Antiradical and Anti-H_2O_2 Properties of Polyphenolic Compounds from an Aqueous Peppermint Extract

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Polyphenolic compounds such as eriocitrin, luteolin-7-*O*-rutinoside, diosmin, hesperidin, narirutin, isorhoifolin, rosmarinic and caffeic acids were identified in an aqueous extract (Ex) obtained from peppermint leaves (*Menthae × piperitae folium*). The content of polyphenols in Ex was as follows: eriocitrin 38%, luteolin-7-*O*-rutinoside 3.5%, hesperidin 2.9%, diosmin 0.8%, isorhoifolin 0.6%, narirutin 0.3%, rosmarinic acid 3.7% and caffeic acid 0.05%. The strongest antiradical activity (determined as $DPPH^{\bullet}$ scavenging features) was observed for luteolin-7-*O*-rutinoside, eriocitrin and rosmarinic acid. Caffeic acid and hesperidin revealed a lower antiradical activity while isorhoifolin, narirutin and diosmin showed the lowest activity. The strongest anti-H_2O_2 activity was observed for eriocitrin, a little lower for rosmarinic acid. The rate of hydrogen peroxide scavenging activity displayed by luteolin-7-*O*-rutinoside and caffeic acid was lower than that of rosmarinic acids. Hesperidin appeared to be a very weak scavenger of hydrogen peroxide. Almost no anti-H_2O_2 activity was demonstrated for diosmin, narirutin and isorhoifolin. Among examined flavonoids, the strongest antiradical and anti-H_2O_2 activity was shown for compounds with two hydroxy groups bound to the B-ring in *ortho* position in relation to each other. Replacement of one hydroxy group in the B-ring with a methoxy group or removing one hydroxy group leads to decrease of antiradical and anti-H_2O_2 activity of flavonoids. Our results suggest that eriocitrin is a powerful peppermint antioxidant and a free radical scavenger.

Key words: Antiradical Activity, Plant Phenolics, *Mentha × piperita*

Introduction

Many metabolic processes taking place in the human or animal body have radical character and proceed with the participation of reactive oxygen species (ROS) such as the hydrogen peroxide and superoxide anion (Halliwell and Gutteridge, 1999a, b). These reactive oxygen species or free radicals when in excess (chronic inflammatory states) lead to many pathological events in the human and animal body (Werns and Lucchesi, 1989; Marnett, 2002). The most common diseases related to the excessive intensity of free radical processes are arteriosclerosis and cancer (Wiseman *et al.*, 1995; Renier *et al.*, 1996).

These injurious effects of reactive oxygen species and free radicals can be effectively quenched by plant extracts or separated plant polyphenolic compounds. The antiradical or antioxidant activities of plant extracts are connected with the qualitative and quantitative content of polyphenols. A majority of plant polyphenolics has antiradical and antioxidant features but the intensity of their activity strongly depends on the chemical structure of individual compounds (Rice-Evans *et al.*, 1996; Chen and Ho, 1997; Miyake *et al.*, 1997a; Sroka and Cisowski, 2003; Nenadis *et al.*, 2004).

Peppermint (*Mentha × piperita* L.), belonging to the labiates, is a popular medicinal herb in Europe used as a spasmolytic, carminative, and cholagogue drug as well as a spice in a variety of beverages and foods. Its biological action is mainly due to the content of volatile oil, but presumably flavonoid glycosides and caffeetannins (caffeic acid derivatives) also play an important biological role (Guedon and Pasquier, 1994; Inoue *et al.*, 2002; Kosar *et al.*, 2004). Peppermint leaves yield volatile oil to the amount of 0.5–4.0% (Bisset and Wichtl, 1994). Besides, this widely used herbal drug obtains up to 21.7% of polyphenolic compounds such as rosmarinic acid and both, flavone and flavanone glycosides (Guedon and Pasquier, 1994; Areias *et al.*, 2001; Inoue *et al.*, 2002). Previous studies revealed the high antioxidant capacity of peppermint expressed as an ORAC value (Zheng and Wang, 2001). Kosar *et al.* (2004) and Dorman *et al.* (2003) examined radical scavenging

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compounds in deodorized aqueous extracts from several species of *Mentha* genus using a postcolumn derivatization method (HPLC-DPPH$^{\bullet}$) and other antioxidant and antiradical assays. On the basis of collected information peppermint leaves could be considered as a potential source of polyphenolic compounds with strong antioxidant and free radical scavenging activities.

