



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

Antioksidantna i citotoksična aktivnost ekstrakta ploda štavolja (*Rumex crispus* L., Polygonaceae)

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### 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.

### Apstrakt

**Uvod/Cilj:** *Rumex crispus* (štavelj) je višegodišnja zeljasta biljka, koja se smatra bogatim izvorom biološki aktivnih molekula sa antioksidantnom i citotoksičnom aktivnošću. Cilj rada bio je procena antioksidantnog kapaciteta i ispitivanje antitumorske aktivnosti vodenog ekstrakta ploda štavolja. **Metode.** Antioksidantna aktivnost vodenog ekstrakta ploda štavolja procenjena je na osnovu *in vitro* testova: ferric-reducing antioxidant power (FRAP), sposobnosti ekstrakta da neutrališe slobodne radikale NO•, OH• i 2,2-difenil-1-pikril hidrazil (DPPH) i utiče na lipidnu peroksidaciju u lipozomima. Cytotoksičnost ispitivanog ekstrakta je određena *in vitro* na tumorskim ćelijskim linijama: humani karcinom cerviksa (HeLa), adenokarcinom (HT-29) i adenokarcinom dojke (MCF7). **Rezultati.** Testirani ekstrakt pokazao je potencijalnu antioksidantnu aktivnost manifestovanu velikom moći u neutralizaciji slobodnih radikala, kao i sposobnost da smanji lipidnu peroksidaciju u lipozomima. Ustanovljena je tkivno-selektivnu citotoksičnost ekstrakta ploda štavolja *in vitro*. Najizraženija antitumorska aktivnost primećena je prema HeLa i MCF7 ćelijskim linijama. **Zaključak.** Vodeni ekstrakt ploda štavolja ima potencijalnu antioksidantnu i citotoksičnu aktivnost, sa nekrozom kao glavnim mehanizmom indukovane ćelijske smrti. Za dalja istraživanja, preporučuju se i druge metode ekstrakcije ploda štavolja.

### 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<sup>1-4</sup>. 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<sup>1, 4, 5</sup>. Apart from being considered a seriously invasive weed, young leaves of curly dock are edible and often used as vegetables or salad<sup>3, 6</sup>. 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<sup>6-8</sup>. 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, spasmolytic 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<sup>2, 7, 9</sup>. Its dried roots are a gentle and safe laxative, particularly useful for treatment of mild constipation, due to anthraquinone content which gives the roots yellow colored pigment<sup>1, 3, 6, 10</sup>. Therefore, its roots are a well-known antidote for stomach disorders<sup>3</sup>. Curly dock is also useful for treating a wide range of skin problems such as fungal disorders, spring eruption and scrofula<sup>6, 10</sup>.

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 antifungal activities, offering remarkable protection against any damage<sup>2, 3, 6, 11</sup>. The thin layer chromatography (TLC) survey confirmed the presence of polyphenols such as flavonoids, phenolic acids and procyanidins in the investigated methanol extract of curly dock fruits<sup>6</sup>. 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<sup>6</sup>. Furthermore, a recent study revealed that curly dock seed extracts may play an important role in reactive oxygen species (ROS) scavenging against oxidative stress<sup>2</sup>. Moreover, methanolic extract of *R. crispus* root has shown a high level of inhibition of the HT-29 cell growth, and also has inhibited  $\alpha$ -glucosidase and amylase effectively. Conclusively, above mentioned extract can be considered as a potent carbohydrase inhibitor, anticancerous and antioxidant<sup>12</sup>. In addition, the extracts of curly dock root have demonstrated the high xanthine oxidase inhibitory activity<sup>13</sup>. The ethanol extracts of roots, leaves and fruits of curly dock have been screened for its cytotoxic activity *in vitro* and it has been found remarkable cytotoxic activities on leukemic 1301 – human T cell leukemia lymphoblast, and EOL-1 cell

