Phenolic Content and Antioxidant Activity of a Raspberry Leaf Dry Extract

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Abstract

Polyphenols are known for their beneficial effects towards chronic disease caused by oxidative stress (diabetes mellitus, cancer, atherosclerosis). In the present study, a raspberry (Rubus idaeus L.) leaf dry extract was evaluated for its phenolic content and antioxidant activity. Spectrophotometric and HPLC-UV/HPLC-MS methods were used for polyphenols determination and 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+), ferric reducing ability (FRAP), oxygen radical absorbance capacity (ORAC) methods for antioxidant activity. 2’,7’dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay was used to investigate the cytoprotective effect against oxidative stress in human HepG2 cell line. It was found that the extract is a rich source of flavonoids and phenolcarboxylic acids (43.08 mg% quercitrin, 14.33% isoquercitrin, 43.40 mg% p-coumaric acid, 17.51 mg% caffeic acid, 11.41 mg% ferulic acid, 220.26 mg% quercetin) and has a modest antioxidant activity (EC50 = 0.0991 mg/mL - DPPH assay, EC50 = 0.2274 mg/mL - ABTS•+, EC50 = 0.0682 mg/mL - FRAP and 7243.7 μM trolox/g - ORAC) compared with standards (ascorbic acid, gallic acid). The dry extract showed a dose-response antioxidant activity against allooxan, induced oxidative stress in HepG2 cells, with a maximum effect at 0.01 mg/mL. Due to its phenolic content, the extract might be used for hepatic disorders treatment, associated with oxidative stress.

Key words: Rubus idaeus L., tannins, HepG2 cells, oxidative stress

1. Introduction

The role of oxidative stress and free-radicals mechanisms in the pathology of diabetes mellitus, cancer, atherosclerosis, non-alcoholic fatty liver and neurodegenerative diseases (Alzheimer) has become an area of intense interest.

Reactive oxygen species (ROS) including free radicals (hydroxyl-OH•; superoxide-O2•; peroxyl-RO2•; nitric oxide-NO•) and non-radicals (hypoclorous acid-HOCl; hydrogen peroxide -H2O2; singlet oxygen -¹O2; peroxynitrite-ONO0•) are constantly formed in the human body from different metabolic pathways. Oxidative stress is considered as an imbalance between the production of various ROS and the ability of the organism’s natural antioxidant defense (superoxid dismutase = SOD, catalase, glutathione peroxidase) to fight...
against reactive compounds and prevent oxidation of lipids, proteins and nucleic acids (P. MONOGHAIN & al. [1]; K.B. PANDEY & al. [2]).

Over the past several decades, a series of research workers have investigated the potential role of natural compounds (polyphenols, carotenoids, mineral elements etc.) in the prevention of chronic disease, caused by oxidative stress. Polyphenols (flavonoids, phenolicarboxylic acids, tannins, proanthocyanidins) are well known for their hypoglycaemic, cardioprotective, antiproliferative and antioxidant activities. They also enhance the human body antioxidant system since they behave as ROS scavengers, metal chelators and enzyme modulators (K.B. PANDLEY & al. [2]).

Raspberry (Rubus idaeus L.) is a shrub that belongs to the Rosaceae family and is widely grown in Europe, Asia and America. Leaves (Rubus idaei folium), fruits (Rubus idaei fructus) and seeds (Rubus idaei semen) are used for therapeutic purposes (TÂMAŞ [3]).

Raspberry leaves are used as a folk medicine to treat wounds, diarrhea and colic pain, due to their antibacterial, haemostatic and antispasmodic properties (BARNES [4], WICHTL [5]). Recent studies have shown cytotoxic (on leukemic cells HL60) (K. SKUPIEN & al. [6]), anti-inflammatory (D. JEAN-GILLES & al. [7]), antibacterial (V.S. NIKITINA & al. [8]) and uterine relaxant properties (L. HOLST & al. [9]).

