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Antioxidant activity of selected phenols estimated by ABTS and FRAP methods*

Aktywność przeciwutleniająca wybranych fenoli oznaczona testem ABTS i FRAP

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B Data Collection
C Statistical Analysis
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Summary

Introduction: Phenols are the most abundant compounds in nature. They are strong antioxidants. Too high level of free radicals leads to cell and tissue damage, which may cause asthma, Alzheimer disease, cancers, etc. Taking phenolics with the diet as supplements or natural medicines is important for homeostasis of the organism.
Materials and methods: The ten most popular water soluble phenols were chosen for the experiment to investigate their antioxidant properties using ABTS radical scavenging capacity assay and ferric reducing antioxidant potential (FRAP) assay.
Results and discussion: Antioxidant properties of selected phenols in the ABTS test expressed as IC_{50} ranged from 4.332 μM to 852.713 μM (for gallic acid and 4- hydroxyphenylacetic acid respectively). Antioxidant properties in the FRAP test are expressed as μmol Fe^{2+}/ml. All examined phenols reduced ferric ions at concentration 1.00 x 10^{-3} mg/ml. Both methods are very useful for determination of antioxidant capacity of water soluble phenols.

Keywords: antioxidants • phenols • ABTS • FRAP

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Introduction

Phenols (and other antioxidant compounds) are very significant for human health. They are widely present in fruits, vegetables, and beverages: coffee, tea, fruit juices, wines and other products [12,18,21,22,31,36]. In plants, phenolic acids occur mainly in bound forms, as components of complex structures such as lignins and hydrolysable tannins or derivatives of sugars and organic acids [14,17]. Two main classes of phenolic acids exist: hydroxybenzoic acids (e.g. gallic, p-hydroxybenzoic, vanillic, syringic, protocatechuic acids) and hydroxycinnamic acid (e.g. coumaric, caffeic, ferulic, sinapic acid). The antioxidant activity of phenolic acids is related to the quantity, number and position of hydroxyl groups in the molecule [4,27]. Electrochemical reduction potentials determined by cyclic voltammetry give information about the capacity of reducing compounds and their results indicate that reactions involving phenols are not always reversible [8]. Phenolic compounds can act in many different ways [6]:

- chelating metals such as iron and copper, which can prevent their involvement in Fenton reactions that can generate high concentrations of hydroxyl radicals,
- breaking the chain of reactions triggered by free radicals,
- slowing down or accelerating enzyme activity.

Phenolic acids are involved in repair and adaptive systems, and can act preventively and sometimes therapeutically in various diseases: cardiovascular, respiratory, dermatological and digestive system diseases [16,25,32]. The relationship between total phenolic and flavonoid contents and antioxidant, anti-inflammatory or antibacterial activities is strong in many plants [10,11,13,23]. The main antioxidants of olive oil reduce the activity of xanthine oxidase, an enzyme potentially involved in carcinogenesis [35]. Oleuropein and p-hydroxy benzoic, vanillic and p-coumaric acids exhibit antimicrobial activity. They completely inhibit the growth of *Escherichia coli*, *Klebsiella pneumoniae* and *Bacillus cereus* [1]. UV radiation damages the skin by increasing levels of free radicals. For their elimination antioxidants contained in creams and diet are helpful [37]. Pollution, cigarette smoke, drugs, illnesses, stress etc. can increase levels of free radicals in the human body and disrupt homeostasis. The consequent damage may contribute to the development of Alzheimer's disease, Parkinson's disease, dementia, atherosclerosis and asthma [3,9,15,20,34,38]. Several methods are available to evaluate antioxidant activities of natural compounds.

The aim of this research was to estimate the antioxidant activity of selected water-soluble phenols, supplied in food of vegetable origin. These compounds differ in structure, especially the number and location of substitution of hydroxyl groups, which has a strong influence on their antioxidant properties. Two methods with different mechanisms were used: ABTS assay with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) based on free radical scavenging [26] and FRAP (ferric reducing antioxidant power) assay based on reduction of ferric ion by phenol [2]. In addition, comparison of the efficiency of these two assays was done.

