric acid, its content in the extracts of samples may be notable. The protocatechuic acid content in all extracts is also noticeable. Chlorogenic acid content was detected in two samples (Pervari and Sor). The contents of vanillic acid (minimum 6.5 μg kg^{-1} and maximum 127 μg kg^{-1}) in all extracts studied were found to be in significant amounts. Although all the extracts contained a certain amount of caffeic acid (minimum 0.13 μg kg^{-1} and maximum 1.56 μg kg^{-1}), the amount of this phenolic acid is higher than the content of chlorogenic acid but lower than the contents of other phenolic acids. Furthermore, all the extracts contain vanillic acid, *tr*-ferulic acid, salicylic acid and *p*-coumaric acid in a certain range, not in a very high concentration. The salicylic acid in the extract was determined in three regions (Sor, Rash and Qalandar). In this study, salicylic acid was identified as a minor component in the quantitative analysis of methanol extracts of the samples by LC–MS/MS. Rosmarinic acid was a famous bioactive phenolic acid and was determined in extracts of the Pervari and Sor regions (73.6 and 2.38 mg kg^{-1}, respectively). It might be said that the rosmarinic acid contents of these species vary, depending on the growing conditions and extraction conditions. Gallic acid was highly abundant in methanol extracts of samples. Besides, several other extracts contained minor amounts of gallic acid. The high antioxidant activity of these species was mainly attributed to the quercetin content as the gallic acid content of the species was not examined. Therefore, it can be said that this high antioxidant potential of samples is related to its high gallic acid content rather than its quercetin content.

While the contents of rutin and isoquercitrin flavonoids in all extracts of samples were determined, nicotiflorin, hesperidin, quercetin and naringenin flavonoids were determined in some extracts of samples. Contents of quercetin in four extracts (Pervari, Şirvan, Sor and Rash) are notable. Also, the isoquercitrin content of extracts found in the Gara region was much higher than in other regions. As for nicotiflorin, its content was determined in four extracts of samples (Pervari,

Şirvan, Sor and Gara) and nicotiflorin content of extracts in Sor is higher than the others.

In the study conducted by Eman *et al.*, the amount of gallic acid in the ethanol and water extract of Rhubarb root was found to be 761 and 400 µg/100 g d.w, respectively.[29] Compared to this study, it was found to be higher only in Sor and Karajar regions. It was found to be less than the amount of gallic acid found in other regions. In terms of protocatechuic acid, it was found to be much higher than the values found in this study. In the study conducted by Eman *et al.*, it was observed that the amounts of protocatechuic acid, chlorogenic acid, caffeic acid, vanilic, hesperidin, quercetin and rosmarinic were much higher than the values found in this study.[29] However, as seen in Table II, the result of the rutin analysis of ethanol and water (7 µg/100 g d.w) extract performed by Eman *et al.* was found to be lower than the rutin results found in this study, as seen in Table II.[29]

The optimized LC–MS/MS method was applied to qualify and quantify the 25 phytochemicals in rhubarb samples. A detailed analytical method validation procedure was conducted comprising the inter-day and the intra-day precision (repeatability), accuracy (recovery), the limits of detection and quantification (*LOD*, *LOQ*), linearity, and the relative standard uncertainty. This method provides a rapid, sensitive and accurate LC–MS/MS method for the simultaneous quantitative determination of 25 types of phenolic compounds in rhubarb samples. The phenolic compound contents varied significantly within the same species. The variations in the phenolic compound contents were primarily due to the geographic distribution and different altitudes where the plant was grown. It was determined that there were significant amounts of important phytochemicals such as gallic, quinic, cinnamic and malic acids, and rutin, hesperidin and ioquercitrin flavonoids in the extracts of the studied species. It has been found that the various parts of the species have very different biological effects and chemical content. The extracts from rhubarb samples were found to be more active, especially in terms of antioxidant capacity. According to the results of LC–MS/MS, it seems that the chemical contents are also different. The various parts of the plant to be studied must be pursued separately, as well as the plant chemistry studies should be pursued on different solvent and extraction varieties.

CONCLUSION

To conclude, the development of a straightforward and accessible methodology for characterizing the bioactive components of *Rheum ribes* has revealed its immense therapeutic potential and significance in the realm of natural medicine.

Through the determination of crucial parameters such as total phenolics, flavonoids, and antioxidant activity, this study sheds light on the richness of the bioactive compounds present in *R. ribes* extracts. The observed variation in phenolic and flavonoid contents underscores the diverse chemical composition of these

extracts across different samples. Moreover, the robust antioxidant activities exhibited by the extracts, as demonstrated by FRAP, DPPH and iron chelating assays, highlight their efficacy in scavenging free radicals and preventing oxidative damage. LC–MS/MS analysis further elucidated the chemical composition of the extracts, identifying key phenolic and flavonoid compounds, such as malic acid and rutin, as major contributors to their biological activities.

Overall, these findings present the potential of *R. ribes* extracts as valuable sources of natural antioxidants, paving the way for the further exploration of their therapeutic applications and integration into pharmaceutical and nutraceutical industries.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/12981, or from the corresponding author on request.

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ИЗВОД

ЕФИКАСАН И JEDNOSTAVAN ПРИСТУП ОДРЕЂИВАЊА БИОАКТИВНИХ *Rheum ribes* У ПРОВИНЦИЈИ КУРДИСТАН У ИРАКУ И РЕГИОНУ СИИРТ У ТУРСКОЈ

İBRAHIM TEGIN[1], BAKHTIYAR MAHMOOD FATTAH FATTAH[1], MEHMET FIDAN[2], ORHAN ACAR[3] и ERDAL YABALAK[4]

[1]Siirt University, Faculty of Arts and Science, Department of Chemistry, Siirt, Turkey, [2]Siirt University, Faculty of Arts and Science, Department of Biology, Siirt, Turkey, [3]Gazi University, Science Faculty, Chemistry Department, Ankara, Turkiye и [4]Mersin University, Department of Nanotechnology and Advanced Materials, Mersin, Turkey

Развијање поједностављене и приступачне методе за идентификацију биоактивних компоненти *Rheum ribes* (рабарбаре) доприноси откривању његовог терапеутског потенцијала и унапређењу истраживања у природној медицини. Одређене су биоактивне компоненте рабарбаре, као што су укупни феноли и флавоноиди, као и антиоксидативна активност њеног метанолног екстракта. Укупни садржај фенола у екстрактима био је између 84,02 and 387,53 mg/L еквивалента галне киселине (GAE). Укупни садржај флавоноида у екстрактима, одређен колориметријском методом, је од 69,98 to 935,75 mg L^{-1} еквивалената рутина (PE). Антиоксидативна активност је одређена применом метода антиоксидативног потенцијала који редукује гвожђе (FRAP) и 1,1-дифенил-2-пикрилхидразил (DPPH). Резултати FRAP методе су показали да је највећа антиоксидативна

активност (IC_{50}) 25,18±0,04 mg L^{-1}. У DPPH методи, максимални проценат инхибиције је 88,11 %. Активности хелирања гвожђа у узорцима биле су изнад 70 %. Садржај хемијских једињења у екстрактима је одређен помоћу LC–MS/MS. Квалитативно и квантитативно је анализирано укупно 25 фенолних и флавоноидних једињења у екстрактима. Као главна фитохемијска једињења у екстракту су јабучна киселина (15,72±0,53 mg kg^{-1}) и рутин (76,93±0,03 mg kg^{-1}). Резултати потврђују да рабарбара има потенцијалну биолошку активност и да се може да буде важан извор природних антиоксиданата.

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