SUPPLEMENTARY MATERIAL

Bioactive Contents, In vitro Antiradical, Antimicrobial and Cytotoxic Properties of Rhubarb (Rheum ribes L.) Extracts

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Abstract

Rheum ribes L. (rhubarb) is belonging to Polygonaceae, and its roots and fresh shoots are consumed as vegetable in Turkey. This plant is considered to be one of the most important pharmaceutical raw materials in Middle East. In this study, the antiradical, antimicrobial, cytotoxic and bioactive properties of water, ethanol, and methanol extracts of R. ribes stems were determined. R. ribes stems water, ethanol and methanol extracts are better scavenged ABTS•+ (99.27, 99.91, and 99.88%), DPPH• (83.11, 81.42, and 83.26%), and OH• radicals (93.49, 94.21, 95.86%) than standard antioxidant BHA (95.32, 80.49, and 93.78%). Stems of R. ribes abundantly include bioactive compounds, dominated by rutin, catechin, caffeic acid, ferulic acid, α-tocopherol and vitamin D. These extracts show effective cytotoxic properties against PC-3, A2780, HCT-116 and MCF-7 cancer cell lines at 24h. It is found that R. ribes contain high amount important bioactive contents, and has effective antiradical and cytotoxic properties.

Keywords: Rhubarb, antiradical, antimicrobial, cytotoxicity, phytosterol

1. Experimental

1.1. Chemicals: All chemical compounds were obtained from Sigma-Aldrich (Germany).

1.2. Extraction procedures: R. ribes were collected in May-August 2016 from Elazig in Turkey. Voucher specimen number is Turkoglu 4870. Voucher specimen was stored in the herbarium of Firat University, Science Faculty, Department of Biology, Elazig, Turkey. The stems of R. ribes were dried at dark and room temperature. The parts were pulverized using a mechanic grinder, and then 10 g of the powdered samples was extracted with 100 mL of solvent (water, ethanol and methanol) in the Soxhlet extractor (Gerhardt Soxtherm SOX-402, Germany). After filtering of solvents, the supernatants were concentrated in a rotary evaporator to dry. The standard antioxidants and dried extracts were dissolved in DMSO (μg/mL).

1.3. Determination of Antiradical Activities: The ABTS•+, OH• and DPPH• radical scavenging activities (RSAs) were determined by the methods of Re et al. (1999), Halliwell et al. (1987) and Brand-Williams et al. (1995), respectively.

1.3.1. ABTS•+ Radical Scavenging: The ABTS•+ radical scavenging properties of extracts were carried out according to the method of Re et al. (1999). Briefly, ABTS•+ was produced by reacting 2 mM ABTS in H2O with 2.45 mM K2S2O8, and it was stored for 2 h at room temperature in the dark. The ABTS•+ solution was diluted to
give an absorbance of 0.750 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH=7.4). Then, 1 mL of ABTS•⁺ solution was added to 3 mL of extract at 500 µg/mL concentrations. After 0.5 h, absorbance was recorded at 734 nm. The extent of decolourization was calculated as percentage reduction of absorbance.

1.3.2. **OH• Radical Scavenging:** The hydroxyl radical scavenging property was determined according to the method of Halliwell et al. (1987). The reaction mixture contained 500 µL of extract, 500 µL of 3.6 mM 2-deoxy-D-ribose in KH₂PO₄–KOH buffer (20 mM, pH=7.4), 200 µL of 100 µM FeCl₃, 200 µL of 104 mM EDTA, 100 µL of 1.0 mM H₂O₂ and 100 µL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 37 °C for 1 h. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to tubes. The samples were vortexed and heated in a water bath at 50 °C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm.

1.3.3. **DPPH• Radical Scavenging:** The DPPH• radical scavenging property of extracts was measured the method of Brand-Williams et al. (1995). A solution of 25 mg/L DPPH in methanol was prepared and 4.0 mL of this solution was mixed with 500 µg/mL of extract. The reaction mixture was stored in darkness at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm in a spectrophotometer. All tests were repeated thrice and the average values were computed. The radical scavenging activity percentages (RSA%) were estimated by the following equation:

\[
\text{RSA} \% = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

Where, A₀ and A₁ are the absorbance of the control and the sample, respectively.