Materials and methods

Phenols shown in Fig. 1 were obtained from Extrasynthesis (4-hydroxyphenylacetic acid (10) and 3,4-dihydroxyphenylacetic acid (3)), Fluka (3-caffeoylquinic acid= chlorogenic acid (6); 3,4-dihydroxybenzoic acid= protocatechuic acid (7); 3,5-dihydroxybenzoic acid= α- resorcylic acid (8); 2,3-dihydroxybenzoic acid= o-pyrocatechuic acid (4); 2,4- dihydroxybenzoic acid= β-resorcylic acid (9); 2,5-dihydroxybenzoic acid= gentisic acid (5)), Mallinckrodt GmbH (1,2,3-trihydroxybenzene= pyrogallol (1)), Polskie Odczynniki Chemiczne (3,4,5-trihydroxybenzoic acid= gallic acid (2)). Stock solutions of phenols were prepared by dissolution in distilled water. All measurements were done on the Cecil CE spectrophotometer model 3021.

ABTS ASSAY

Chemicals: Trolox (Hoffman-La Roche) (6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid; Sigma Aldrich) was used an antioxidant standard. Trolox (2.5 mM) was prepared in methanol for use as a stock standard. Working standards were prepared daily on dilution with methanol. ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and potassium persulfate (di-potassium peroxdisulfate) were obtained from Sigma-Aldrich and HPLC grade methanol from POCh.

Assay: Experiments were performed according to [26] with small modifications. ABTS and potassium persulfate were dissolved in distilled water to a final concentration of 7 mM and 2.45 mM respectively. These two solutions were mixed and the mixture allowed to stand in the dark at room temperature for 16 h before use in order to produce ABTS radical ($ABTS^{\bullet+}$). For the study of phenolic compounds the ABTS radical solution was diluted with distilled water to an absorbance of 1.00 at 734 nm.

Phenols (final concentrations 0.0001-0.01 mg/ml) or Trolox standards (final concentration 0-20 mM) were

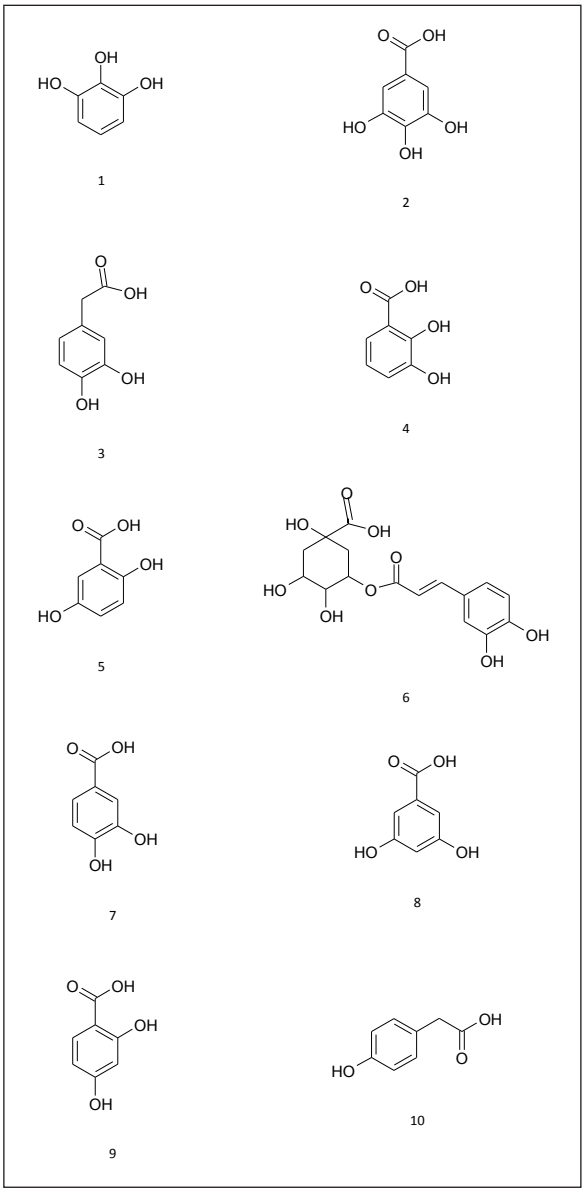


Fig. 1. Structures of selected phenols

added to diluted ABTS•+ solution and the absorbance reading was taken 6 min after mixing using the spectrophotometer. Results are presented as the ability of phenols to scavenge 50% of free radical ABTS • + (IC_{50}) and TEAC (Trolox equivalent antioxidant capacity). Parameters IC_{50} (μM) and TEAC (μM) were determined with a relative uncertainty of less than five percent.

