Antioxidant and cytotoxic activities of curly dock (Rumex crispus L., Polygonaceae) fruit extract

Tatjana Ćehović*, Dunja Jakovljević*, Zoran Maksimović†, Snežana Djordjević‡§, Sanja Jakovljević*, Dragana Ćetojević-Simin∥
University of Novi Sad, Faculty of Medicine, *Biochemistry Department, Novi Sad, Serbia; University of Belgrade, Faculty of Pharmacy, †Institute of Pharmacognosy, Belgrade, Serbia; Military Medical Academy, ‡National Poison Control Center, Belgrade, Serbia; University of Defence, §Faculty of Medicine od the Military Medical Academy, Belgrade, Serbia; University of Novi Sad, Faculty of Medicine, ∥Oncology Institute of Vojvodina, Sremska Kamenica, Serbia

Abstract

Background/Aim. Rumex crispus (curly dock) is a wild perennial herbaceous plant, which products are considered as a rich source of biologically active molecules with antioxidant and cytotoxic activities. The aim of this study was to estimate of antioxidant and cytotoxic activities of aqueous extract of curly dock fruits. Methods. The aqueous extract of curly dock fruits was evaluated for its antioxidant activity by in vitro assays for ferric-reducing antioxidant power (FRAP), NO•, OH• and 2,2-diphenyl-1-picrylhydrazyl (DPPH)-free radical scavenging activities and the influence on lipid peroxidation in liposomes. The cytotoxicity of tested extract was examined in vitro in human cervix carcinoma (HeLa), colon adenocarcinoma (HT-29) and breast adenocarcinoma (MCF7) cells. Results. The tested extract showed a potential antioxidant activity manifested in scavenging of free radicals as well as an ability to decrease lipid peroxidation in liposomes. The results indicated tissue-selective cytotoxicity of R. crispus fruit extract in vitro. The most prominent antitumor activity was observed towards HeLa and MCF7 cell lines. Conclusion. The investigated aqueous fruit extract of R. crispus had potential antioxidant and cytotoxic activities, with necrosis as a main mechanism of induced cell-death. Different methods of extraction of R. crispus fruits, apart from aqueous, are recommended for further investigations.

Key words: antioxidants; lipid peroxidation; phytotherapy; plants, medicinal; polygonaceae.

Antiksidantna i citotoksična aktivnost ekstrakta ploda štavelja (Rumex crispus L., Polygonaceae)


Ključne reči: antioksidansi; lipidi, peroksidacija; fitoterapija; biljke, lekovite; polygonaceae.
Introduction

*Rumex crispus* (curly dock) is a wild perennial herbaceous plant in the family Polygonaceae. Its basal leaves are narrowly lanceolate, curly in the edges. The young leaves of curly dock appear in spring, and its flowering season starts from May to August. The seeds are harvested in the summer. On the stalk, flowers and seeds are produced in clusters on branched stems. Getting caught in wool and animal fur, or carried by the wind, the seeds have been spreading to new locations. Growing almost everywhere, the curly dock can be found especially in grassy areas, marshes, waste places, near roads, mostly in acid soils, and represents a real threat for crops as noxious weed species worldwide. Apart from being considered a seriously invasive weed, young leaves of curly dock are edible and often used as vegetables or salad. Furthermore, the use of its fruits has been described in Serbian and Turkish traditional medicine in treatment of stomach complaints such as dysentery for their astringent activity. 

Regarding its biological effects, the roots, leaves and fruits of *R. crispus* have been widely consumed in traditional medicine for many centuries as a tonic, laxative, spasmylic and cholagogue agent in bilious complaints, as well as an astringent for hemorrhoids and bleeding. The curly dock has also been used in folk medicine in order to control fungal and peptic disorders. Its dried roots are a gentle and safe laxative, particularly useful for treatment of mild constipation, due to anthaquinone content which gives the roots yellow colored pigment. Therefore, its roots are a well-known antdote for stomach disorders. Curly dock is also useful for treating a wide range of skin problems such as fungal disorders, spring eruption and scrofula.