Regarding their chemical composition, Rubus idaeus L. leaves are a rich source of:

- flavonoids (quercetin-3-O-rutinoside = rutin - 52-234.6 mg/kg; quercetin-3-O-galactoside = hyperoside - 34.4-720.2 mg/kg; isorhamnetin-3-O-rutinoside - 176.4-2914.2 mg/kg; kaempferol-3-O-glucoside = astragalin - 266.9-1260.9 mg/kg; kampferol-3-O-β-D-(6'-p-coumaroyl) glucopyranoside = tiliroside) and phenolicarboxylic acids (caffeic acid - 3.28-22.61 mg/kg; chlorogenic acid - 29.04-77.23 mg/kg; rosmarinic acid - 6.57-30.82 mg/kg; gentisic acid - 6.40-155.4 mg/kg and ellagic acid - 20.26-127.4 mg/kg) (M. PELC & al. [10]).
- Raspberry leaves have high amounts of ellagitannins (2.06-15% such as sanguin H6, lambertianin C), sterols (stigmasterol, β-sitosterol), ascorbic acid (A.V. PATEL & al. [11]), proanthocyanidins B1 (L. BUŘIČOVA & al. [12]), oligoelements (selenium - 21-189 μg/kg; vanadium 138-1958 μg/kg; zinc - 28.60 mg/kg; manganese -182.75 mg/kg) (D.S. ANTAL & al. [13-14], Z. KARAKLAJIĆ-STAJIĆ & al. [15]) and volatile compounds (2-hexenal, Z-3-hexenol) (WICHTL [5]).

The aim of our work was the determination of phenolic content and the evaluation of antioxidant activity of a Rubi idaei folium (raspberry leaf) dry extract. For the antioxidant activity we performed experiments in both noncellular and cellular systems. For non-cellular assays we have used several well-known methods: DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS’+ (2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), FRAP (ferric reducing antioxidant activity) and ORAC (oxygen radical absorbance capacity). In order to study the antioxidant activity in cellular system (HepG2 cells), we have quantified the intracellular ROS production with DCFH-DA (2',7'-dichloro-dihydro-fluorescein diacetate) in correlation with MTT viability assay. Aloxan, a widely used reagent for induction of experimental diabetes, was chosen as an oxidant for cellular system experiments, since it also affects the liver. The formation of ROS is preceeded by alloxan reduction to dialuric acid, that is re-oxidised to alloxan, establishing a redox cycle with generation of superoxide radicals. Further, these can reduce Fe³⁺ or undergo dismutation to H₂O₂, that can lead to OH⁻ formation through Fenton reaction (T. SZKUDELSKI [16]).
2. Material and methods

Plant material

Rubus idaeus L. (raspberry) leaves were collected from Jilava (private garden), Ilfov county, Romania, in April 2011, before flowering. Leaves were air-dried in the shade, at room temperature. Herbarium voucher (no.528) samples were deposited in the Department of Pharmacognosy, Phytochemistry, Phytotherapy, School of Pharmacy, ”Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania.

Reagents and solvents

All chemicals were purchased from Fluka (Switzerland), unless otherwise stated. Gallic acid, ascorbic acid, hyperoside, tannic acid, chlorogenic acid were from Roth (Germany); dimethylthiazol-diphenyltetrazolium bromide = MTT, alloxan, trolox(((S)-(−)-6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid)) = TE, ferrous sulphate, phosphate buffered saline = PBS were purchased from Sigma-Aldrich (Germany) and potassium persulphate, 2,4,6-tripyridyl-s-triazine = TPTZ were from Merck (Germany). HepG2 (human hepatocarcinoma) cell line (ATCC, HB-8065) was acquired from LGC Standards (U.K.).

Preparation of raspberry leaf dry extract (RE)

300 g of dried raspberry leaves were heated twice with 20% ethanol (v/v) under a reflux condenser for 30 min., using a 1:30 herbal product/solvent ratio for the first extraction and 1:15 for the second one. After cooling, the combined filtrates were concentrated using a rotary evaporator (Buchi R 210-215) to remove the solvent, and then freeze-dried (using a Christ Alpha 1-2/B Braun, Biotech-International lyophylizator) to yield 51 g extract (RE).

Spectrophotometric determination of polyphenols

Spectrophotometric determination of total phenolic content (expressed as tannic acid equivalent) was performed with Folin-Ciocalteu reagent according to V.L. SINGLETON & al. [17] modified by H.P.S. MAKKAR & al. [18]. Flavonoids and phenolcarboxylic acids contents (expressed as hyperoside and chlorogenic acid equivalents) were estimated based on the chelating reaction with aluminum chloride and formation of oxymes with Arnow reagent, respectively (EUROPEAN PHARMACOPOEIA 7th ed.[19]). For all determinations a Jasco V-530 (Jasco, Japan) spectrophotometer was used. Calibration curves of tannic acid (linearity range: 2.04-9.18 μg/mL, R² = 0.9994, n = 8), hyperoside (linearity range: 7.32-34.16 μg/mL, R² = 0.9991, n = 12) and chlorogenic acid (linearity range: 0.0113-0.0527 mg/mL, R² = 0.9998, n = 6) were used to calculate the percentage of active substances. All spectrophotometric determinations were performed in triplicate using a 0.1% RE solution.