lines – human eosinophilic leukemia. The analysis of morphological changes showed that the mechanism of cell-death was apoptosis<sup>9</sup>. 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<sup>7</sup>. 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<sup>11</sup>. Recently, it has been reported that an extract of *R. crispus* roots exhibits antifungal activity due to chrysophanol and parietin, both anthraquinones, and nepodin, that were isolated from roots<sup>14</sup>. 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 fenarimol, at similar concentrations<sup>3, 14</sup>. 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<sup>15</sup>. 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 IC<sub>50</sub> 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 acetone (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 ( $\text{AlCl}_3$ ; Merck, Darmstadt, Germany) solution was added and made to volume with acetic acid (5%; Zorka Pharma, Šabac, Serbia) in methanol (Zorka Pharma, Šabac, Serbia). Preparing the blank solution was as follows: an aliquot of 10 mL working solution was transferred to 25 mL volumetric flask and completed to volume with acetic acid (5%) in methanol. After 30 min the absorbance of the test solution was measured (Evolution 300 UV-VIS spectrophotometer, Thermo Scientific, Madison, WI, USA) at 425 nm against the blank solution. The result was expressed as the percentage of flavonoids (%)<sup>16</sup>.

#### *In vitro antioxidant tests*

##### The ferric-reducing antioxidant power (FRAP) assay

Appropriately diluted aqueous extract of *R. crispus* fruits (100  $\mu\text{L}$  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  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (LaChema, Neratovice, Czech Republic)] were combined. After incubation at 37°C for 30 min, the intensive blue complex  $\text{Fe}^{2+}$ -TPTZ was formed and the absorbance was recorded at 593 nm (Evolution 300 UV-VIS spectrophotometer, Thermo Scientific, Madison, WI, USA) against the blank, containing 100  $\mu\text{L}$  of resembling solvent. The absorbance was measured to test the amount of iron reduced and correlated with the amount of antioxidants present in the extracts. The absorbance of the samples was compared to the calibration curve of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (Kemika, Zagreb, Croatia) standard solutions, covering the concentration range 100–1000  $\mu\text{mol/L}$ ; the FRAP values were expressed as FRAP units, where one FRAP unit was 100  $\mu\text{mol/L}$   $\text{Fe}^{2+}$ <sup>6-18</sup>.

##### 2,2-diphenyl-1-picrylhydrazyl (DPPH)-free radical scavenging activity (DPPH assay)

Samples of the extract, and a standard substance Trolox<sup>®</sup> (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) were put into the set of test tubes and made up to 4.0 mL by the addition of methanol (Merck, Darmstadt, Germany). Finally, 1 mL of 0.5 mmol/L methanol DPPH (Sigma Aldrich GmbH, Steinheim, Germany) solution was put into each test tube. Decolorisation percentage was obtained spectrophotometrically at 517 nm (Evolution 300 UV-VIS spectrophotometer, Thermo Scientific, Madison, WI, 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<sup>®</sup><sup>6, 18-20</sup>.

##### Inhibition of lipid peroxidation in liposomes by thiobarbituric acid (TBA assay)

In brief, 10  $\mu\text{L}$  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  $\mu\text{L}$  of liposomes [commercial product of proliposomes “Pro-Lipo S” (Lucas Meyer GmbH & Co., Hamburg, Germany)] with 30% of phosphatidylcholine of soya, pH 5–7, 20  $\mu\text{L}$  of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1 mol/L, Kemika, Zagreb, Croatia) and 20  $\mu\text{L}$  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, Millipore S.A., Molsheim, France)] and 0.2 mL ethylenediaminetetraacetic acid (EDTA) (0.1 mol/L, Merck, Darmstadt, Germany)]. After heating at 100°C for 15 min, cooling and centrifugation, 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<sup>6, 21, 22</sup>.

##### Inhibition of lipid peroxidation in liposomes (TBA assay) combined with carbon tetrachloride $\text{CCl}_4$

The experiment was repeated with  $\text{CCl}_4$  ( $\text{CCl}_4$ , 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<sup>23, 24</sup>.