The plant products are considered as a rich source of biologically active molecules, so the extracts of *R. crispus* have been declared to possess an antioxidant, antimicrobial and anti-fungal activities, offering remarkable protection against any damage. The thin layer chromatography (TLC) survey confirmed the presence of polyphenols such as phenolic acids and procyanidins in the investigated methanol extract of curly dock fruits. Previous studies have shown a high antioxidant activity of leaf and seed of this plant, but there is a lack of reports about potency of its fruits. Only its methanol fruits extract has shown a specific antioxidant potential, suggesting that it can be used as hepatoprotective agent and an active agent in the treatment of other diseases caused by oxidative stress. Furthermore, a recent study revealed that curly dock seed extracts may play an important role in reactive oxygen species (ROS) scavenging against oxidative stress. Moreover, methanolic extract of *R. crispus* root has shown a high level of inhibition of the HT-29 cell growth, and also has inhibited α-glucosidase and amylase effectively. Conclusively, above mentioned extract can be considered as a potent carbohydrate inhibitor, anticancerous and antioxidant. In addition, the extracts of curly dock root have demonstrated the high xanthine oxidase inhibitory activity. The ethanol extracts of roots, leaves and fruits of curly dock have been screened for its cytotoxic activity on leukemic cell lines – human eosinophilic leukemia. The analysis of morphological changes showed that the mechanism of cell-death was apoptosis. It has been proven that the extract of entire plant of *R. crispus* possess high antimalarial activity in vitro and in vivo against *Plasmodium falciparum* chloroquine-sensitive and *Plasmodium falciparum* chloroquine-resistant. The antimalarial activity of curly dock originates from isolated compound nepodin (a naphthalene derivative), which appears in high concentration in roots of the plant. In addition, it has been discovered that ether extracts of the leaf and the seed and an ethanol extract of the leaf of curly dock have antimicrobial activities on *Staphylococcus aureus* and *Bacillus subtilis*, which are Gram-positive bacteria. Recently, it has been reported that an extract of *R. crispus* roots exhibits antifungal activity due to chrysophanol and paretin, both anthraquinones, and nepodin, that were isolated from roots. These compounds were screened for in vivo antifungal activity against several various plant pathogenic fungi and were effective in controlling of the disease development comparable to that of synthetic fungicide fenamidon, at similar concentrations. The overall activities of extracts of curly dock support the traditional use of extracts from all parts of the mentioned plant in the treatment of various disorders.

The aim of this study was to measure amount of flavonoids, which are present in aqueous extract of *R. crispus* fruits, and known to possess a potential antioxidant activity. Furthermore, this study was based on estimation of antioxidant capacity and potentiality of investigated extract. The aim of this study also was to evaluate in vitro antitumor activity of the aqueous extract of curly dock fruits and to determine in vitro mechanism of cell-death induced by this extract in human tumor cell lines. The aim also was a determination of the non-tumor/tumor IC50 effects obtained after treatment with mentioned extract.

Methods

Extraction

Ripe fruits of *R. crispus* (voucher specimen number 3874) were collected during July 2012 from a meadow at Kumodraž, the suburbs of Belgrade. The identification was confirmed by Prof. Dr Radiša Jančić, Faculty of Pharmacy, University of Belgrade, Serbia. The voucher specimen was deposited in the herbarium of Faculty of Pharmacy, University of Belgrade, Serbia. Harvested fruits were dried in room temperature in airy place avoiding sunlight. Dried plant sample was reduced to a fine powder and defatted in a Soxhlet-type apparatus with n-hexane and chloroform until exhausted. Residual plant material was extracted for 3 h at 50°C with 10-fold quantity of water and filtered. Resulting aqueous extract was evaporated under reduced pressure in order to produce a deep brownish-red powder.

Quantification of flavonoids

The plant material (600 mg of dry extract of curly dock fruits) was extracted under reflux conditions (80°C) with 20 mL aceton (LaChema, Neratovice, Czech Republic), 1 mL solution

of urotropine (5 g/L; Merck, Darmstadt, Germany) and 2 mL hydrochloric acid (25%; Zorka Pharma, Šabac, Serbia) during 30 min. The extract was cooled to room temperature and filtered. The residue was reextracted under the same conditions. Both extracts were combined and the volume was completed to 100 mL of acetone solution, resulting in the stock solution. An aliquot of 20 mL of the stock solution was transferred to a separatory funnel, mixed with 20 mL of water and shaken once with 15 mL and three times more with 10 mL ethyl acetate (Zorka Pharma, Šabac, Serbia). All ethyl acetate solutions were merged and shaken twice with 50 mL of water in a separatory funnel, then transferred to flask and completed to 50 mL of ethyl acetate – the working solution. Preparing the test solution was performed as follows: an aliquot of 10 mL working solution was transferred to 25 mL volumetric flask, a volume of 1 mL 2% aluminium chloride (AlCl₃; Merck, Darmstadt, Germany) solution was added and made to volume with acetate buffer (Sigma Aldrich GmbH, Steinheim, Germany) in 40 mmol/L HCl (Sigma Aldrich GmbH, Steinheim, Germany), + 2.5 mL of 20 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma Aldrich GmbH, Steinheim, Germany), 300 mmol/L, pH 3.6, + 2.5 mL of 10 mmol/L FeCl₃•6H₂O (Sigma Aldrich GmbH, Steinheim, Germany) in 800 mL of distilled water containing 100 μL of trichloroacetic acid (TCA) (TCA, LaChema, Neratovice, Czech Republic), + 10.4 mL of perchloric acid (Sigma Aldrich GmbH, Steinheim, Germany). The reaction was ended by adding 2.3 mL of TBA-reagent [3 g TBA acid (TBA, Reanal, Budapest, Hungary) + 120 g of trichloroacetic acid (TCA) (TCA, LaChema, Neratovice, Czech Republic) + 10.4 mL of perchloric acid (Sigma Aldrich GmbH, Steinheim, Germany) in 800 mL of distilled water (obtained from a Simplicity 185 purification system, Milli pore Inc., USA) after 60 min incubation at room temperature in the dark, against methanol (Merck, Darmstadt, Germany) as the blank. The percent of scavenging was calculated against the control, containing only methanol instead of the extract, and Trolox®