All determinations were carried out in triplicate.

FRAP ASSAY

Chemicals: TPTZ (= 2,4,6-tripyridyl-s-triazine) (Fluka Analytical) was dissolved in HCl (POCh) for a few hours. $FeSO_4$* $7H_2O$ (= iron (II) sulfate heptahydrate) (Acros Organics) dissolved in deionized water was used as standard solution. $FeCl_3$*$6H_2O$ (= iron (III) chloride hexahydrate) was dissolved in deionized water for a few minutes prior to analysis.

Assay: Experiments were done according to [2] with modifications. FRAP working solution was prepared freshly each time: 0.3 M acetate buffer (pH=3.6), 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04 M HCl and 0.02 M $FeCl_3$*$6H_2O$ were mixed in 10:1:1 (v/v/v) and kept away from light. 0.075 ml of phenols (final concentration 0.0001-0.01 mg/ml) or $FeSO_4$* $7H_2O$ (final concentration 0-1.8 μmol Fe^{2+} /ml) solution were added to 2.25 ml FRAP working solution and 0.225 ml of deionized water. The mixture was vortexed and incubated at 37°C for 30 min away from light. Absorbance was measured at 593 nm using the spectrophotometer. FRAP working solution with deionized water instead of a sample was used as a blank. All determinations were carried out in triplicate.

Results

Structurally different water-soluble phenols were compared: phenylacetic acids, cinnamic acids, hydroxybenzoic acid derivatives and ester of caffeic acid with quinic acid. At least three different concentrations were chosen for each tested compound in both methods in order to avoid speculation of how sample concentration influences the antioxidant capacity [7].

Table 1. Antioxidant properties of selected phenolics expressed as the ability to scavenge 50% of free radical ABTS•+ (IC_{50}) and TEAC (Trolox equivalent antioxidant capacity)

No.	Phenol	IC_{50}(μM)	TEAC(μM)
1	pyrogallol	5.28	3.465
2	gallic acid	4.332	4.328
3	3.4-dihydroxyphenylacetic acid	8.538	2.123
4	o-pyrocatechuic acid	10.728	1.930
5	gentisic acid	15.562	1.323
6	chlorogenic acid	17.285	0.962
7	protocatechuic acid	20.668	0.854
8	α-resorcylic acid	41.853	0.221
9	β-resorcylic acid	169.439	0.060
10	4-hydroxyphenylacetic acid	852.713	0.030

Table 1 shows the antioxidant activity of phenols expressed as the ability to scavenge 50% of free radical ABTS•+ (IC_{50}) and TEAC (Trolox equivalent antioxidant capacity). Phenols with a higher number of hydroxyl groups

showed higher antioxidant capacity, which is expressed as the lowest IC_{50} value, 4.332 μM and 5.228 μM respectively for gallic acid and pyrogallol. In contrast, the highest IC_{50} value was obtained for 4-hydroxyphenylacetic acid (852.713 μM), almost 200 times greater, which confirms the stronger antioxidant activity of phenols with a higher number of hydroxyls [30].

Antioxidant activity of selected phenols determined by FRAP is depicted in Fig. 2a,b.