In our work, an aqueous extract (Ex) was obtained from peppermint leaves. Then flavonoid glycosides and caffeic acid derivatives were identified qualitatively and quantitatively in this extract using chromatographic techniques. The antioxidant activity as well as capability for hydrogen peroxide scavenging were measured for Ex and individual compounds were identified in Ex. The percentage of contribution of flavonoid glycosides and caffeetannins to the antiradical and anti-H_2O_2 effectiveness of Ex was also calculated.

Materials and Methods

Material and extract preparation

The dried leaves of peppermint (*Mentha* × *piperita* L.) were purchased from the pharmaceutical industry (KAWON, Gostyn, Poland). To obtain an aqueous extract (Ex), boiling distilled water (200 ml) was poured over the powdered peppermint leaves (5 g); the mixture was placed in an ultrasonic bath for 15 min and passed through a filter (Whatman No 1). After filtration, 100 ml of aqueous extract were acidified with 0.5 ml of 98% formic acid to pH 3 and applied to the octadecyl column (2 × 5 cm, J. T. Baker, USA), which was then washed with 100 ml of methanol. The elute was evaporated (Büchi system) to dryness to obtain Ex.

The detailed quantitative and qualitative analysis of flavonoid glycosides and caffeic acid derivatives in Ex was performed with a HPLC method.

Standard substances

Rosmarinic acid, eriocitrin, luteolin-7-*O*-rutinoside, diosmin and hesperidin were isolated in our laboratory from peppermint leaves (300 g). The 50% aqueous acetone extract of peppermint was subjected to a combination of adsorption and partition chromatography (octadecyl, Sephadex LH-20) with various solvent systems containing water, MeOH and acetone. Structures of isolated compounds were elucidated by comparison of their UV, ^{1}H NMR, ^{13}C NMR and ESI-MS data with literature values (Markham and Chari, 1982; Markham and Geiger, 1994; Lu and Foo, 1999). Standards of isorhoifolin and narirutin (identified in Ex) were bought from Extrasynthese (France). Caffeic acid was purchased from Koch-Light Laboratories (UK).

Stock standard solutions (1 mg/ml) were prepared by dissolving 5 mg of each compound in 5 ml of HPLC grade methanol (Merck, Germany) and filtered through membrane filters (Millipore, 0.22 μm). Working standard solutions, 0.05–0.50 mg/ml, were prepared by dilution with methanol.

HPLC analysis

The HPLC separation of polyphenolic compounds was performed on a Knauer system (Germany) equipped with two pumps (type 64), a sample injector and variable wavelength UV detector (type 87.00). The column used was a Beta Basic-18, 5 μm C_{18} (250 × 4.6 mm I.D.) from Thermo Hypersil (UK). The detection was carried out at 280 and 330 nm. The flow rate was set to 1.0 ml/min. Peppermint compounds were analyzed using an acetonitrile/water gradient with formic acid addition according to the solvent program: solvent A, 1.5% formic acid in acetonitrile; solvent B, 1.5% formic acid in water; starting from 15% A up to 45% A in B by 25 min. The injection volume for all samples was 20 μl. Gradient grade acetonitrile was used (Merck, Germany) for HPLC analyses. Water was glass-distilled and deionized. Solvent solutions were vacuum degassed with sonication prior to the usage. All experiments were performed at 20 °C. Contents of analyzed compounds in Ex were measured in triplicate as the mean of three independent extraction processes. The standard deviation was calculated.

Calibration graphs were generated using five calibration solutions. All graphs were linear in the examined range (0.05–0.50 mg/ml). The minimum detectable concentration (MDC) values (μg/ml) were calculated from calibration equations. Correlation coefficients *r* for graphs of peppermint polyphenols and their MDC values are given in Table I.