**In vitro antioxidant tests**

The ferric-reducing antioxidant power (FRAP) assay

Appropriately diluted aqueous extract of *R. crispus* fruits (100 μL with final concentrations 0.01 mg/mL, 0.02 mg/mL, 0.03 mg/mL, 0.04 mg/mL, 0.05 mg/mL and 0.10 mg/mL) and 3.0 mL of freshly prepared FRAP-reagent [25 mL of the acetate buffer (Sigma Aldrich GmbH, Steinheim, Germany), 300 mmol/L, pH 3.6, + 2.5 mL of 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma Aldrich GmbH, Steinheim, Germany) in 40 mmol/L HCl (Sigma Aldrich GmbH, Steinheim, Germany), + 2.5 mL of 20 mmol/L FeCl₃•6H₂O (LaChema, Neratovice, Czech Republic)] were combined. After incubation at 37°C for 30 min, the intensive blue complex Fe²⁺–TPTZ was formed and the absorbance was recorded at 593 nm (Evolution 300 UV–VIS spectrophotometer, Thermo Scientific, Madison, WI, USA). The result was expressed as the percentage of flavonoids (%)\(^\text{16}\).

**Inhibition of lipid peroxidation in liposomes**

by thiobarbituric acid (TBA assay)

In brief, 10 μL of appropriately diluted extract (final concentrations 0.01 mg/mL, 0.02 mg/mL, 0.03 mg/mL, 0.04 mg/mL, 0.05 mg/mL and 0.10 mg/mL) was put into screw-capped glass test tubes and incubated for 60 min at 25 °C with 60 μL of liposomes [commercial product of proliposomes “Pro-Lipo S” (Lucas Meyer GmbH & Co., Hamburg, Germany)] with 30% of phosphatidylcholene of soya, pH 5–7, 20 μL of FeSO₄•7H₂O (0.1 mol/L, Kemika, Zagreb, Croatia) and 20 μL of ascorbic acid (0.1 mol/L, Sigma Aldrich GmbH, Steinheim, Germany). The reaction was ended by adding 2.3 mL of TBA-reagent [3 g TBA acid (TBA, Reanal, Budapest, Hungary) + 120 g of trichloroacetic acid (TCA) (TCA, LaChema, Neratovice, Czech Republic) + 10.4 mL of perchloric acid (Sigma Aldrich GmbH, Steinheim, Germany) in 800 mL of distilled water (obtained from a Simplicity 185 purification system, Milli pore Inc., USA) after 60 min incubation at room temperature in the dark, against methanol (Merck, Darmstadt, Germany)] against methanol (Merck, Darmstadt, Germany) as the blank. The percent of scavenging was calculated against the control, containing only methanol instead of the extract, and Trolox®

The experiment was repeated with CCl₄ (CCl₄, in 50% ethanol, Sigma Aldrich GmbH, Steinheim, Germany) introduced to the system just before adding the diluted extracts (final concentrations 0.01 mg/mL, 0.02 mg/mL, 0.03 mg/mL, 0.04 mg/mL, 0.05 mg/mL and 0.10 mg/mL) in order to evaluate possible antioxidant activity of the examined extract. The intensity of lipid peroxidation was expressed in nmol MDA/mL of liposomes.\(^{6,21,22}\)

**Hydroxyl radical (OH•) scavenging activity**

In brief, 60 μL of liposomes [commercial product of proliposomes “Pro-Lipo S” (Lucas Meyer GmbH & Co., Hamburg, Germany)] with 30% of phosphatidylcholene of soya, pH 5–7, was put into screw-capped glass test tubes with 20 μL of FeSO₄•7H₂O (0.1 mol/L, Kemika, Zagreb, Croatia) and 2 mL of water. The reaction was incubated for 60 min and the absorbance of red colored adduct was measured at 532 nm (Evolution 300 UV–VIS spectrophotometer, Thermo Scientific, Madison, WI, USA). The intensity of lipid peroxidation was expressed in nmol MDA/mL of liposomes.\(^{23,24}\)

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Hydroxyl radical (OH•) scavenging activity combined with CCl4

In order to evaluate possible antioxidant activity of the examined extract, the experiment was repeated with the same diluted extracts added after introducing the CCl4 (CCl4, in 50% ethanol, Sigma Aldrich GmbH, Steinheim, Germany) to the system. The production of OH• was expressed in nmol MDA/mg of deoxyribose 23, 24.

