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EVALUATION OF ANTIOXIDANT POTENTIAL OF FLAVONOIDS: AN *IN VITRO* STUDY

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In recent years, there is an increasing interest in antioxidants. The main reason for this interest is the protection of cells, their organelles (especially membranes) and metabolic pathways against oxygen free radicals and their reactive derivatives (ROS). They participate in formation of chronic inflammation states and other diseases associated with oxidative stress, such as cancer, hypertension, atherosclerosis, cardiovascular and neurodegenerative diseases (1). Antioxidant compounds from dietary sources are receiving tremendous attention. Among them are flavonoids – the widely distributed group of secondary metabolites in the plant kingdom (2, 3). Flavonoids are inseparable elements of human diet. Their main sources are presented in Table 1. The most commonly-consumed flavonoids are quercetin, luteolin and apigenin. They appear mainly in the glycosidic forms with residues such as: D-glucose, L-rhamnose, galactose, arabinose (eg., quercetin-3-glucoside, quercetin-3-rhamnoside, quercetin-3-galactoside, quercetin-3-rutinoside (rutin)) (4). Flavonoids exhibit different biological activities influencing on numerous metabolic pathways. Due to their radical scavenging, antioxidant, anti-inflammatory, anti-allergic, anticancer, antiatherosclerotic, antiaggregational and detoxification activities they might be useful for prevention and treatment of many human diseases.

Because of the wide spectrum of biological activities and the prevelance of flavonoids in daily life we decided to determine in present study an antioxidant activity of these compounds in *in vitro* model. Antioxidant activities of numerous flavonoids in plant extracts or natural formulations had intensively been studied (6–8), but little is known about properties of pure flavonoids. In this

Table 1. Flavonoids in human diet (4, 5).

Flavonoids	**Food sources**
Flavonols	onion, broccoli, curly kale, leek, lettuce, string bean, "cherry" tomato, broad bean, apples, cherries, apricots, blueberries, strawberries, redcurrants, grapes red wine, tomato juice
Flavones	parsley, celery carrot, chicory, lettuce
Flavanones	citrus fruit (oranges, lemons and grapefruits), tomatoes, aromatic plants eg. mint
Isoflavones	leguminous plants (soy and its products)
Flavanols	green tea, red wine, wine, beer, cider, chocolate, apricot, grapes, peaches, kakis, apples
Anthocyanins	blackcurrants, blackberries, blueberries, plum, cherries, strawberries, aubergines, rhubarb, cabbage, beans, radish and onions, red wine, cereals

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study we have examined the antioxidant activity of five pure flavonoids.

EXPERIMENTAL

Materials

The flavonoids from two classes: flavonols (quercetin, rhamnetin, isorhamnetin) and flavones (apigenin, luteolin) were from Sigma-Aldrich. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) – a synthetic analogue of vitamin E, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. All solvents were purchased from POCH (Gliwice, Poland).

The tested flavonoids have a common structure: a double bond between C2 and C3 in C ring and a catechol moiety also in the same ring. They differ in the number and location of hydroxyl moieties. Two of them (rhamnetin and isorhamnetin) have an additional methoxy moieties (Fig. 1).

Flavonoids dissolved in DMSO were tested at concentrations: 0.1–50 mg/sample.

In this study vitamin C dissolved in water and Trolox dissolved in DMSO were used as control standard antioxidants at the same concentrations as tested compounds.

Method

2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$) radical scavenging assay

Antioxidant activity of tested flavonoids was measured using the stable radical chromogen 2,2-diphenyl-1-picrylhydrazide photometric assay (9, 10). The antioxidant activity of tested compounds was determined by measuring the percent of DPPH$^{\bullet}$ neutralization by them and compared to the standard antioxidants (vitamin C and Trolox).

One and a half milliliter of 50 μM DPPH ethanol solution were mixed with 0.25 mL of the investigated compounds. In 10 min after mixing, the absorbance at 517 nm against a blank sample (without tested flavonoids) was spectrophotometrically measured (Shimadzu UV-1202 UV-Vis spectrophotometer).