Measurement of antiradical activity

The antiradical activity of substances was measured in triplicate according to the method of Brand-Williams *et al.* (1995). A solution I was

freshly prepared by dissolving 2 mg of DPPH• (1,1-diphenyl-2-picrylhydrazyl, Sigma) in 54 ml of MeOH. A solution II was obtained by dissolving the investigated compounds or extract in MeOH at 0.87 mg/ml concentration. Then 40 μl of II were added to 1460 μl of I at room temperature. The absorbance at 515 nm was measured in a 1-cm glass cuvette at 0 (start) and after 10 min of the reaction versus blank (40 μl of II added to 1460 μl of MeOH). The control sample was prepared in the following way: 40 μl of MeOH were added to 1460 μl of I and the absorbance was measured at 515 nm at 0 and after 10 min of the reaction.

The radical scavenging activity (Rs%) was expressed in percentages as a DPPH• elimination.

All measures were made in triplicate and the standard deviation was calculated.

The percentage of contribution (CRs%) of identified compounds to the antiradical effectiveness of Ex was also calculated.

For comparison of peppermint polyphenols their antiradical activity was also calculated as a percentage of DPPH• radical elimination (Rsm%) at 0.05 mM of a compound concentration (Table II).

Measurement of scavenging of hydrogen peroxide

The hydrogen peroxide scavenging activity was measured by the method of Pick and Keisari (1980), modified later by Bahorun *et al.* (1996). A 100 μl of water solution of investigated substance was added to a test tube at the concentration of 0.21 mg/ml for eriocitrin, luteolin-7-*O*-rutinoside, rosmarinic acid, caffeic acid, Ex or 0.26 mg/ml for diosmin, hesperidin, narirutin and isorhoifolin. 100 μl of water (without substance) were added to the control sample. Then 100 μl of 0.002% H_2O_2 and 0.8 ml of 0.1 M phosphate buffer (Na_2HPO_4: KH_2PO_4), pH 7.4, with 100 mM NaCl were added. The reaction mixture was preincubated for 10 min at 37 °C. Then 1 ml of 0.1 mg/ml horseradish peroxidase (EC 1.11.1.7; donor: hydrogen-peroxide oxidoreductase, Sigma) in 0.2 mg/ml phenol red dye in 0.1 M phosphate buffer was added. After 15 min 50 μl of 1 M NaOH were added to stop the reaction and the absorbance was measured at 610 nm immediately.

All measurements were three times repeated and the standard deviation was calculated. The hydrogen peroxide scavenging activity (Hs%) was expressed as the percentage of eliminated H_2O_2.

The investigated compounds were taken to the test in different concentrations because of limitations of the test and different activities of substances. Mainly the best results for strong antioxidants (eriocitrin, luteolin-7-*O*-rutinoside, rosmarinic acid, caffeic acid and Ex) were observed at 0.21 mg/ml. When weak antioxidants (diosmin, hesperidin, narirutin and isorhoifolin) were investigated the best results were observed at 0.26 mg/ml. Finally, the activity measured for diosmin, hesperidin, narirutin and isorhoifolin (investigated at 0.26 mg/ml) was multiplied by 0.81 (0.21/0.26 = 0.81) in order to compare to activity of more active compounds examined at 0.21 mg/ml.

For comparison of anti-H_2O_2 activity of peppermint polyphenols their activity was also calculated and expressed as the percentage of H_2O_2 elimination (Hsm%) at 2 mM of compound concentration (Table II).

Results and Discussion

The infusion prepared from dried leaves of *Mentha* × *piperita* L. is a very popular herbal drug, which is indicated in acute and chronic gastritis and enteritis, in colicky disorders of the gastrointestinal trace, and also in chronic cholecystopathies. A peppermint tea usually contains only 21–25% of the volatile oil present in the drug and thus only small amounts of menthol and menthone. About 75% of polyphenolic compounds are extracted using boiling water (Duband *et al.*, 1992; Bisset and Wichtl, 1994). The composition of peppermint polyphenols was early examined by Duband *et al.* (1992), Guedon and Pasquier (1994), and Areias *et al.* (2001). To determine the amount of flavonoid glycosides and caffeic acid derivatives in the aqueous extract Ex from leaves of *Mentha* × *piperita* L., HPLC analysis was used (Fig. 1). Quantification was carried out by the external standard method from integrated peak areas of samples at 280 nm (Fig. 1), the UV absorption maximum of flavanone glycosides (Guedon and Pasquier, 1994). The main individual compounds of Ex were identified as eriocitrin (= eriodictyol-7-*O*-rutinoside) (**1**), luteolin-7-*O*-rutinoside (**4**), hesperidin (= hesperetin-7-*O*-rutinoside) (**2**) and rosmarinic acid (**7**), according to the literature (Duband *et al.*, 1992; Guedon and Pasquier, 1994; Inoue *et al.*, 2002). Narirutin (= naringenin-7-*O*-rutinoside) (**3**), diosmin (= diosmetin-7-*O*-rutinoside) (**5**), isorhoifolin (= apigenin-7-*O*-rutinoside)