Nitric oxide radical (NO•) scavenging assay

NO• scavenging potential of the aqueous extract of curly dock fruits was evaluated using the method described by Garrat. A volume of 2 mL of 10 mM sodium nitroprusside (Sigma Aldrich GmbH, Steinheim, Germany) in phosphate buffer saline (Sigma Aldrich GmbH, Steinheim, Germany), pH 7.4, was combined with 0.5 mL of investigated extract (with final concentrations: 1.000 mg/mL, 0.500 mg/mL, 0.100 mg/mL, 0.050 mg/mL, 0.025 mg/mL and 0.010 mg/mL). After the incubation at 25°C during 150 min, 0.5 mL of incubation solution was withdrawn and mixed with 0.5 mL of Griess reagent [1.0 mL sulfanilic acid reagent (0.33%) prepared in 20% glacial acetic acid (Sigma Aldrich GmbH, Steinheim, Germany)] at room temperature for 5 min with 1 mL of naphthylethylene diamine dihydrochloride (0.1% w/v, Sigma Aldrich GmbH, Steinheim, Germany). The tested mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm (Evolution 300 UV-VIS spectrophotometer, Thermo Scientific, Madison, WI, USA). The percent of inhibition was calculated against the control solution, containing only methanol (Merck, Darmstadt, Germany) instead of test solutions.

Tests on cell lines

Cell lines

For the estimation of cell growth effects, human tumor cell lines HeLa (cervix epitheloid carcinoma, ECACC No. 93021013), MCF7 (breast adenocarcinoma, ECACC No. 86012803), HT-29 (colon adenocarcinoma, ECACC No. 91072201), and MRC-5 (human fetal lung, ECACC 84101801) were used and prepared according to previously described procedures 26–28. Cell lines were cultivated in DMEM medium combined with 4.5% glucose, supplemented with 10% heat-inactivated fetal calf serum (FCS; NIVNS, Novi Sad, Serbia), 100 IU/mL of penicillin and 100 μg/mL of streptomycin (Galenika, Belgrade, Serbia). All tested cell lines grew attached to the surface, cultivated in 25 mL flasks (Corning, New York, USA) at 37°C, provided with 5% CO2 and high humidity, and sub-cultured twice a week. A single cell suspension was obtained using 0.1% trypsin (Serva, UK) with 0.04% EDTA 26–29.

Cell growth activity

According to previous studies, the cell lines were harvested and plated into a volume of 199 μL in 96-well microtitre plates (Sarstedt, Newton, USA) at a seeding density of 3–5 × 103 cells in each well, and preincubated in complete medium provided with 5% FCS, at 37°C for 24 h 26–29.

In order to achieve required final concentrations, serial twofold dilutions of the aqueous extract of R. crispus fruits in DMSO (1 μL) were combined with 199 μL of medium. Equal volume of solvent was added in control wells. After further incubation of microplates at 37°C for 48 h, the cell growth was evaluated by measuring the total protein content by colorimetric sulforhodamine B assay (SRB) according to Skehan et al. 30. Colour development was measured using a Multispec Ascent (Labsystems; Helsinki, Finland) photometer at 540 nm against 620 nm as background 26–29.

The effect on cell growth was calculated as 100 x (AT/AC) (%), where AT and AC are the absorbance of the test sample and the control, respectively, 26, 27. The concentration-cell growth (dose effect) curves were made for each treatment and IC50 values (concentration of extract that inhibits cell growth by 50%) were calculated, using OriginPro 8 SRO (Origin-Lab Corporation, Northampton, USA). The non-tumor/tumor IC50 ratios were calculated for HeLa, MCF-7, HT-29 and MRC-5 cell lines 26, 29.

Cell-death detection

The mechanism of cell-death in human cervix carcinoma (HeLa) and breast adenocarcinoma (MCF7) cell lines was determined by detection of apoptosis and necrosis using Cell Death Detection ELISA+ Apoptosis kit (Roche, Version 11). The enzyme-immunoassay, based on the sandwich principle, qualitatively and quantitatively determines cytoplasmic histone associated DNA fragments. Mouse monoclonal antibodies directed against DNA and histones, allowing specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of the cell, were used. Cell death detection experiments were performed when IC50 < 100 μg/mL criterion was met in cell growth experiments (in HeLa and MCF7 cell lines) 26.
According to study of Četojević-Simini et al. 26, the cell lines (1 × 10⁶ cells) were seeded in a 96-well microplate and preincubated for 24 h. After adding the extract and solvent (negative control) and incubation (about 2 h, precise timing was determined under the microscope) the plate was centrifuged, cell culture supernatants pooled for each treatment (n = 4) and used for the examination of necrosis. Cells were then lysed, centrifuged, lysates pooled (n = 4) and evaluated for apoptosis 26. The supernatant of cell lines (for the evaluation of necrosis) and cell lysis fraction (for the evaluation of apoptosis) (n = 2), both containing cytoplasmic histone-associated DNA fragments, were treated with the anti-histone antibodies and anti-DNA antibodies coupled to peroxidase and incubated for 2 h 26. The microplate was then washed, substrate of the peroxidase was added and colour development measured, using a Multiscan Ascent (Labsystems; Helsinki, Finland) photometer at 405 nm against 492 nm as background 26.