All phenols reduce Fe^{3+} ions to Fe^{2+} ions which are complexed by TPTZ at a concentration of 1.00 $x10^{-3}$ mg/ml. Phenol antioxidant activity at this concentration decreased in the order *o*-pyrocatechuic acid>3,4-dihydroxyphenylacetic acid>pyrogallol>gallic acid>gentisic acid>protocatechuic acid>chlorogenic acid>α-resorcylic acid>4- hydroxyphenylacetic acid> β-resorcylic acid. Inhibitory effects of radicals have been identified for all compounds and their strength depends on the concentration of phenol. No reaction occurred for the concentration of 1.00$x10^{-4}$ mg/ml of chlorogenic acid and gentisic acid. The remaining eight phenols with the same concentration have antioxidant properties in the following decreasing order: pyrogallol> *o*-pyrocatechuic acid>3,4-dihydroxyphenylacetic acid> protocatechuic acid>gallic acid> 4-hydroxyphenylacetic acid>β-resorcylic acid>α-resorcylic acid. In the case of chlorogenic acid at a concentration of 5$x10^{-4}$ mg/ml it also does not absorb at 593 nm but 3,4-dihydroxyphenylacetic acid, *o*-pyrocatechuic acid and pyrogallol are still in the top three most powerful antioxidants. Phenols at concentrations 2.50 $x10^{-3}$, 5.00 $x10^{-3}$, 1.00 $x10^{-2}$, 5.00 $x10^{-2}$, 1.00 $x10^{-1}$ (mg/ml) in most cases showed a very significant reduction of Fe^{3+} ions to Fe^{2+} ions and thus exceeded the scope of the absorbance.

The largest correlation was observed between ABTS method and FRAP method for phenols at a concentration 1 x 10^{-3} (1), 5 x 10^{-4} (2), 1 x 10^{-4} (3) mg/ml as shown in Table 2. The results obtained using linear regression analysis were used to fit a line to a set of experimental results using the "least squares". The correlation coefficient in these cases is 0.746, 0.783, 0.786 and all dependencies can be considered as statistical type I error not more than p=0.0023.

Table 2. Correlation between ABTS method and FRAP method for phenols at concentration 1 x 10^{-3} (1), 5 x 10^{-4} (2), 1 x 10^{-4} (3) mg/ml

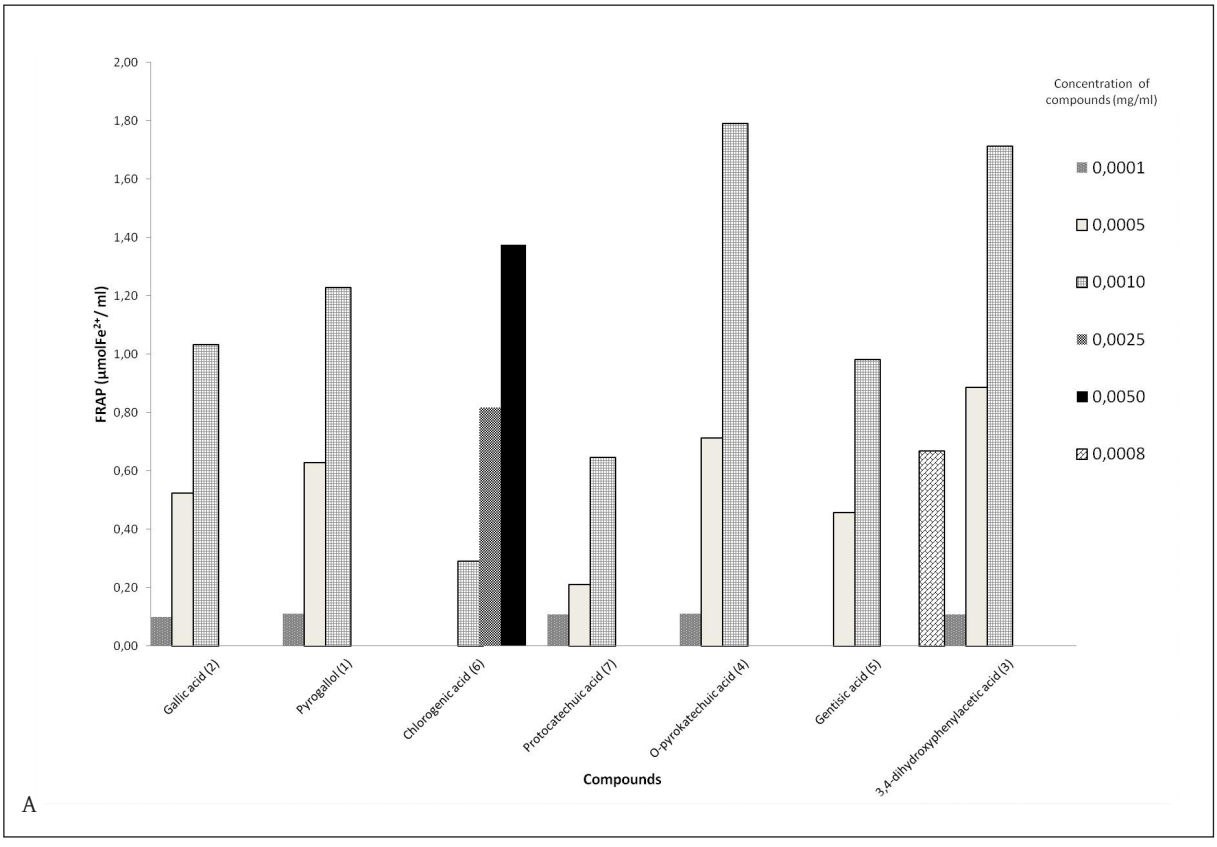
Number	Number of observations	R^2	Type I error
1	10	0.746	0.0009
2	9	0.783	0.0010
3	8	0.786	0.0023