DPPH radical scavenging activity of the flavonoids were calculated according to the formula:

DPPH radical scavenging rate (%) = $(A_{BS} - A_{TS})/A_{BS} \times 100\%$

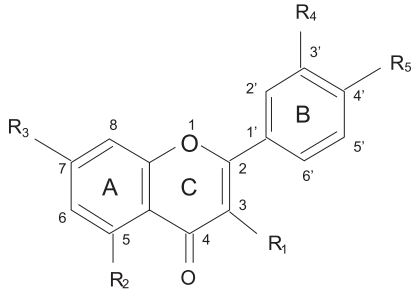
where A_{BS} = the absorbance of blank sample (t = 0 min) and A_{TS} = the absorbance of tested sample (t = 10 min).

The IC_{50} (the concentration of antioxidant which eliminate 50% of DPPH radicals) was defined for all tested flavonoids and control standard antioxidants.

The study was carried out in three independent experiments in triplicate at each concentrations of tested compounds (n = 9).

Data analysis

The results are expressed as the mean values ± standard deviation (SD). The statistical significance of differences between results obtained for flavonoids and standard antioxidants was evaluated



Flavonoids	R_1	R_2	R_3	R_4	R_5
quercetin	OH	OH	OH	OH	OH
rhamnetin	OH	OH	CH_3	OH	OH
isorhamnetin	OH	OH	OH	OH	CH_3
luteolin	-	OH	OH	OH	OH
apigenin	-	OH	OH	-	OH

Figure 1. Structure of tested flavonoids

Table 2. DPPH radical scavenging rate (%) of tested flavonoids and controls at various concentrations.

Concentration	Standard antioxidant		Flavonols			Flavones	
(µg/sample)	Vitamin C	Trolox	quercetin	rhamnetin	isorhamnetin	luteolin	apigenin
0.1	1.7 ± 0.6	1.3 ± 0.2	4.5 ± 0.2 */**	1.3 ± 0.4	1.1 ± 0.3	0.8 ± 0.2 */**	0.6 ± 0.1 */**
0.25	2.1 ± 0.4	1.1 ± 0.2	11.7 ± 2.2 */**	2.1 ± 0.3 **	1.2 ± 0.3 *	2.8 ± 0.7 **	0.8 ± 0.3 */**
0.5	2.9 ± 0.2	2.4 ± 0.7	25.0 ± 2.1 */**	3.5 ± 0.5 **	2.5 ± 0.3	3.5 ± 0.3 */**	0.9 ± 0.2 */**
1	6.8 ± 0.9	5.0 ± 1.3	53.9 ± 2.1 */**	6.8 ± 0.6 **	4.4 ± 1.0 *	6.6 ± 0.4 **	1.0 ± 0.3 */**
2.5	19.9 ± 0.7	12.0 ± 3.0	69.4 ± 0.4 */**	16.2 ± 0.8 */**	9.2 ± 1.9 *	20.9 ± 1.5 **	1.4 ± 0.4 */**
5	40.8 ± 2.1	30.1 ± 4.0	69.0 ± 0.7 */**	34.5 ± 1.6 */**	21.8 ± 1.7 */**	49.8 ± 4.5 */**	1.6 ± 0.5 */**
10	83.6 ± 0.9	56.1 ± 8.9	72.1 ± 1.6 */**	59.1 ± 0.9 *	51.2 ± 2.0 *	55.1 ± 1.8 *	1.8 ± 0.5 */**
25	88.2 ± 4.5	75.9 ± 6.0	74.9 ± 1.3 *	62.0 ± 1.4 */**	60.6 ± 3.2 */**	59.3 ± 2.0 */**	5.2 ± 1.4 */**
50	90.1 ± 0.3	82.3 ± 4.8	79.2 ± 3.9 *	62.5 ± 1.8 */**	60.1 ± 2.3 */**	62.4 ± 0.7 */**	7.3 ± 0.9 */**

Values are average of three separate experiments of three samples (the mean ± SD); * $p \leq 0.05$ *vs.* vitamin C, ** $p \leq 0.05$ *vs.* Trolox