##### Hydroxyl radical ( $\text{OH}^\bullet$ ) scavenging activity

In brief, 60  $\mu\text{L}$  of liposomes [commercial product of proliposomes “Pro-Lipo S” (Lucas Meyer GmbH & Co., Hamburg, Germany) with 30% of phosphatidylcholine of soya, pH 5–7, was put into screw-capped glass test tubes with 20  $\mu\text{L}$  of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1 mol/L, Kemika, Zagreb, Croa-

tia), 10  $\mu\text{L}$  of hydrogen peroxide (0.009 mol/L, Kemika, Zagreb, Croatia) and 20  $\mu\text{L}$  of deoxyribose (0.05 mol/L, Sigma Aldrich GmbH, Steinheim, Germany) and finally 10  $\mu\text{L}$  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 added in the system and incubated for 60 min at 25°C. The reaction was ended by adding 2.3 mL of TBA-reagent [3 g TBA (TBA, Reanal, Budapest, Hungary) + 120 g of TCA acid (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, Millipore S.A., Molsheim, France)] and 0.2 mL EDTA (0.1 mol/L, Merck, Darmstadt, Germany). After heating at 100°C for 15 min, cooling and centrifugation, the absorbance of red colored adduct was measured at 532 nm (Evolution 300 UV-VIS spectrophotometer, Thermo Scientific, Madison, WI, USA). The production of OH• was expressed in nmol MDA/mg of deoxyribose<sup>6,21-24</sup>.

#### Hydroxyl radical (OH•) scavenging activity combined with CCl<sub>4</sub>

In order to evaluate possible antioxidant activity of the examined extract, the experiment was repeated with the same diluted extracts added after introducing the CCl<sub>4</sub> (CCl<sub>4</sub>, in 50% ethanol, Sigma Aldrich GmbH, Steinheim, Germany) to the system. The production of OH• was expressed in nmol MDA/mg of deoxyribose<sup>23,24</sup>.

#### 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<sup>20,25</sup>.

#### 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<sup>26-28</sup>. 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  $\mu\text{g}/\text{mL}$  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% CO<sub>2</sub> 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<sup>26-29</sup>.

##### Cell growth activity

According to previous studies, the cell lines were harvested and plated into a volume of 199  $\mu\text{L}$  in 96-well microtitre plates (Sarstedt, Newton, USA) at a seeding density of 3–5  $\times 10^3$  cells in each well, and preincubated in complete medium provided with 5% FCS, at 37°C for 24 h<sup>26-29</sup>.

In order to achieve required final concentrations, serial twofold dilutions of the aqueous extract of *R. crispus* fruits in DMSO (1  $\mu\text{L}$ ) were combined with 199  $\mu\text{L}$  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.<sup>30</sup>. Colour development was measured using a Multiscan Ascent (Labsystems; Helsinki, Finland) photometer at 540 nm against 620 nm as background<sup>26-29</sup>.

The effect on cell growth was calculated as 100  $\times$  (AT/AC) (%), where AT and AC are the absorbance of the test sample and the control, respectively<sup>26,29</sup>. The concentration-cell growth (dose effect) curves were made for each treatment and IC<sub>50</sub> 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 IC<sub>50</sub> ratios were calculated for HeLa, MCF-7, HT-29 and MRC-5 cell lines<sup>26,29</sup>.

##### 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<sup>PLUS</sup> 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 cytoplasmic fraction of the cell, were used. Cell death detection experiments were performed when IC<sub>50</sub> < 100  $\mu\text{g}/\text{mL}$  criterion was met in cell growth experiments (in HeLa and MCF7 cell lines)<sup>26</sup>.

According to study of Četojević-Simin et al. <sup>26</sup>, the cell lines ( $1 \times 10^4$  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 <sup>26</sup>. 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 <sup>26</sup>. 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 <sup>26</sup>.

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  $EF A/N = EF A / EF N$  <sup>26</sup>.

#### Statistical analysis

All results are expressed as mean  $\pm$  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 1**  
**Ferric reducing antioxidant power (FRAP) of the aqueous extract of *Rumex crispus* fruits**

Group	FRAP units, mean $\pm$ SD
Control	1
Extract, mg/mL	
0.01	1.14 $\pm$ 0.04*
0.02	1.13 $\pm$ 0.03*
0.03	1.11 $\pm$ 0.07
0.04	1.14 $\pm$ 0.06*
0.05	1.20 $\pm$ 0.10*
0.10	1.19 $\pm$ 0.04*

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

\*Statistically significant difference between particular concentrations of the extract and control group,  $p < 0.001$ .