HPLC analysis

HPLC-MS analysis (method 1) was performed using an Agilent 1100 HPLC Series System (Agilent, SUA) and the chromatographic conditions previously described. Calibration curves in 0.5-50 mg/mL range with good linearity (R² > 0.99, n = 5) have been used for determination of polyphenols contents (L. VLASE & al. [20]). Chlorogenic and caffeic acid were not quantified in the present chromatographic conditions, thus we have chosen another method (HPLC-UV = method 2) for their determination (I.NENCU & al. [21]). Calibration curves in 8-170 μg/mL range showed good linearity (R² = 0.998, n = 5).

Preparation of samples: 0.25 g RE was dissolved in 25 mL ethanol 20% (volumetric flask) (SRE). For extraction of free aglycons 1mL HCl 2N was added to 1 mL SRE and heated on a reflux condenser at 80°C for 60 min. (SRH).
Antioxidant activity

1. DPPH radical scavenging assay

DPPH free radical scavenging ability was estimated using the method described by M. OHNISHI & al. [22]. Briefly, the dry extract (RE) was dissolved in 20% ethanol (v/v) in different concentrations ranging from 0.01 mg/mL to 0.2 mg/mL. Each dilution (0.5 mL) was mixed with 3 mL DPPH ethanolic solution (0.1 mM). The mixture was incubated in darkness, at room temperature and the absorbance of the DPPH solution was measured at $\lambda = 517$ nm (Jasco V-530, Japan) before ($A_{\text{control}}$) and 30 min. after adding the extract ($A_{\text{sample}}$). Ascorbic acid and gallic acid (0.008-0.05 mg/mL dissolved in 20% ethanol) were used as positive controls. The ability to scavenge the DPPH free radical was calculated using the following formula:

$$\text{DPPH radical scavenging activity} (%) = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100.$$

The concentration of the extract that inhibited 50% of the DPPH free radical (EC$_{50}$, mg/mL) was determined graphically from the linear regression curve plotted between percent (%) of inhibition and extract concentration. All measurements were done in triplicate.

2. ABTS•+ radical cation scavenging assay

The antioxidant capacity was measured using the method of R. RE & al. [23]. Briefly, the ABTS radical cation was generated by incubation of ABTS diammonium salt (7 mM ) with potassium persulphate (2.45 mM) in the dark, at room temperature for 16 h. The absorbance of the ABTS radical solution was equilibrated to a value of 0.700 ± 0.02 at $\lambda = 734$ nm after dilution with ethanol. The extract was dissolved in 20% ethanol (v/v) in different concentration ranging from 0.01 mg/mL to 0.5 mg/mL. Each dilution (0.1 mL) was mixed with 3 mL reagent and the absorbance of the ABTS cation radical was measured at $\lambda = 734$ nm, before ($A_{\text{control}}$) and 6 min. after adding the extract($A_{\text{sample}}$). Ascorbic acid and gallic acid (0.01-0.7 mg/mL dissolved in 20% ethanol) served as positive controls. The scavenging activity was calculated as:

$$\text{ABTS radical scavenging activity} (%) = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100.$$

In order to calculate TEAC values (trolox equivalent antioxidant capacity) for positive controls and extract, trolox was used as a standard. The percentage of absorbance decrease vs. trolox concentration was plotted (linearity range: 0.01-0.1 mg/mL, $R^2 = 0.9926$, $n = 6$). The antioxidant activity was expressed as mg trolox/mg extract or positive controls. EC$_{50}$ (mg/mL) of RE and positive controls were also determined. All measurements were done in triplicate.

3. Ferric reducing antioxidant assay (FRAP)

The FRAP assay was conducted according to S.P. WONG & al. [24]. The working FRAP reagent was prepared daily by mixing 10 volumes of 300 mM acetate buffer (pH = 3.6) with 1 volume of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM hydrochloric acid and with 1 volume of 20 mM ferric chloride. Briefly, 0.2 mL of RE and positive controls concentrations (dissolved in 20% ethanol) ranging from 0.02 mg/mL to 0.15 mg/mL and 0.004-0.035 mg/mL respectively, were mixed with 3 mL FRAP reagent and incubated for 30 min. at 37°C in a water bath (Raypa BAD 2). The absorbance was measured at $\lambda = 593$ nm against FRAP reagent, used as a blank. A high absorbance of the reaction mixture indicated a strong reducing capacity.