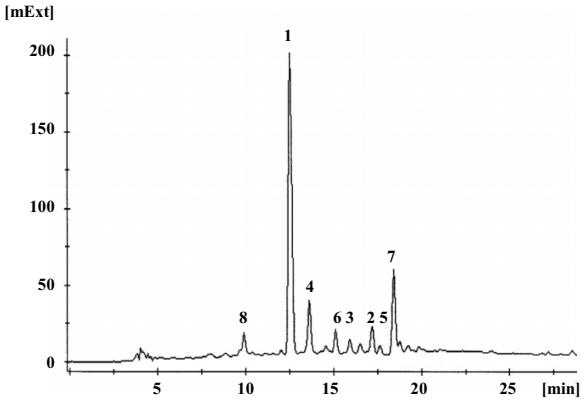

Fig. 1. HPLC chromatogram of aqueous extract from peppermint leaves (Ex) at 280 nm. Peaks: **1**, eriocitrin; **2**, hesperidin; **3**, narirutin; **4**, luteolin-7-*O*-rutinoside; **5**, diosmin; **6**, isorhoifolin; **7**, rosmarinic acid; **8**, caffeic acid.

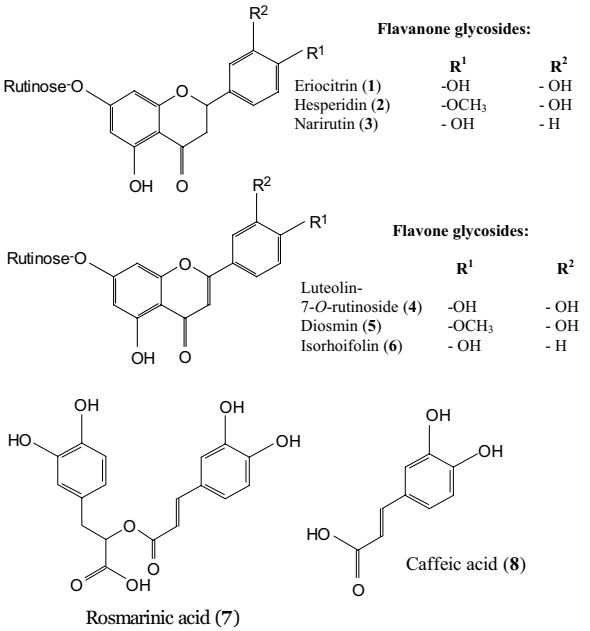

Fig. 2. Structures of polyphenolic compounds separated from peppermint leaves (*Mentha* × *piperita* L.).

(**6**) and caffeic acid (**8**) were detected in a low yield. The similar polyphenolic composition of *Mentha* × *piperita* (a chemical fingerprint) described Areias *et al.* (2001) for plants cultivated in the North of Portugal and Guedon and Pasquier (1994) for the 40 European and North American peppermint clones. The structures of identified flavonoids and caffeetannins are demonstrated in Fig. 2. Amounts of identified compounds in Ex and dried peppermint leaves are given in Table I.

The antioxidant and antiradical activities of polyphenols depend on the position and degree of hydroxylation, polarity and solubility. According to literature evidences, caffeic, dihydrocaffeic and rosmarinic acids are recognized as strong herbal antioxidants (Chen and Ho, 1997; Kosar *et al.*, 2004; Nenadis *et al.*, 2004). Their activity is even much higher than that of α-tocopherol and BHT (Chen and Ho, 1997; Moon and Terao, 1998). Rosmarinic acid is an effective antioxidant in the biological systems through the scavenging of superoxide and other reactive oxygen species. Its scavenging effect is greater than that of ascorbic

Table I. Contents of peppermint polyphenolic compounds and their analytical data (n = 3).