Background value was subtracted from the averages for each treatment. Enrichment factors (EF), both for apoptosis and necrosis, were calculated as EF = AT/AC, where AT was absorbance of the treatment and AC of the negative control (solvent). EF apoptosis/necrosis ratios were calculated as EF A/N = EF A / EF N 26.

Statistical analysis

All results are expressed as mean ± standard deviation (SD). Student’s t-test (presented as t values in results) was used for comparing means of two groups. One-way analysis of variance (ANOVA, presented as F values in results) was used to compare means of more than two groups. Post-hoc analysis was performed by using the Tukey’s test. All values p < 0.05 were considered to be statistically significant. Statistical analysis was performed using IBM SPSS Statistics 21.

Results

Quantification of flavonoids

The concentration of flavonoids in aqueous extract of *R. crispus* fruits, determined using spectrophotometric method with aluminium-chloride, was 0.67%.

In vitro antioxidant test

The results of the FRAP assay are shown in Table 1. The concentration of 0.05 mg/mL of the extract had the highest antioxidant capacity, but among the different concentrations of investigated extract there was no significant difference in antioxidant power (by using ANOVA, F = 1.511, p = 0.224). There were clear differences between the control group and the all concentrations of the extract except the sample that contained 0.03 mg/mL of the extract.

<table>
<thead>
<tr>
<th>Group</th>
<th>FRAP units, mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>Extract, mg/mL</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>1.14 ± 0.04*</td>
</tr>
<tr>
<td>0.02</td>
<td>1.13 ± 0.03*</td>
</tr>
<tr>
<td>0.03</td>
<td>1.11 ± 0.07</td>
</tr>
<tr>
<td>0.04</td>
<td>1.14 ± 0.06*</td>
</tr>
<tr>
<td>0.05</td>
<td>1.20 ± 0.10*</td>
</tr>
<tr>
<td>0.10</td>
<td>1.19 ± 0.04*</td>
</tr>
</tbody>
</table>

Values (FRAP units) represent means ± standard deviation (SD) of six measurements (n = 6).

The results of the DPPH assay (Table 2) were reported as the percentage of scavenged DPPH free radical and as the IC₅₀. The investigated extract was capable of neutralizing DPPH free radicals via hydroxyl donating activity 20 by 84.66%, 83.12%, 64.26%, 52.32%, 34.16% and 14.04% at concentrations 1.000 mg/mL, 0.500 mg/mL, 0.100 mg/mL, 0.050 mg/mL, 0.025 mg/mL and 0.010 mg/mL, respectively. The IC₅₀ value for the investigated extract was 0.046 mg/mL, while the standard substance Trolox® was able to reduce the stable DPPH free radical, reaching 50% reduction with an IC₅₀ 0.216 mg/mL. Analyzing intensity of inhibition of the DPPH free radical under the influence of aqueous extract of *R. crispus* fruits, it was determined significant difference in reduction among concentrations used (F = 169.504, p < 0.001). Furthermore, using post-hoc analysis (Tukey’s test), it was found the presence of statistically significant difference in scavenging power between consecutive concentrations used: 0.500 mg/mL and 0.100 mg/mL (p < 0.01), 0.100 mg/mL and 0.050 mg/mL (p < 0.05), 0.050 mg/mL and 0.025 mg/mL (p < 0.01), 0.025 mg/mL and 0.010 mg/mL (p < 0.01). The statistically significant difference in inhibition of the DPPH free radical was not established only between concentrations 1.000 mg/mL and 0.500 mg/mL (p = 1.00).

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentrations of the extract (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>Trolox®</td>
<td>98.44 ± 1.62</td>
</tr>
<tr>
<td>Extract</td>
<td>84.66 ± 5.31</td>
</tr>
</tbody>
</table>

Note: Results are given as % of DPPH• scavenging and represent as means ± standard deviation (SD) of six measurements (n=6).

* p < 0.05, ** p < 0.01 - statistically significant difference between particular concentrations of the extract and Trolox®

The results of TBA assay are shown in Table 3. A statistically significant difference of intensity of lipid peroxidation between the control group and the group with 0.01 mg/mL of the investigated extract, in absence of CCl₄ was determined, while there was no significant difference between the control and the extract group (0.01 mg/mL) in the presence of CCl₄. The comparisons between concentrations of the examined extract and both control groups (with and with CCl₄) are given in Table 3. Analyzing intensity of lipid peroxidation affected by the examined extract, it was concluded that there was a statistically significant difference in means of the investigated parameter related to concentration, in both conditions: in absence and in presence of CCl₄ (F = 429.220, p < 0.001).

Note: Values represent means ± standard deviation (SD) of six measurements (n = 6).

*Statistically significant difference between particular concentration of the extract and control, p < 0.001.

**Statistically significant difference between particular concentration of the extract and control in the presence of CCl₄, p < 0.001.