1.4. **Determination of Phytochemical Compounds:**

1.4.1. **Total Phenolic Contents:** Total polyphenolic contents in the extracts were determined according to Slinkard and Singleton’s method (1977). Briefly, 1 mL of the extract solution in a volumetric flask diluted with distilled water (46 mL). One milliliter of Folin–Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 mL of Na₂CO₃ (2%) was added and then it was intermittent shaken for 2 h. The absorbance was measured at 760 nm. The results are expressed as gallic acid equivalent.

1.4.2. **Total Flavonoid Content:** The determination of total flavonoid was performed according to the colorimetric assay reported by Kim et al. (2003). Distilled water (4 mL) was added to 0.5 mL extracts. Then, 5% sodium nitrite solution (0.3 mL) was added, followed by 10% aluminum chloride solution (0.3 mL). Test tubes were incubated at the room temperature for 5 min, and then 2 mL of 1 M sodium hydroxide were added to the mixture. The mixture was thoroughly vortexed and the absorbance of the pink color developed was measured at 510 nm. The results are expressed as catechin equivalent.

1.4.3. **Proanthocyanidin Content:** Determination of proanthocyanidin was carried out according to method described by Amaeze et al. (2011). A volume of 0.5 mL of extract was mixed with 1.5 mL of 4% vanillin-methanol solution and 0.75 mL concentrated hydrochloric acid. The mixture was allowed to stand for 15 min after which the absorbance was measured at 500 nm. The results are expressed as catechin equivalent.

1.4.4. **Chromatographic Conditions for Flavonoid and Phenolic Acids Analysis:** Chromatographic analyses of flavonoid and phenolic acids in the R. ribes stems were done using the method of Zu et al. (2006). Chromatographic analysis was carried out using PREVAIL C 18 reversed-phase column (150 x 4.6 mm x 5 µm) diameter particles. The mobile phase was methanol/water/acetonitrile (46/46/8, v/v/v) containing 1.0% acetic acid. This phase was filtered through a 0.45 µm membrane filter (millipore), then deaerated ultrasonically prior to use. Rutin, myricetin, morin, kaempferol, catechin, quercetin, naringin, naringenin, resveratrol, gallic acid,
vanillic acid, hydroxycinnamic acid, ferulic acid, caffeic acid and rosmarinic acid were quantified by DAD following RP-HPLC. Flow rate and injection volume were 1.05 mL/min and 10 μL, respectively. The peaks in the chromatograms of the extract were confirmed by comparing to their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of the peak using the external standard method. All chromatographic operations were carried out at 25 °C. The results of the analyses were expressed as mg/g.

1.4.5. Fatty Acids Analyses: Fatty acids in the extracts (%) were analyzed by GC according to Christie’s method (1992). The fatty acid methyl esters were extracted with n-hexane. The methyl esters were then separated and quantified by gas chromatography and flame-ionization detection (Shimadzu GC 17 Ver.3) coupled to Glass GC 10 computer software. Chromatography was performed with a capillary column (25 m in length and 0.25 mm in diameter) (Permabound 25, Macherey-Nagel, Germany) using nitrogen as a carrier gas (flow rate 0.8 mL/min.). The temperatures of the column, detector and injection valve were 130-220, 240, and 280 °C, respectively. Identification of the individual methyl esters was performed by frequent comparison with authentic standard mixtures that were analyzed under the same conditions. The results of the analyses were expressed as percent.

1.4.6. Analyses of Lipid Soluble Vitamins and Phytosterols: Phytosterols and lipid-soluble vitamins were extracted from the R. ribes according to the HPLC method of Sánchez-Machado et al. (2002) and Lopez-Cervantes et al. (2006). The extract was dissolved in acetonitrile/methanol (75/25 v/v) and 10 μL was injected into the HPLC instrument (Shimadzu, Kyoto Japan). The column used was a Supelcosil™ LC18 (250 x 4.6 mm, 5 μm, Sigma, USA). The mobile phase was acetonitrile/methanol (75/25, v/v) and the elution was performed at a flow rate of 1 mL/min. The temperature of the analytical column was kept at 40 °C. Identification of the individual vitamins and phytosterols were performed by frequent comparison with authentic external standard mixtures analyzed under the same conditions. Class VP 6.1 software assisted in the workup of the data. The results of the analyses were expressed as mg/g.