Identified compound	Content of compounds [mg/g dry wt of Ex extract]	Content of compounds [mg/g dry wt of peppermint leaves]*	[a]MDC [μg/ml]	[a]Calibration coefficient r
Eriocitrin (**1**)	383.3 ± 0.80	30.0 ± 0.06	38.9	0.999
Hesperidin (**2**)	29.0 ± 0.14	2.3 ± 0.01	12.1	0.993
Narirutin (**3**)	2.9 ± 0.04	0.2 ± 0.01	3.5	0.988
Luteolin-7-*O*-rutinoside (**4**)	35.5 ± 0.12	2.8 ± 0.01	6.8	0.998
Diosmin (**5**)	7.8 ± 0.05	0.6 ± 0.00	11.9	0.993
Isorhoifolin (**6**)	6.4 ± 0.08	0.5 ± 0.01	7.1	0.989
Rosmarinic acid (**7**)	37.4 ± 0.22	2.9 ± 0.02	2.6	0.994
Caffeic acid (**8**)	0.5 ± 0.05	0.04 ± 0.00	4.4	0.999

Mean values of three independent experiments ± standard deviation.
* Mean values calculated on the basis of extract yield.
[a] Calibration graphs were generated using five calibration solutions. All graphs were linear in the examined range (0.05–0.50 mg/ml). The minimum detectable concentration (MDC) values (μg/ml) were calculated from calibration equations.

Table II. Antiradical and anti-H_2O_2 activities of aqueous extract Ex and peppermint polyphenols ($n = 3$).

Identified compounds	Rs%	Hs%	Rsm%	Hsm%	CRs%	CHs%
Eriocitrin (**1**)	57 ± 0.6	60 ± 1.85	77 ± 0.81	68 ± 2.04	78 ± 13	78 ± 6.5
Hesperidin (**2**)	17 ± 0.89	9 ± 2.74	24 ± 1.22	10 ± 3.05	1.7 ± 0.34	0.9 ± 0.34
Narirutin (**3**)	1.3 ± 0.55	1.2 ± 0.00	1.7 ± 0.73	1.3 ± 0.00	0.013 ± 0.008	0.01 ± 0.001
Luteolin-7-*O*-rutinoside (**4**)	80 ± 0.82	31 ± 6.7	108 ± 1.08	35 ± 7.56	10 ± 1.7	4 ± 1.0
Diosmin (**5**)	0.37 ± 0.64	0 ± 0.82	0.5 ± 0.89	0 ± 0.99	0.01 ± 0.02	0 ± 0.00
Isorhoifolin (**6**)	5.1 ± 0.55	0.8 ± 0.69	6.5 ± 0.70	0.88 ± 0.76	0.12 ± 0.032	0.02 ± 0.017
Rosmarinic acid (**7**)	75 ± 1.51	79 ± 2.76	61 ± 1.23	54 ± 1.92	10 ± 1.8	11 ± 0.90
Caffeic acid (**8**)	100 ± 0.5	94 ± 6.78	41 ± 0.21	32 ± 2.32	0.17 ± 0.04	0.16 ± 0.04
Aqueous extract Ex	57 ± 0.83	59 ± 6.18	–	–	–	–

Mean values of triplicate measurement ± standard deviation.
Rs%, radical scavenging activity; Hs%, hydrogen peroxide scavenging activity; Rsm%, antiradical activity calculated per 0.05 mM of compound concentration; Hsm%, anti-H_2O_2 activity of polyphenols calculated per 2 mM of compound concentration; CRs%, percentage of contribution of identified compounds to the antiradical effectiveness of Ex; CHs%, percentage of contribution of identified compounds to the anti-H_2O_2 effectiveness of Ex.