The results of OH• scavenging activity are shown in Table 4. There was no statistically significant difference in intensity of the production of OH• between the control group and the group with 0.01 mg/mL of the aqueous extract of curly dock fruits, in both experiments: without and in the presence of CCl₄ (p = 0.224, p = 0.719, respectively). The comparisons between other concentrations of the investigated extract and both control groups (control and control + CCl₄) are given in Table 4. Analyzing intensity of forming OH• influenced by mentioned extract, it was concluded that there was a statistically significant difference in means of the investigated parameter related to concentration, in both conditions: in absence and in presence of CCl₄ (F = 220.891, p < 0.001; F = 83.174, p < 0.001, respectively).

Note: The production of OH• was expressed in nmol MDA/mg of deoxyribose; values (nmol MDA/mg of deoxyribose) represent means ± standard deviation (SD) of six measurements (n = 6).

*Statistically significant difference between particular concentration of the extract and control, p < 0.001.

**Statistically significant difference between particular concentration of the extract and control in the presence of CCl₄, p < 0.001.

The results of NO• scavenging activity are shown in Table 5. The investigated extract significantly inhibited generation of NO• and it was related to different concentrations of the extract used (F = 220.891, p < 0.001).

Note: Values (% of NO• inhibition) represent means ± standard deviation (SD) of six measurements (n = 6).

**Tests on cell lines**

The results of an influence of the extract tested on tumor cell growth are given in Table 6. The most pronounced antitumor activity was observed towards cervix carcinoma cell line (HeLa) with IC₅₀ 16.9 µg/mL and breast adenocarcinoma cell line (MCF7) with IC₅₀ 19.3 µg/mL. The non-tumor/tumor IC₅₀ ratios were calculated for HeLa, MCF-7 and HT-29 cell lines and they were NT/T = 1.54, NT/T = 1.35, NT/T < 0.42, respectively.

Table 3

**Influence on lipid peroxidation (LPx) in liposomes of the aqueous extract of Rumex crispus fruits with or without CCl₄ addition**

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol/mL of liposomes), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without CCl₄</td>
</tr>
<tr>
<td>Control</td>
<td>76.62 ± 1.61 *</td>
</tr>
<tr>
<td>Extract, mg/mL</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>73.64 ± 1.39 **</td>
</tr>
<tr>
<td>0.02</td>
<td>60.60 ± 2.79 **</td>
</tr>
<tr>
<td>0.03</td>
<td>55.16 ± 1.69 **</td>
</tr>
<tr>
<td>0.04</td>
<td>41.52 ± 2.17 **</td>
</tr>
<tr>
<td>0.05</td>
<td>21.98 ± 2.56 **</td>
</tr>
<tr>
<td>0.10</td>
<td>19.44 ± 3.02 **</td>
</tr>
</tbody>
</table>

Note: Values (nmol MDA/mL of liposomes) represent means ± standard deviation (SD) of six measurements (n = 6).

*Statistically significant difference between particular concentration of the extract and control in the presence of CCl₄, p < 0.001.

**Statistically significant difference between particular concentration of the extract and control in the presence of CCl₄, p < 0.001.

Table 4

**Influence of the aqueous extract of Rumex crispus fruits on production of hydroxyl radical**

<table>
<thead>
<tr>
<th>Extract, mg/mL</th>
<th>MDA (nmol/mg of deoxyribose), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.17 ± 0.13 *</td>
</tr>
<tr>
<td>0.01</td>
<td>2.08 ± 0.06 **</td>
</tr>
<tr>
<td>0.02</td>
<td>2.05 ± 0.09 **</td>
</tr>
<tr>
<td>0.03</td>
<td>1.95 ± 0.07 **</td>
</tr>
<tr>
<td>0.04</td>
<td>1.82 ± 0.12 ***</td>
</tr>
<tr>
<td>0.05</td>
<td>1.52 ± 0.17 ***</td>
</tr>
<tr>
<td>0.10</td>
<td>1.59 ± 0.18 ***</td>
</tr>
</tbody>
</table>

Note: Values (nmol MDA/mg of deoxyribose) represent means ± standard deviation (SD) of six measurements (n = 6).

*Statistically significant difference between particular concentration of the extract and control, p < 0.001.

**Statistically significant difference between particular concentration of the extract and control in the presence of CCl₄, p < 0.001.

Table 5

**Influence of the aqueous extract of Rumex crispus fruits on production of nitric oxide radical**

<table>
<thead>
<tr>
<th>Concentration of the extract, mg/mL</th>
<th>NO• (% of inhibition), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
<td>-15.54 ± 0.77</td>
</tr>
<tr>
<td>0.500</td>
<td>-7.26 ± 1.92</td>
</tr>
<tr>
<td>0.100</td>
<td>-4.36 ± 0.34</td>
</tr>
<tr>
<td>0.050</td>
<td>-3.66 ± 0.72</td>
</tr>
<tr>
<td>0.025</td>
<td>-0.81 ± 0.45</td>
</tr>
<tr>
<td>0.010</td>
<td>-0.03 ± 0.14</td>
</tr>
</tbody>
</table>

Note: Values (% of NO• inhibition) represent means ± standard deviation (SD) of six measurements (n = 6).