The antioxidant activity of RE, ascorbic acid, gallic acid was expressed as mmol Fe$^{2+}$/mg extract or positive controls based on a calibration curve prepared with FeSO$_4 \times 7$H$_2$O (linearity range: 0.04-0.2 mg/mL, $R^2 = 0.9937$, $n = 9$). The EC$_{50}$ value (the concentration of extract or positive control for which the absorbance is 0.5) was also determined. All measurements were done in triplicate.
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4. Oxygen radical absorbance capacity (ORAC)

The ORAC assay was conducted as previously described by A-R. LUPU & al. [25].

Briefly, using a 96 wells flat-bottom plate, 150 µl of 4 nM fluorescein sodium salt solution in phosphate buffer pH=7.4 were mixed with 25µl phosphate buffer saline pH=7.4 for „blank” wells; 25µl of the corresponding solutions for trolox standard curve (in phosphate buffer saline pH=7.4, concentrations 100µM, 50µM, 25µM, 12.5µM, 6.25µM, and 3.125µM) and 25 µl sample (vegetal extract). All sample types were processed in quadruplicate.

The test plate was incubated for 30 min at 37°C in a Fluoroskan FL (Thermo) equipment. Then, a fluorimetric reading (ex. 485nm and em. 523nm) was performed to detect autofluorescence and 25 µl AAPH (2,2’-azobis-2-amidino-propane dihydrochloride) 0.25M in phosphate buffer saline pH=7.4 were added to each well (automatic dispensing). 50 fluorimetric readings (ex. 485nm, em. 523nm, Δtime between readings = 1 min) were taken. The samples corresponding values were calculated against a Trolox standard curve (linearity range: 3.125µM – 100 µM, n = 6. R² = 0.9871) and expressed as Trolox equivalents/g (TE/g); 1 TE = 1 µM Trolox. The final results are obtained from three independent experiments.

In vitro tests on human HepG2 cell line

a. Cell culture and treatment

HepG2 cells were seeded in 24-well culture plates at a density of 2.5 x 10⁴ cells/ml in DMEM F12 culture medium/well. Cells were treated with concentrations ranging from 0.001-0.1 mg/mL RE and cultured in standard conditions (37°C, 95% relative humidity, 5% CO₂) for 24, 48 and 72 h.

In our experiment we needed to use RE concentration with no significant cytotoxic or proliferative effects. To clarify this aspect, HepG2 cells were treated with 0.001, 0.005, 0.01, 0.05 and 0.1mg/mL RE respectively. After 24, 48 and 72h, MTT cell viability assay was performed as described below. Untreated cells were considered as control.

In order to establish the alloxan concentration that provides recoverable cell damage (approx. 50%), cells were treated with different concentrations of alloxan ranging from 4-200 mM, for 24 h. Cell viability was determined using the MTT assay. Control sample was represented by untreated cells.

To evaluate the cytoprotective effect of the extract, cells were simultaneously treated (co-treatment) with different RE concentrations (0.001, 0.005, 0.01, 0.05 and 0.1mg/mL RE respectively) and 50 mM alloxan for 24, 48 and 72 h. Cell viability was assessed using MTT test. Results were reported as percentage from the cell control (cells treated with alloxan only).

b. MTT cell viability test

MTT (dimethylthiazol-diphenyltetrazolium bromide), a water-soluble tetrazolium salt, is reduced to a deep-purple formazan by mitochondria dehydrogenases and NADPH oxidases in living cells (P.R. TWENTYMAN & al.[26], BERRIDGE & al. [27]). The absorbance of resulted colored solution is related to the number of viable cells in the tested sample.