acid, a common used water-soluble radical scavenger, or other simple phenolic acids (Nakamura *et al.*, 1998). Flavonoid rutinosides such as eriocitrin, hesperidin, narirutin and diosmin were first reported to exist in the *Citrus* fruits and juice (*C. limon* Burm., *C. aurantium* L., *C. macrophylla* L.). Lemon flavonoids are also known as antioxidants (Miyake *et al.*, 1997a, b; Gil-Izquierdo *et al.*, 2004). Their activity was examined *in vitro* using linoleic acid autoxidation, the liposome oxidation system and the low-density lipoprotein (LDL) oxidation system (Miyake *et al.*, 1997a, b). Eriocitrin and its metabolites by intestinal bacteria (eriodictyol, dihydrocaffeic acid and phloroglucinol) possess the strongest antioxidant activity among analyzed lemon flavonoids in the LDL oxidation system, even higher than α-tocopherol (Miyake *et al.*, 1997a, b). Moreover peppermint polyphenols were examined by Inoue *et al.* (2002) for antiallergic properties. We evaluated antiradical and anti-H_2O_2 effects of the peppermint aqueous extract Ex and its individual polyphenolic components. The antiradical activity (Rs%) of Ex and identified phenolic compounds expressed as the percentage of free radical elimination is given in Table II. For comparison, the antiradical activities of all peppermint polyphenols were calculated for 0.05 mM substance concentration (Rsm%) (Table II). The Rsm% values in decreasing order are as follows: luteolin-7-*O*-rutinoside (**4**) > eriocitrin (**1**) > rosmarinic acid (**7**) > caffeic acid (**8**) > hesperidin (**2**) >> isorhoifolin (**6**) > narirutin (**3**) > diosmin (**5**). The hydrogen peroxide scavenging activities (Hs%) of Ex, flavonoid glycosides and caffeic acid derivatives are shown in Table II. Anti-H_2O_2 activity of peppermint polyphenols calculated per 2 mM of compound concentration (Hsm%) are demonstrated in Table II. The Hsm% presented in decreasing order are as follows: eriocitrin (**1**) > rosmarinic acid (**7**) > luteolin-7-*O*-rutinoside (**4**) > caffeic acid (**8**) > hesperidin (**2**) >> narirutin (**3**) > isorhoifolin (**6**) > diosmin (**5**). Taking into account the antiradical activity of each polyphenolic compound (Rs%) in relation to their content in Ex, there was calculated the following decreasing order of the contribution percentage (CRs%) of each compound to the antiradical effectiveness of Ex: eriocitrin (**1**) >> luteolin-7-*O*-rutinoside (**4**) = rosmarinic acid (**7**) > hesperidin (**2**) >> caffeic acid (**8**) > isorhoifolin (**6**) > narirutin (**3**) > diosmin (**5**). The percentage of contribution of polyphenols to the anti-H_2O_2 effectiveness of Ex (CHs%) was as

follows: eriocitrin (**1**) >> rosmarinic acid (**7**) > luteolin-7-*O*-rutinoside (**4**) > hesperidin (**2**) >> caffeic acid (**8**) > isorhoifolin (**6**) > narirutin (**3**) > diosmin (**5**) (Table II).

As we can see in our experiments the most active antiradical compounds appeared to be luteolin-7-*O*-rutinoside (**4**) and eriocitrin (**1**) with two hydroxy groups bound to the aromatic ring in *ortho* position in relation to each other. Similarly rosmarinic acid (**7**) and caffeic acid (**8**) exhibited strong antiradical features. When one hydroxy group in the B-ring of the flavonoids luteolin-7-*O*-rutinoside (**4**) or eriocitrin (**1**) was replaced by the methoxy one giving, respectively, diosmin (**5**) and hesperidin (**2**), the antiradical activity was weaker. Besides the flavanone rutinoside **2** was much more active than the flavone rutinoside **5**. When one hydroxy group was removed from the B-ring of luteolin-7-*O*-rutinoside (**4**) or eriocitrin (**1**) giving, respectively, isorhoifolin (**6**) and narirutin (**3**) the antiradical activity decreased and the flavone rutinoside **6** was a little more active than the flavanone rutinoside **3**.

The strongest anti-H_2O_2 agent appeared to be eriocitrin (**1**) and rosmarinic acid (**7**). However, strong anti-H_2O_2 activity was measured for luteolin-7-*O*-rutinoside (**4**) and a little weaker for caffeic acid (**8**). The methoxy-derivatives such as hesperidin (**2**) exhibited a low activity and diosmin (**5**) was completely inactive. Derivatives with only one hydroxy group in the B-ring such as narirutin (**3**) and isorhoifolin (**6**) exhibited weak anti-H_2O_2 features (Table II).

In conclusion it can be stated that the flavanone eriocitrin (**1**) had the greatest percentage of contribution (78%) in the antiradical and anti-H_2O_2 activity of Ex. Rosmarinic acid (**7**) and luteolin-7-*O*-rustinoside (**4**) were less effective and other examined polyphenols were almost completely ineffective.

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