**Tests on cell lines**

The results of an influence of the extract tested on tumor cell growth are given in Table 6. The most pronounced antitumor activity was observed towards cervix carcinoma cell line (HeLa) with IC₅₀ 16.9 µg/mL and breast adenocarcinoma cell line (MCF7) with IC₅₀ 19.3 µg/mL. The non-tumor/tumor IC₅₀ ratios were calculated for HeLa, MCF-7 and HT-29 cell lines and they were NT/T = 1.54, NT/T = 1.35, NT/T < 0.42, respectively.

Table 6

**Influence of the aqueous extract of Rumex crispus fruits on cell growth in selected human cell lines**

<table>
<thead>
<tr>
<th>Group</th>
<th>IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
</tr>
<tr>
<td>Extract (doxorubicin)</td>
<td>16.88 ± 3.08</td>
</tr>
<tr>
<td>Control</td>
<td>0.25 ± 0.09*</td>
</tr>
</tbody>
</table>

Note: Values represent means ± standard deviation (SD) of eight measurements (n = 8) obtained in 3.91 to 1,000 µg/mL concentration range.

*Rewritten from Ćetojević-Simin et al. 26
Apoptosis and necrosis were expressed as enrichment factor (EF) and apoptosis/necrosis ratios (A/N) obtained in HeLa and MCF7 cell lines after treatment with the investigated extract. *R. crispus* fruit extract slightly decreased apoptosis and significantly increased necrosis in both MCF7 (EFA = 0.77; EFN = 2) and HeLa (EFA = 0.97; EFN = 4) cell lines giving high overall decrease in apoptosis/necrosis ratios compared to the control (EFA/N = 0.24–0.39) (Table 7).

Table 7 Apoptosis and necrosis expressed as enrichment factor (EF) and apoptosis/necrosis ratios (A/N) obtained in HeLa and MCF7 cell lines after treatment with the aqueous extract of *Rumex crispus* fruits

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EF A</th>
<th>EF N</th>
<th>EF A/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>0.97</td>
<td>4.00</td>
<td>0.24</td>
</tr>
<tr>
<td>MCF7</td>
<td>0.77</td>
<td>2.00</td>
<td>0.39</td>
</tr>
</tbody>
</table>

EF A – enhancement factor for apoptosis (EF for the control is 1); EF N – enhancement factor for necrosis (EF for the control is 1); EF A/N = EF A/EF N.

**Discussion**

**Quantification of flavonoids**

The interest in possible health benefits of flavonoids has increased owing to their potent antioxidant and free radical scavenging activities observed in vitro. According to numerous previous studies, flavonoids might be responsible for many biological activities of curvy dock, such as antiviral, antibacterial, anticancer, antioxidant activities and they might be capable to activate antioxidant enzymes. In the present study, there was no significant difference in antioxidant power among all the different concentrations of investigated extract. The results suggested that the mentioned extract might have an ability to reduce Fe$^{3+}$, and thus, evident ability to donate electrons due to clear differences between the control and the samples containing 0.01 mg/mL, 0.02 mg/mL, 0.04 mg/mL, 0.05 mg/mL and 0.10 mg/mL of the extract. The FRAP value of investigated extract at concentration of 0.05 mg/mL was 1.20 FRAP units [120 μmol/L Fe(II)], which could be comparable to ascorbic acid and vitamin E, known as antioxidants, with FRAP values 14.61 μg/mL Fe(II) (about 2.62 FRAP units) and 12.39 μg/mL Fe(II) (about 2.22 FRAP units), respectively.

The free radical scavenging activity of the investigated extract was measured using DPPH assay. The unpaired electron of the stable free radical DPPH$^+$ determines the appearance of a purple color, with an absorption at maximum 517 nm. Generation of the reduced (molecular) form (DPPH) is accompanied by the disappearance of the violet color and the vanishing of absorption. Taking into account that IC$_{50}$ was the concentration of the extract or Trolox$^e$ necessary to decrease the initial DPPH concentration by 50%, it was concluded that the mentioned extract was more potent than standard substance Trolox$^e$ in scavenging the stable DPPH free radical, due to the fact that IC$_{50}$ of the extract was lower than that of the standard substance, Trolox$^e$. The scavenging effect of the examined extract on the DPPH radical was comparable to previous study of extracts of *R. crispus* seeds, but the ascorbic acid with IC$_{50}$ = 9.51 μg/mL was more potent than the examined extract (IC$_{50}$ = 46 μg/mL).

The measurement of the end product of lipid peroxidation, malondialdehyde (MDA), is one of the most widely accepted assays for oxidative damage and has been used as an indicator of lipid peroxidation. The effect of the aqueous extract of curvy dock fruits on lipid peroxidation in liposomes was evaluated by the TBA test after lipid peroxidation induced by the Fe$^{3+}$/ascorbate system. This assay is based on the reactivity of MDA with TBA to generate a red chromo-mogen complex MDA-TBA with an absorption maximum at 532 nm. Lipid peroxidation caused by reactive oxygen species (ROS) plays an important role in the damaging mechanism of many disorders and it is used as an indicator of oxidative stress in cells and tissues. In the present study, there was no statistically significant difference between values of 0.10 mg/mL of the extract (19.44 nmoL MDA/mL of liposomes) and the control group without CCL$_4$ (76.62 nmoL MDA/mL of liposomes). The concentration of 0.05 mg/mL of the extract combined with CCL$_4$ was the most prominent. Taking into account that there was a statistically significant difference between the control group without CCL$_4$ (76.62 nmoL MDA/mL of liposomes) and 0.05 mg/mL of the extract combined with CCL$_4$ (91.76 nmoL MDA/mL of liposomes), the examined extract had a low ability to decrease lipid peroxidation in the presence of CCL$_4$ and it was not able to completely neutralize damaging effects of CCL$_4$.