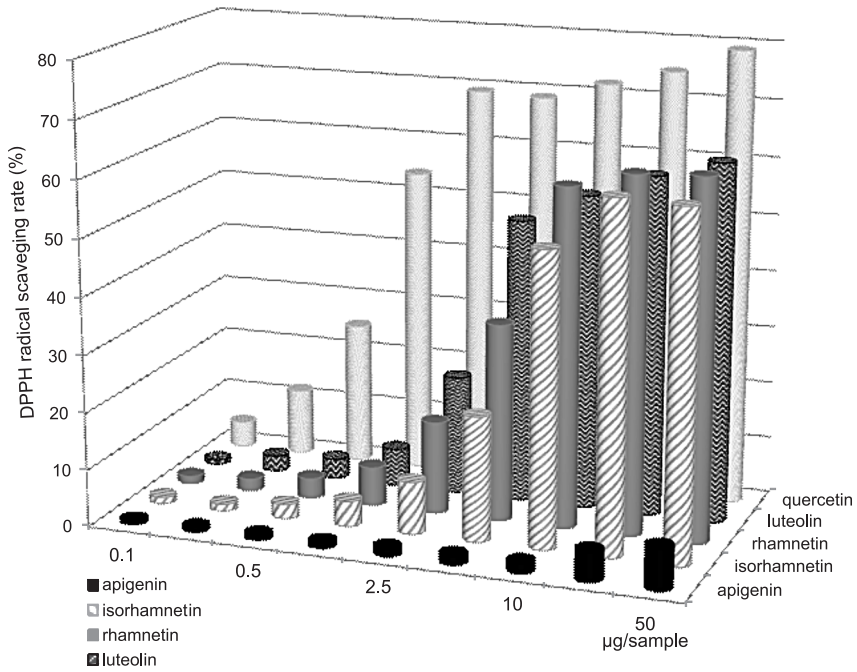


Figure 2. Antioxidant profile of tested flavonoids.

by Student *t*-test; $p \leq 0.05$ was accepted as statistically significant. All statistical calculations were performed using statistical package Statistica 6.0 programme (StatSoft 6.0).

RESULTS

Results obtained in this study indicate different DPPH radical scavenging rate of tested flavonoids. Table 2 and Figure 1 illustrate a significant decrease of DPPH radical due to the scavenging ability of tested compounds which correlates with a dose – effect dependence. The higher concentration of flavonoids the higher percentage of DPPH radical scavenging rate.

Among tested flavonols, quercetin reaches the highest radical scavenging activity already at concentration 1 µg/sample; it is almost eight times high-

er (53.9%) than rhamnetin (6.8%) and twelve times higher than isorhamnetin (4.4%). Rhamnetin and isorhamnetin have the similar antioxidant activity. The difference in antioxidant potential between quercetin and two methylated flavonols is maintained at every tested concentration. At 50 µg/sample quercetin reaches 79.2% of DPPH radical scavenging rate while rhamnetin and isorhamnetin only 62.5% and 60.1%, respectively.

Compared to the standard antioxidants (vitamin C and trolox) quercetin has the highest antioxidant potential at lower concentration (0.1 – 5 µg/sample), at concentrations 10–50 µg/sample vitamin C is a stronger antioxidant than quercetin. Rhamnetin and isorhamnetin have similar activity as trolox at concentrations 0.1–5 µg/sample. At higher concentrations (25–50 µg/sample) both standard antioxidants show higher scavenging activity than tested methylated flavonols (Table 2).

The results obtained for tested flavones show a huge difference of DPPH radical scavenging rate between them. Luteolin is several times stronger than apigenin (even 20 times at 2.5 µg/sample). Both standard antioxidants have similar antioxidant activity as luteolin only at lower concentrations (0.1– 5 µg/sample) (Table 2).

On the basis of results obtained for all tested flavonoids quercetin has the highest DPPH radical scavenging rate, and then in the following order luteolin nad rhamnetin, isorhamnetin, apigenin (Fig. 2).

Comparing IC_{50} values of tested compounds we observed that quercetin is the most effective antioxidant, only 8.5 µg/mL is needed to eliminate 50% of DPPH radical. It has stronger radical scavenging activity than controls – standard antioxidants: vitamin C (19.6 µg/mL) and Trolox (36.9 µg/mL). Luteolin and rhamnetin have similar antioxidant activity, 53 and 59 µg/mL respectively. The IC_{50} value for isorhamnetin is 93.4 µg/mL. The weakest radical scavenging potential against DPPH shows apigenin.