1.5. Determination of Antimicrobial Properties: Bacillus megaterium DSM 32, Escherichia coli ATCC 25922, Bacillus subtilis IMG 22, Proteus vulgaris FMC 1, Listeria monocytogenes SCOTTA, Pseudomonas aeruginosa DSM 50071, Staphylococcus aureus COWAN 1, Klebsiella pneumonia FMC 5 bacteria and Candida albicans FMC 17 were used as test organisms. Collins and Lyne’s method (1989) was used for the antimicrobial tests using the disc diffusion method. The disc diffusion method of using 100 μL of suspension containing 10⁶ per/mL of bacteria, and 10⁴ per/mL yeast, inoculated into Mueller Hinton Agar (Difco), and Malt Extract Agar (Difco), respectively. The discs (6 mm in diameter) were then impregnated with 100 μL of extract and then placed on the inoculated agar. Petri dishes were prepared at 4 °C for 2 h. Then, the inoculated plates were incubated at 37±0.1 °C for 24 h for bacterial strains and at 25±0.1 °C for 72 h for yeast. At the end of the incubation period, the inhibition zones were measured. Streptomycin sulfate (10 mg/disc) and nystatin (30 mg/disc) were used as standard antibiotics.

1.6. Determination of Cytotoxic Properties:
1.6.1. Cell Culture: The cell lines of human prostate cancer (PC-3), human breast cancer (MCF-7), human ovarian cancer (A2780) and human colon cancer (HCT-116) were employed in this study. The PC-3, A2780, HCT-116 and MCF-7 cell lines were retrieved from American Type Culture Collection (ATCC).
1.6.2. MTT Assay: The water, ethanol and methanol extracts of *R. ribes* were screened for their cytotoxic properties against different type cancer cell lines (PC-3, A2780, HCT-116 and MCF-7). The viability of the cells was determined using 0.4% trypan blue. Experiments were not started when the cell viability was below 90%. Cells were counted by hemocytometer and $1.5 \times 10^4$ cells were put in all wells. These cells were treated with different concentrations (1, 5, 25, 50 and 100 $\mu$g/mL) of *R. ribes* extracts in DMSO, then cells were incubated for 24 h. The effects of the % cell viability of *R. ribes* extracts were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Denizot and Lang, 1986; Mosmann, 1983).

1.7. Statistical Analyses: SPSS Statistics 22.0 for Windows software was used for statistical analysis. The antiradical results were evaluated using the analysis of variance (ANOVA). For antiproliferative activity tests, normal distribution was obtained using Kolmogorov Smirnov test ($p<0.05$). The IC$_{50}$ values were calculated by using % cell viabilities of extracts.

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### Table S1. ABTS\(^{**}\), OH\(^{-}\), and DPPH\(^{•}\) radicals scavenging activities, total flavonoid, total proanthocyanidin and total phenolic contents of *Rheum ribes* extracts

| Samples       | ABTS\(^{**}\) Scavenging (%) | OH\(^{-}\) Scavenging (%) | DPPH\(^{•}\) Scavenging (%) | Total Flavonoid (µg/g) | Total Proanthocyanidin (µg/g) | Total Phenolic (µg/g) |
|---------------|-------------------------------|---------------------------|----------------------------|------------------------|-----------------------------|----------------------|
| *R. ribes* water | 99.27±0.05\(^{a}\)           | 83.11±0.82\(^{a}\)       | 93.49±0.79\(^{a}\)         | 2681.49±3.54           | 1306.33±2.98               | 118.76±0.75          |
| *R. ribes* ethanol | 99.91±0.01\(^{a}\)           | 81.42±0.75\(^{b}\)       | 94.21±0.25\(^{b}\)         | 2345.85±2.84           | 1827.44±1.85               | 125.07±0.64          |
| *R. ribes* methanol | 99.88±0.02\(^{b}\)           | 83.26±0.96\(^{b}\)       | 95.86±0.34\(^{b}\)         | 1239.74±2.49           | 4473.00±5.27               | 136.82±0.37          |
| BHA           | 95.32±0.17\(^{a}\)           | 80.49±0.27\(^{a}\)       | 93.78±0.11\(^{a}\)         | -                      | -                           | -                    |

Within a column, different superscripts are significantly different at p<0.001. The antiradical activity results were calculated for 500 µg/mL extract concentrations. Total flavonoid and total proanthocyanidin contents were expressed as µg catechin equivalent/g extract, and total phenolic content was expressed as mg gallic acid equivalent/g extract.