The results of the DPPH assay (Table 2) were reported as the percentage of scavenged DPPH free radical and as the  $IC_{50}$ . The investigated extract was capable of neutralizing DPPH free radicals via hydrogen donating activity <sup>20</sup> 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  $IC_{50}$  value for the investigated extract was 0.046 mg/mL, while the standard substance Trolox<sup>®</sup> was able to reduce the stable DPPH free radical, reaching 50% reduction with an  $IC_{50}$  0.216 mg/mL. Analyzing intensity of inhibition of the DPPH free radical under the influence of the 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 2**  
**2,2-diphenyl-1-picrylhydrazyl(DPPH)-free radical scavenging activity of the aqueous extract of *Rumex crispus* fruits**

Group	Concentrations of the extract (mg/mL)					
	1.000	0.500	0.100	0.050	0.025	0.010
Trolox <sup>®</sup>	98.44 $\pm$ 1.62	56.72 $\pm$ 29.71	48.64 $\pm$ 5.34	31.20 $\pm$ 5.32	14.68 $\pm$ 1.06	11.38 $\pm$ 0.83
Extract	84.66 $\pm$ 5.31**	83.12 $\pm$ 7.56**	64.26 $\pm$ 3.12**	52.32 $\pm$ 5.39**	34.16 $\pm$ 3.09**	14.04 $\pm$ 1.88*

Note: Results are given as % of DPPH• scavenging and represent as means  $\pm$  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<sup>®</sup>

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 CCl<sub>4</sub> (by using Student's test,  $t = 3.127$ ,  $p = 0.014$ ) was determined, while there was no significant difference between the control and the extract group (0.01 mg/mL) in the presence of CCl<sub>4</sub>. The comparisons between concentrations of the examined extract and both control groups (without and with CCl<sub>4</sub>) are given in Table 3. Analyzing intensity of lipid peroxidation influenced by the examined extract, it was concluded that there was a statistically significant difference in means of the investigated parameter related to the extract concentrations, in both conditions: in absence and in presence of CCl<sub>4</sub> ( $F = 429.220$ ,  $p < 0.001$ ;  $F = 83.174$ ,  $p < 0.001$ , respectively).

**Table 3**

**Influence on lipid peroxidation (LPx) in liposomes of the aqueous extract of *Rumex crispus* fruits with or without CCl<sub>4</sub> addition**

Group	MDA (nmol/mL of liposomes), mean $\pm$ SD	
	without CCl <sub>4</sub>	with CCl <sub>4</sub>
Control	76.62 $\pm$ 1.61**	220.66 $\pm$ 18.93*
Extract, mg/mL		
0.01	73.64 $\pm$ 1.39**	198.46 $\pm$ 15.75*
0.02	60.60 $\pm$ 2.79**	193.38 $\pm$ 12.60**
0.03	55.16 $\pm$ 1.69**	157.12 $\pm$ 14.00***
0.04	41.52 $\pm$ 2.17**	107.92 $\pm$ 9.42**
0.05	21.98 $\pm$ 2.56**	91.76 $\pm$ 6.89**
0.10	19.44 $\pm$ 3.02**	98.86 $\pm$ 9.84**

**Note:** Values (nmol MDA/mL of liposomes) represent means  $\pm$  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 and control combined in the presence of CCl<sub>4</sub>,  $p < 0.001$ .

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 CCl<sub>4</sub> ( $p = 0.224$ ,  $p = 0.719$ , respectively). The comparisons between other concentrations of the investigated extract and both control groups (control and control + CCl<sub>4</sub>) 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 CCl<sub>4</sub> ( $F = 18.576$ ,  $p < 0.001$ ;  $F = 84.554$ ,  $p < 0.001$ , respectively).

**Table 6**

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

Group	IC <sub>50</sub> ( $\mu$ g/mL)			
	HeLa	MCF7	MRC-5	HT-29
Extract	16.88 $\pm$ 3.08	19.26 $\pm$ 3.45	25.98 $\pm$ 5.38	n.a. (IC <sub>32</sub> = 62.5)
Control (doxorubicin)	0.25 $\pm$ 0.09*	0.26 $\pm$ 0.02*	0.40 $\pm$ 0.03*	0.38 $\pm$ 0.04*

**Note:** Values represent means  $\pm$  standard deviation (SD) of eight measurements ( $n = 8$ ) obtained in 3.91 to 1,000  $\mu$ g/mL concentration range.