Briefly, at 24h (48/72 h) after cells were incubated with different concentrations of RE and/or alloxan according to previously described protocols, they were washed with PBS, treated in the dark with 300 µL MTT solution (1mg/mL in PBS) and incubated at 37°C. After 2 h, the water insoluble formazan crystals were solubilized in 300 µL DMSO. The mixtures were brought in 96-well cell culture plates (100 µL/well, 4 wells/sample) and the absorbance was measured at λ = 540 nm using a Mutiskan EX (Thermo) spectrophotometer. Results were reported as percentage from the corresponding control sample, considered as 100% viable cells.
c. Quantification of intracellular ROS production

The assay was based on the fluorimetric determination of intracellular ROS production using 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA), which is a good indicator of the overall oxidative status of the cell. DCFH-DA penetrates into the cell and it hydrolysed by cellular esterase to dichloro-dihydro-fluorescein (DCFH), that can be oxidised to its fluorescent product 2′,7′-dichlorofluorescein (DCF) (H.WANG & al. [28]). Briefly, after 24 h of co-treatment between 0.001-0.01 mg/mL RE and alloxan 50 mM, the culture medium was replaced with 1 mL DMEM-F12 containing 25 mg/mL DCFH-DA (0.2 μL/1 mL culture medium) and cells were incubated at 37°C for 30 min. Then, the culture medium was replaced, cells were trypsinised with 2.5% trypsin and 0.53 mM EDTA, suspended in PBS and centrifuged (1500 rpm/min) at 4°C for 10 min. Cells were suspended in 400-500 μL PBS and brought to 96-wells cell culture plates. Emission at λ = 530 nm was measured after excitation at λ = 485 nm using a Fluoroskan FL plate reader.

In order to verify if induction of intracellular ROS production by alloxan was efficient, untreated cells were considered as 100% and alloxan only – treated cells were compared with this value. The data corresponding to RE + alloxan treated cells were analyzed with respect to alloxan only-treated cells.

Statistical analysis

For spectrophotometric and antioxidant assays data are reported as mean ± SD of three replicates. For cell culture tests (MTT assay) results represent the average ± SD of three independent experiments. The coefficient of variation (CV) for each sample (cell culture tests) was less than 20%. The data analysis was performed using GraphPad version 5.00 (for Windows) and Microsoft Office (Excel 2007).

3. Results and discussion

Spectrophotometric determination of polyphenols

Our results pointed out that raspberry leaf dry extract (RE) is a rich source of polyphenols, among them tannins were the most representative (table 1). Regarding the tannins content, our results are lower compared to the ones reported by D. DUDZINSKA & al. [29], while the flavonoids content are higher. However, the total phenolic content determined by us is similar to the one reported by other authors (D. DUDZINSKA & al. [29]).

<table>
<thead>
<tr>
<th>Raspberry leaf dry extract (RE)</th>
<th>Total phenolic content (g% tannic acid)</th>
<th>Tannins (g% tannic acid)</th>
<th>Flavonoids (g% hyperoside)</th>
<th>Phenolcarboxylic acids (g% chlorogenic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32.3202 ± 1.5124</td>
<td>23.2808 ± 1.4220</td>
<td>7.6040 ±1.0413</td>
<td>5.4642 ±0.4402</td>
<td></td>
</tr>
</tbody>
</table>

HPLC analysis

Using HPLC-MS analysis (method 1, table 2) we identified several phenolic compounds, also mentioned by scientific literature (M. PELC & al. [10]). Quercetin content in SRE solution (8.55 mg%) was high compared to M.PELC & al. [10] results that found 0.76-6.8 mg%; while kaempferol content was much lower. In contrast to scientific literature (M. PELC & al. [10]) hyperoside was not identified. The flavonoids aglycons content increased after hydrolysis. Based on our results, quercetin came from quercitrin, isoquercitrin and other hetersides (quercetin-3-O-galactoside), which are also mentioned by scientific literature.
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(BARNES [4]). Due to lack of standards we couldn’t establish if kaempferol comes from astragaline, tiliroside or kaempferol-3-α-rhamnosyl-[6-O-3-hydroxy-3-methylglutaryl]-β-galactoside (BARNES [4], M.PELC & al. [10], E.A. PORTER & al.[30]).

Chlorogenic acid, caffeic acid, gentisic acid, ferulic acid and p-coumaric acid were identified in SRE and SREH solutions, so we concluded that they exist in both free, esterified and glycosidic forms.

Chlorogenic and caffeic acids weren’t quantified in the present chromatographic conditions (method 1) due to overlapping. Using HPLC-UV (method 2) we found 2.66 mg% chlorogenic acid and 17.51 mg% caffeic acid (table 2). Chlorogenic acid concentration was low compared to M.PELC & al. [10] results (29.74-60 mg%), nevertheless caffeic acid content was similar (4-22.61 mg%).