### Table S2. Contents and composition of flavonoids, phenolic acids, vitamins, phytosterols and fatty acids in *Rheum ribes*

| Flavonoids and Phenolic Acids | (mg/g) |
|------------------------------|--------|
| Rutin                        | 15.90±0.85 |
| Myricetin                    | 8.10±0.55  |
| Morin                        | 0.05±0.00  |
| Quercetin                    | 0.05±0.00  |
| Kaempferol                   | 0.05±0.00  |
| Catechin                     | 24.85±0.95 |
| Naringin                     | 5.45±0.25  |
| Naringenin                   | 0.05±0.00  |
| Resveratrol                  | 1.50±0.10  |
| Vanillic Acid                | 5.85±0.20  |
| Gallic Acid                  | 2.35±0.15  |
| Caffeic Acid                 | 302.45±2.65|
| Ferulic Acid                 | 269.25±1.90|
| Rosmarinic Acid              | 1.30±0.05  |

| Vitamin and Phytosterols    | (mg/g) |
|------------------------------|--------|
| Retinol                      | 0.10±0.00  |
δ-Tocopherol  
α-Tocopherol  
Vitamin K  
Vitamin D  
Ergosterol  
Stigmasterol  

| Fatty Acids (FA) | (%) |
|-----------------|-----|
| 16:0            | 15.22±0.42 |
| 16:1            | 10.17±0.23 |
| 18:0            | 5.67±0.06  |
| 18:1            | 10.29±0.47 |
| 18:2            | 20.40±0.81 |
| 18:3            | 29.24±0.17 |
| 20:5            | 9.01±0.08  |
| Saturated FA    | 20.89     |
| Unsaturated FA  | 79.11     |

Table S3. The antimicrobial activities of *Rheum ribes* extracts (mm zone)

| Samples                | *R. ribs* (water) | *R. ribes* (ethanol) | *R. ribes* (methanol) | Standard |
|------------------------|-------------------|----------------------|-----------------------|----------|
| *Escherichia coli*     | 8                 | 9                    | 11                    | 10       |
| *Proteus vulgaris*     | 8                 | 9                    | 10                    | 10       |
| *Pseudomonas aeruginosa* | 8             | 9                    | 10                    | 15       |
| *Listeria monocytogenes* | -               | 8                    | 9                     | 8        |
| *Klebsiella pneumonia* | -                 | 8                    | 10                    | 9        |
| *Bacillus subtilis*    | 8                 | 9                    | 10                    | 9        |
| *Bacillus megaterium*  | -                 | 8                    | 9                     | 12       |
| *Staphylococcus aureus* | 8                | 9                    | 10                    | 12       |
| *Candida albicans*     | 8                 | 8                    | 9                     | 10       |

Streptomycin sulfate (10 mg/disc) and Nystatin (30 mg/disc) were used as standard antibiotic discs. The diameter of the paper discs was 6 mm.
Table S4. The IC₅₀ values of *R. ribes* extracts against PC-3, A2780, MCF-7 and HCT-116 cancer cell lines for the cytotoxicity activity assay

| Samples (μg/mL)   | PC-3  | A2780 | MCF-7 | HCT-116 |
|-------------------|-------|-------|-------|---------|
| *R. ribes* water  | 39.59 | 19.67 | 26.63 | 18.38   |
| *R. ribes* ethanol| 38.11 | 18.20 | 26.80 | 14.18   |
| *R. ribes* methanol| 42.18 | 16.76 | 25.46 | 14.87   |

Figure S1. The relative cell viability (%) of HCT-116 (A), PC-3 (B), MCF-7 (C), A2780 (D) cancer cells after 24-hour treatment with *Rheum ribes* stem extracts. The changes on the cell viability (%) caused by *Rheum ribes* stem water, ethanol and methanol extracts are compared with the control data. Each data point is an average of 10 viability (n=10, *p<0.05).