\*Rewritten from Četojević-Simin et al. <sup>26</sup>

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

**Table 4**

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

Group	MDA (nmol/mg of deoxyribose), mean $\pm$ SD	
	without CCl <sub>4</sub>	with CCl <sub>4</sub>
Control	2.17 $\pm$ 0.13**	5.44 $\pm$ 0.29*
Extract, mg/mL		
0.01	2.08 $\pm$ 0.06**	5.37 $\pm$ 0.28*
0.02	2.05 $\pm$ 0.09**	5.09 $\pm$ 0.07*
0.03	1.95 $\pm$ 0.07**	4.75 $\pm$ 0.25***
0.04	1.82 $\pm$ 0.12***	4.16 $\pm$ 0.12***
0.05	1.52 $\pm$ 0.17***	3.49 $\pm$ 0.21***
0.10	1.59 $\pm$ 0.18***	3.43 $\pm$ 0.18***

**Note:** The production of OH• was expressed in nmol MDA/mg of deoxyribose; values (nmol MDA/mg of deoxyribose) represent means  $\pm$  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 CCl<sub>4</sub>,  $p < 0.001$ .

**Table 5**

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

Concentration of the extract, mg/mL	NO• (% of inhibition), mean $\pm$ SD
1.000	-15.54 $\pm$ 0.77
0.500	-7.26 $\pm$ 1.92
0.100	-4.36 $\pm$ 0.34
0.050	-0.36 $\pm$ 0.72
0.025	0.81 $\pm$ 0.45
0.010	-0.03 $\pm$ 0.14

**Note:** Values (% of NO•inhibition) represent means  $\pm$  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 IC<sub>50</sub> 16.9  $\mu$ g/mL and breast adenocarcinoma cell line (MCF7) with IC<sub>50</sub> 19.3  $\mu$ g/mL. The non-tumor/tumor IC<sub>50</sub> 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.

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 (EF A/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**

Cell line	EF A	EF N	EF A/N
HeLa	0.97	4.00	0.24
MCF7	0.77	2.00	0.39

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*<sup>30</sup>. According to numerous previous studies, flavonoids might be responsible for many biological activities of curly dock, such as antiviral, antibacterial, anticancer, antioxidant activities and they might be capable to activate antioxidant enzymes<sup>2, 3, 6, 7, 9, 11–14</sup>. However, most interest of this study was devoted to the antioxidant activity of flavonoids and investigated extract, due to their ability to reduce free radical formation and to scavenge free radicals<sup>31</sup>.

### *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<sup>3+</sup> complex with TPTZ (Fe<sup>3+</sup>-TPTZ), to the Fe<sup>2+</sup> complex (Fe<sup>2+</sup>-TPTZ) by antioxidants in acidic medium<sup>6, 17, 18</sup>. 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<sup>3+</sup>, 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<sup>32</sup>.

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<sup>6, 18–20</sup>. Taking into account that IC<sub>50</sub> was the concentration of the extract or Trolox<sup>®</sup> necessary to decrease the initial DPPH concentration by 50%, it was concluded that the mentioned extract was more potent than standard substance Trolox<sup>®</sup> in scavenging the stable DPPH free radical, due to the fact that IC<sub>50</sub> of the extract was lower than that of the standard substance, Trolox<sup>®</sup>. 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<sub>50</sub> = 9.51 μg/mL was more potent than the examined extract (IC<sub>50</sub> = 46 μg/mL)<sup>2, 32, 33</sup>.

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 curly dock fruits on lipid peroxidation in liposomes was evaluated by the TBA test after lipid peroxidation induced by the Fe<sup>2+</sup>/ascorbate system. This assay is based on the reactivity of MDA with TBA to generate a red chromogen 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<sup>6, 21, 22</sup>. The investigated extract showed a potential ability to decrease lipid peroxidation in absence of CCl<sub>4</sub>, especially at concentration of 0.10 mg/mL, because there was a 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<sub>4</sub> (76.62 nmoL MDA/mL of liposomes). The concentration of 0.05 mg/mL of the extract combined with CCl<sub>4</sub> was the most prominent. Taking into account that there was a statistically significant difference between the control group without CCl<sub>4</sub> (76.62 nmoL MDA/mL of liposomes) and 0.05 mg/mL of the extract combined with CCl<sub>4</sub> (91.76 nmoL MDA/mL of liposomes), the examined extract had a low ability to decrease lipid peroxidation in the presence of CCl<sub>4</sub> and it was not able to completely neutralize damaging effects of CCl<sub>4</sub><sup>23, 24</sup>. CCl<sub>4</sub>, probably showing prooxidative activity, caused the oxidative stress (generation of ROS) and lipid peroxidation in liposomes as an *in vitro* model of cell membrane. The examined extract combined with CCl<sub>4</sub> might inhibit lipid peroxidation, probably as antioxidant agent, scavenging ROS<sup>23, 24</sup>.