<table>
<thead>
<tr>
<th>Table 2. HPLC-MS/HPLC-UV analysis of RE</th>
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<tbody>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>gentisic acid</td>
</tr>
<tr>
<td>chlorogenic acid</td>
</tr>
<tr>
<td>caffeic acid</td>
</tr>
<tr>
<td>ferulic acid</td>
</tr>
<tr>
<td>p-coumaric acid</td>
</tr>
<tr>
<td>hyperoside</td>
</tr>
<tr>
<td>quercitrin</td>
</tr>
<tr>
<td>isorquercitrin</td>
</tr>
<tr>
<td>quercetin</td>
</tr>
<tr>
<td>kaempferol</td>
</tr>
</tbody>
</table>

Legend: „+” - identified by MS, „-” not identified by MS, „NA”- not applicable, „ND”- not determined

Results are expressed as mg%

Antioxidant activity using DPPH, ABTS+, FRAP and ORAC assays

The antioxidant activity was determined by means of well-known methods: DPPH, ABTS+, FRAP and ORAC, which are frequently used for evaluation of herbal products antioxidant activity (S. DUDONNÉ & al. [31]).

For all assays, both extract and positive controls (ascorbic acid, gallic acid) were dissolved in the same solvent, since the antioxidant activity is influenced by solvent type, polarity and polyphenols chemical structure (S.E. CELIK & al. [32]).

As shown in fig. 1 both extract and positive controls (gallic acid, ascorbic acid) scavenged DPPH free radical in a concentration dependent manner. The DPPH and ABTS+ scavenging capacity of RE increased significantly from 4.28 ± 0.5939% (for 0.01 mg/mL) to 90.85 ± 0.2687% (for 0.2 mg/mL) and from 9.775 ± 1.8314 % (for 0.01 mg/mL) to 92.02 ± 0.9410% (for 0.5 mg/mL) respectively. Positive controls scavenging capacity continuously increased up to 0.1 mg/mL. Our results are similar to P.R.VENSKUTONIS & al. [33] that reported a scavenging activity between 20.5-82.5% for DPPH reaction system and 8-42.7% for ABTS+ assay.

Reducing power of RE (FRAP method) increased from 0.137 ± 0.0088 (for 0.02 mg/mL) to 1.058 ± 0.0046 (for 0.15 mg/mL). Positive controls showed high antioxidant activity within the concentration range of 0.004-0.030 mg/mL (fig. 1).

For DPPH, ABTS+ and FRAP methods the EC50(mg/mL) and TEAC/mmol Fe+2 values of RE were high and respectively low compared to ascorbic acid and gallic acid, suggesting a
higher antioxidant activity for positive controls (table 3). Regarding ORAC results (trolox calibration curve $R^2 = 0.9871$, $n = 6$), ours (table 3) were higher compared to those of S.Y. WANG. & al. [34], that found 366-7728 μM trolox/g extracts obtained from leaves of other Rubus species.

![Graphs showing antioxidant activity](image_url)

**Fig. 1.** Radical scavenging capacity and reducing power of RE and positive controls

**Table 3.** Antioxidant activity of RE

<table>
<thead>
<tr>
<th>Extract/positive control</th>
<th>DPPH method EC$_{50}$ (mg/mL)</th>
<th>ABTS$^+$ method EC$_{50}$ (mg/mL)</th>
<th>FRAP method mmol Fe/mg extract/positive control</th>
<th>ORAC method TE/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE</td>
<td>0.0991±0.008</td>
<td>0.2274±0.0275</td>
<td>0.2043±0.1094</td>
<td>7243.7±686.1</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.0317±0.004</td>
<td>0.0531±0.0029</td>
<td>0.8552±0.1429</td>
<td>-</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.0062±0.003</td>
<td>0.0191±0.0015</td>
<td>3.3266±0.4856</td>
<td>-</td>
</tr>
</tbody>
</table>

*In vitro tests on human HepG2 cell line*

a. *Cell culture and treatment/ b. MTT cell viability test*

To exclude a possible cytotoxic effect of Rubi idaei folium (raspberry leaf) dry extract, several concentrations (0.001-0.1 mg/mL) were tested on HepG2 cells. As shown in fig. 2 cells treatment for 24, 48 and 72 h with different concentrations of RE induced a dose-dependent effect upon cell viability. After 72 h, high concentrations (0.05-0.1 mg/mL) decreased cell viability up to 50.45 ± 1.026% (fig.2) compared with control (untreated cells, viability 100%).
The viability decrease is probably the outcome of pro-oxidant activity of polyphenols, that can react with transitional metals (in the culture medium) and lead to quinones and ROS (O₂•, H₂O₂) with cytotoxic activity (B. HALIWELL [35]).