**In vitro antioxidant tests**

Several in vitro methods are known to measure the total antioxidant capacity of biological samples. One of them, the FRAP method, is based on the reduction of a ferroin analog, the Fe$^{3+}$ complex with TPTZ (Fe$^{3+}$-TPTZ), to the Fe$^{2+}$ complex (Fe$^{2+}$-TPTZ) by antioxidants in acidic medium. In the present study, there was no significant difference in antioxidant power among all the different concentrations of investigated extract. The results suggested that the mentioned extract might have an ability to reduce Fe$^{3+}$, and thus, evident ability to donate electrons due to clear differences between the control and the samples containing 0.01 mg/mL, 0.02 mg/mL, 0.04 mg/mL, 0.05 mg/mL and 0.10 mg/mL of the extract. The FRAP value of investigated extract at concentration of 0.05 mg/mL was 1.20 FRAP units [120 μmol/L Fe(II)], which could be comparable to ascorbic acid and vitamin E, known as antioxidants, with FRAP values 14.61 μg/mL Fe(II) (about 2.62 FRAP units) and 12.39 μg/mL Fe(II) (about 2.22 FRAP units), respectively.

The intensity of the production of OH• radicals was determined following reaction of degradation of deoxyribose, with main product MDA. Decreased absorbance of the reaction mixture indicated increased OH• radicals scavenging activity of the tested extract. Analyzing the results, in this assay, the investigated extract might exhibit a hydroxyl radical scavenging activity. The OH• radical scavenging activity of the extract may be due to the presence of phenolic com-

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pounds. There is lack of reports in the literature of the OH• scavenging activity of any standard substances.

Taking into account the results, the aqueous extract of curly dock fruits might have a potential activity to scavenge NO•. The concentration of 1 mg/mL od the extract showed the highest potential to neutralize the NO• radical. In addition, there is no report available in the literature on NO• scavenging activity of any standard substances.

**Tests on cell lines**

In this study, the cytotoxic effect of the aqueous fruit extract of *R. crispus* in cancer cell lines and its potential to inhibit their growth were evaluated. Dose-dependent activity was confirmed in all tested cell lines. The effect of the aqueous extract of *R. crispus* fruits on non-cancerous cells MRC-5 was lower (IC50 = 25.98) (Table 6), suggesting its lower toxicity towards healthy cells. The IC50 value in colon adenocarcinoma HT-29 cells was not reached, but IC25 value that was obtained at still low concentration (IC25 = 62.5 µg/mL) suggests its activity towards this cell type, as well. The results showed high and favourable tissue-selective antitumor activity of the curly dock fruit extract in vitro.

Favorable non-tumor/tumor ratios were obtained in cervix carcinoma (NT/T = 1.35) and breast adenocarcinoma (NT/T = 1.54) cell lines. The highest non-tumor/tumor IC50 ratio was obtained in HeLa cell line, suggesting its high effectiveness towards this cancer type compared to non-tumor cells (MRC-5) (NT/T value was above 1). In colon adenocarcinoma HT-29 cell line, NT/T was < 0.42, which was below 1, suggesting higher activity of the extract towards healthy tissue (MRC-5) compared to colon cancer cells.

Taking into account that the contemporary cytotoxic therapy is based on the higher activity of antitumor drugs towards a tumor than to healthy tissue, this quality might favor the use of the curly dock extract in potential antitumor therapy.

Further tests were performed to evaluate the mechanism of cell-death for the examined extract. Enhancement factor ratios (EF A/N), when above 1, indicate favorable apoptosis/necrosis ratio, i.e. that dominant mode of cell death is apoptosis. Values of this ratio, when below 1, indicate that necrosis is dominant mode of cell death. Analysis of cytotoxicity mechanism showed that the necrosis was main mechanism of induced cell death in cervix (HeLa) and breast (MCF7) tumor cell lines after the *R. crispus* fruits water extract treatment.

**Conclusion**

The results of this study suggest that the aqueous extract of *R. crispus* fruits, containing flavonoids, might have a potential antioxidant activity and free radicals scavenging power. It was also concluded that the examined extract could have the cytotoxic activity, but with necrosis as a main mechanism of induced cell death. Different method of extraction of *R. crispus* fruits, apart from aqueous, is recommended in order to pinpoint possible active principles with lower necrotic and higher apoptotic potential that will also retain high antitumor potential.

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