DISCUSSION

Oxygen free radicals and their reactive derivatives are generated endogenously in cells during physiological processes eg., within the respiratory tract, by phagocyte cells or by oxidative enzymes like xanthine oxidase, lipoxygenases and cyclooxygenases. The exogenous sources of free radicals are eg., smoking, air pollution, UV radiation and metabolism of xenobiotics. In excess they can cause multiple damages by attacking biomolecules like proteins, lipids, DNA etc. (11, 12). Naturally, there is a dynamic balance between the amount of ROS generated and degraded in cells. They are degraded to non-reactive forms by enzymatic and non-enzymatic antioxidant defenses produced in cells or by others supplied with the diet. If there is a depletion of these compounds, the oxidative stress can lead to tissue injury and subsequent diseases such as pathogenesis of ageing, degenerative diseases like arteriosclerosis, cardiovascular disease, diabetes and cancer (13–16).

Serious health hazard at high levels of ROS in cells led to the development of research on the occurrence and biological activity of natural antioxidants which are able to eliminate them (17). The role of antioxidants in the inhibition of antioxidant processes occurring in living organisms consists of: scavenging free radicals and quenching singlet oxygen, disconnection of radical reactions, chelate metals which catalyze the oxidation process, inhibition of certain enzymes (eg., oxidases). Flavonoids are active in all these processes (18).

It has been demonstrated in various *in vitro* assays that these phenolic compounds, extracted from plants, have antioxidative properties (19–22). It is little known about antioxidant potential of pure flavonoids.

In the present study, the free radical scavenging activity of five pure flavonoids was evaluated through their ability to quench the synthetic 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The assay has been used worldwide as a screen to determine the free radical scavenging capacity of various diet compounds because of its simplicity and requiring relatively short time compared to other methods. Antioxidant activity of flavonoids was observed in the first minute of their incubation with DPPH radical, suggesting rapid kinetics of interaction of these compounds. Scavenging activity differs between tested compounds. It is shown that chemical structure has an important impact on radical scavenging activity. The more hydroxyl moieties the higher antioxidant activity. Two hydroxyl groups in B ring are required to stronger antioxidant potential, lack of one of them (apigenin) significantly reduces this activity. Methyl groups in rhamnetin and isorhamnetin decrease the radical scavenging properties at lower concentrations compared to the strongest flavonoid – quercetin. The location of methyl group also affect an antioxidant potential, rhamnetin, which has this group at C7 in A ring and possesses higher antioxidant activity than isorhamnetin (methyl group at C4' in B ring).

Our observations indicate that effectiveness of flavonoids in DPPH radical scavenging depends

largely on their structure, hydrophobicity, biological and oxidative activity. Mentioned above ability and disconnection of radical chain reactions by flavonoids is mainly dependent on the presence in B ring of at least two *o*-hydroxyl groups. It enables the formation of intramolecular hydrogen bond between hydroxyl groups, which increases the stability of the phenoxyl radicals.

Biological and pharmacological properties of flavonoids depends on their antioxidant activity (23–28). Pro-health effects of these compounds in human diet can also result from their actions already in the lumen of the digestive tract, where they can protect other easily assimilated antioxidants and the epithelial cell membrane walls against the attack of ROS.

CONCLUSION

The presented results indicate high antioxidant potential of pure flavonoids. The highest level of radical scavenging properties at all concentrations among tested flavonoids exhibits quercetin and in the following order luteolin, rhamnetin, isorhamnetin and apigenin. The strong antioxidant potential could allow to administer flavonoids for prevention of numerous free radical based diseases or as an additive element to food and pharmaceutical industry.

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In the paper: Synthesis of some new 2-(substituted phenyl)-5-(N,N-diphenylaminomethyl)-1,3,4-oxadiazoles: A safer anti-inflammatory and analgesic agents. Vol. 68, No. 3, p. 381, the name of one of the authors was misprinted. Instead of Rashid Haider, should be Rashiduddin Haider.