Moreover, ellagitannins from red raspberry leaves (sanguin H6, lambertianin C, D) are well-known for their anti-cancer properties (J.A. ASCACIO-VALDÉS& al. [36]). Based on these data, we selected the optimal range of non-cytotoxic concentrations of RE (0.001-0.01 mg/mL), used in further experiments.

It is also known that MTT enter in the cell through endocytosis process and it is metabolised by NADPH oxidase (M.V. BERRIDGE & al. [36]). Flavonoid compounds are able to inhibit NADPH oxidase activity (A.L. TAUBER & al. [37], Y. STEFFEN & al. [38]). Based on these data, we can presume that lower values obtained for high concentrations of RE could be the result of cell reduced capacity to metabolize MTT rather than lower number of viable cells.

The dose of 50 mM alloxan (that reduced HepG2 cells viability by 50%) was used for further experiments (fig 3). In order to evaluate the cytoprotective effect of RE against alloxan induced oxidative stress upon HepG2 cells, co-treatment was carried out and results are presented in fig. 4. Co-treatment with RE (0.001-0.01 mg/mL) and 50 mM alloxan for 24, 48, 72 h presented similar or lower viability (%) compared to control (cells treated with alloxan only). From our results, it could be suggested that co-treatment induced an increase of cell damage.

c. Quantification of intracellular ROS production

In order to see if RE is able to reduce the pro-oxidant effect of alloxan, we also determined intracellular levels of ROS for cells treated with alloxan and RE within the concentration range of 0.001-0.01 mg/mL. As shown in fig. 5, the production of intracellular ROS decreased in the presence of RE (0.001-0.01 mg/mL and alloxan) compared to cells treated with alloxan only.

The decrease of ROS can be assigned to phenolic compounds (flavonoids, tannins, phenolcarboxylic acids) (K.B. PANDEY & al. [2]).
Among flavonoids (identified by HPLC-MS/HPLC-UV), quercitrin, isoquercitrin, quercetin and kaempferol permeate the membrane by passive pathways; the highest permeability coefficient was observed for kaempferol (X-Y. Tian & al. [40]). Results from in vitro experiments (Coco-2 monolayer model) showed that quercitrin and isoquercitrin have limited permeabilities compared to their aglycone (quercetin) (Z. Zuo & al. [41]). In cells, flavonoid aglycones (quercetin, kaempferol) occur mostly as metabolites (methylated, glucuronide conjugates), however the best scavenger activity was observed for free aglycones, due to free hydroxyl groups (K. Kanazawa & al. [42]).

Phenolic acids (chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid) might be responsible for ROS reduction, since they can permeate the membrane by active pathways (for chlorogenic acid and gentisic acid - MCT = „monocarboxylic acid transporter” and for ferulic acid - SMCT = „sodium dependent monocarboxylic acid transporter”) (S. Lafay & al. [43]). Ellagitannins from RE might also be responsible for ROS decrease, since they are hydrolysed to ellagic acid, a natural compound with strong antioxidant activity (A. Scalbert & al. [44]).

Taking into consideration ROS production decrease and the insignificant variations of cells viability, which were incubated with 0.001-0.01 mg/mL RE for 24h, we can assume that RE has antioxidant activity and the RE + alloxan co-treatment results are not associated with additional pro-oxidant activity. We presume that the viability decrease for co-treatment is probably the consequence of other mechanisms involved in polyphenols effect upon cancer cells: changes in cellular signaling, induction of cell cycle arrest, inhibition of protein kinase C, Bcl-2 phosphorylation and NF-κB activity (K.B. Pandey & al. [2]).

4. Conclusions

*Rubi idaei folium dry extract* is an importance source of phenolic compounds with antioxidant activity. *In vitro* tests upon HepG2 cells showed encouraging premises for therapeutic use of raspberry leaf dry extract in hepatic disorders, associated with oxidative stress. Still, *in vivo* tests are necessary in order to determine the accurate therapeutic range of concentrations, since high doses had cytotoxic properties.
References


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