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<sup>22</sup>. 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-

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 of 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 (IC<sub>50</sub> = 25.98) (Table 6), suggesting its lower toxicity towards healthy cells. The IC<sub>50</sub> value in colon adenocarcinoma HT-29 cells was not reached, but IC<sub>32</sub> value that was obtained at still low concentration (IC<sub>32</sub> = 62.5 µg/mL) suggests its activity towards this cell type, as well. The results showed high and favourable tissue-selective anti-tumor 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 IC<sub>50</sub> 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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#### R E F E R E N C E S

- Mertoglu-Elmas G, Gunaydin K, Ozden O. Environmental friendly alkaline sulfite anthraquinone-methanol (ASAM) pulping with *Rumex crispus* plant extract of woody materials. *J Environ Biol* 2012; 33(5): 941–4.
- Sub HJ, Lee KS, Kim SR, Shin MH, Park S, Park S. Determination of singlet oxygen quenching and protection of biological systems by various extracts from seed of *Rumex crispus* L. *J Photochem Photobiol B* 2011; 102(2): 102–7.
- Kim JC, Choi GJ, Lee SW, Kim JS, Chung KY, Cho KY. Screening extracts of *Achyranthes japonica* and *Rumex crispus* for activity against various plant pathogenic fungi and control of powdery mildew. *Pest Manag Sci* 2004; 60(8): 803–8.
- Zaller JG. Ecology and non-chemical control of *Rumex crispus* and *R. obtusifolius* (Polygonaceae): a review. *Weed Res* 2004; 44: 414–32.
- Křišťalová V, Hejman M, Červená K, Pavlu V. Effect of nitrogen and phosphorus availability on the emergence, growth and over-wintering of *Rumex crispus* and *Rumex obtusifolius*. *Grass Forage Sci* 2011; 66(3): 361–9.
- Maksimović Z, Kovačević N, Lakušić B, Čebović T. Antioxidant Activity of Yellow Dock (*Rumex crispus* L., Polygonaceae) Fruit Extract. *Phytother Res* 2011; 25(1): 101–5.
- Lee KH, Rhee KH. Antimalarial activity of nepodin isolated from *Rumex crispus*. *Arch Pharm Res* 2013; 36: 430–5.
- Jančić R, Janjatorić V, Obradović M, Pavlović S, Lukić P, Krstić-Parlović N. *Rumex*. In: Sarić M, editor. *Medicinal Plants of SR Serbia*. Belgrade: Serbian Academy of Sciences and Arts; 1989. p. 494–7. (Serbian)
- Węgięra M, Smolarz H, Bogucka-Kocka A. *Rumex* L. species induce apoptosis in 1301, EOL-1 and H-9 cell lines. *Acta Pol Pharm* 2012; 69(3): 487–99.
- Baskan S, Dant-Ozdemir A, Gunaydin K, Erim FB. Analysis of anthraquinones in *Rumex crispus* by micellar electrokinetic chromatography. *Talanta* 2007; 71(2): 747–50.
- Yildirim A, Mavi A, Kara AA. Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *J Agric Food Chem* 2001; 49(8): 4083–9.
- Shivani S, Singh NK, Wang MH. Carbohydrase inhibition and anti-cancerous and free radical scavenging properties along with DNA and protein protection ability of methanolic root extracts of *Rumex crispus*. *Nutr Res Pract* 2012; 6(5): 389–95.
- Orban-Gyapai O, Lajter I, Hobmann J, Jakab G, Vasas A. Xanthine oxidase inhibitory activity of extracts prepared from Polygonaceae species. *Phytother Res* 2015; 29(3): 459–65.
- Choi GJ, Lee SW, Jang KS, Kim JS, Cho KY, Kim JC. Effects of chrysophanol, parietin, and nepodin of *Rumex crispus* on barley and cucumber powdery mildews. *Crop Protection* 2004; 23: 1215–21.



15. Djeradi H, Rahmouni A, Cheriti A. Antioxidant activity of flavonoids: a QSAR modeling using Fukui indices descriptors. *J Mol Model* 2014; 20(10): 2476.
16. Petrović S, Maksimović Z, Kundaković T. Chemical analysis of herbal drugs and ingredients. 2<sup>nd</sup> ed. Belgrade: University of Belgrade, Faculty of Pharmacy; 2013.
17. Pellegrini N, Serafini M, Colombi B, Del Rio D, Salvatore S, Bianchi M, et al. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J Nutr* 2003; 133(9): 2812–9.
18. Antolovich M, Prenzler DP, Patsalides E, McDonald S, Robards K. Methods for testing antioxidant activity. *Analyst* 2002; 127(1): 183–98.
19. Cuendet M, Hostettmann K, Potterat O, Dyatmiko W. Iridoid glucosides with free radical scavenging properties from *Fagraea blumei*. *Helv Chim Acta* 1997; 80(4): 1144–52.
20. Deepa B, Prema G, Sai KB, Cheriai KM. Antioxidant and free radical scavenging activity of triphala determined by using different in vitro models. *J Med Plants Res* 2013; 7(39): 2898–905.
21. Mimica-Dukić N, Božin B, Soković M, Simin N. Antimicrobial and antioxidant activities of *Melissa officinalis* L. (Lamiaceae) essential oil. *J Agric Food Chem* 2004; 52(9): 2485–9.
22. Buege AL, Aust DS. Microsomal lipid peroxidation. In: Fleisher S, Parker L, editors. *Methods in Enzymology*. New York: Academic Press; 1978. p. 306–10.
23. Čebović T. In vitro and in vivo antioxidant properties testing of *Viscum album* L. and *Allium cepa* L. [thesis]. Novi Sad: Faculty of Sciences; 2003. (Serbian)
24. Čebović T. The influence of the extracts (*Viscum album* L.) from different hosts on the formation of free radicals of oxygen and the activity of antioxidant enzymes [dissertation]. Belgrade: University of Belgrade, Faculty of Pharmacy; 2008. (Serbian)
25. Garrat DC. The quantitative analysis of drugs. 3<sup>rd</sup> ed. Japan, Tokyo: Chapman and Hall International Edition 1964.
26. Četojević-Simin D, Velićanski A, Cvetković D, Markov S, Četković G, Tumbas Šaponjac V, et al. Bioactivity of Meeker and Willamette raspberry (*Rubus idaeus* L.) pomace extracts. *Food Chem* 2015; 166: 407–13.
27. Beara I, Lesjak M, Četojević-Simin D, Marjanović Ž, Ristić J, Mrkonjić Z, et al. Phenolic profile, antioxidant, anti-inflammatory and cytotoxic activities of black (*Tuber aestivum* Vittad.) and white (*Tuber magnatum* Pico) truffles. *Food Chem* 2014; 165: 460–6.
28. Četojević-Simin D, Velićanski A, Cvetković D, Markov S, Mrđanović J, Bogdanović V, et al. Bioactivity of Lemon Balm Kombucha. *Food Bioprocess Technol* 2012; 5(5): 1756–65.
29. Četojević-Simin D, Čanadanović-Brunet J, Bogdanović G, Dilas S, Četković G, Tumbas V, et al. Antioxidative and Antiproliferative Activities of Different Horsetail (*Equisetum arvense* L.) Extracts. *J Med Food* 2010; 13(2): 452–9.
30. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; 82(13): 1107–12.
31. Procházková D, Boušová I, Wilbelmová N. Antioxidant and prooxidant properties of flavonoids. *Fitoterapia* 2011; 82(4): 513–23.
32. Pietta PG. Flavonoids as antioxidants. *J Nat Prod* 2000; 63(7): 1035–42.
33. Avotunde OS, Dhanabal SP, Rajeshkumar R, Chasitainya MV. Antioxidant activities of Hydro-ethanol and Saponin extracts of *Terminalia schimperi* root. *Der Pharmacia Lettre* 2017; 9(11): 47–53.

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