The correlation coefficient between ABTS method and FRAP method for three phenols at a concentration 2.5 x 10^{-3} (1) mg/ml is 0.997 and type I error p=0.0259. For the other, dependence on type I error is greater than 0.05; this means that no correlation exists between the two methods.

Discussion

Antioxidant activity depends on the number and position of the hydroxyl groups of the aromatic ring binding site and the type of substituent. The strongest antioxidant activity in the ABTS method was achieved by phenols with 3 hydroxyl groups from all phenols tested in this experiment. The carboxylic group slightly increases this activity (lower IC_{50} for gallic acid compared to IC_{50} for pyrogallol). The next antioxidants in line are two hydroxylated phenolic acids with the strongest,3,4-dihydroxyphenylacetic acid, among them. It was proved that the type of spacer between the carboxylic acid and benzene ring influences their activity, which increases for the methylenic group in phenolic acids [27] Our results confirmed this thesis. What is more, phenols with two hydroxyl groups linked to the aromatic ring at the ortho position more strongly quench ABTS•+ radical than compounds with hydroxyl groups substituted at the meta position [30]. Also presence of a carbonyl group like ester (chlorogenic acid) enhances antioxidant activity [5]. So far, the great majority of antioxidant assays have been based on methanol as a solvent. The present results are in agreement with this, which makes the ABTS method equal for methanolic and watery solutions. Pyrogallol, o-pyrocatechuic acid and 3,4 dihydroxyphenylacetic acid have the strongest antioxidant properties identified by the FRAP method. The amount of Fe^{3+} ions reduced to Fe^{2+} ions varies for different concentrations of phenols. Phenols with two hydroxyl groups bonded to the aromatic ring in the *ortho* position, such as *o*-pyrocatechuic acid and 3,4 dihydroxyphenylacetic acid, showed strong antioxidant anti-radical activity at a concentration of 1 x 10^{-3} and 5 x 10^{-4} mg/ml but pyrogallol with three hydroxyl groups bonded to the aromatic ring at a concentration of 1 x 10^{-4} mg/ml. 3,5-Dihydroxybenzoic (α- resorcylic) and 2,4-dihydroxybenzoic (β-resorcylic) acids with two hydroxyls bonded in the *meta* position in relation to each other are weak antioxidants. The results confirm earlier studies [30]. 4-Hydroxyphenylacetic acid behaves like the previous two acids but is involved in scavenging reactive oxygen and nitrogen species both *in vitro* and *in vivo* [19,33].

To sum up, these two methods complement one another and give full information of antioxidative capacity of water-soluble phenols. The correlation between ABTS and FRAP methods was indicated by Nilsson et al. [24]. The ABTS method for investigating hydroxybenzoic and hydroxycinnamic acids takes advantage of the fact that they probably act as radical scavengers owing to their hydrogen and electron donating capacity and their ability to delocalize/stabilize the resulting phenoxyl radical within the structure [28,29], while the FRAP method is based on Fe^{3+} being reduced to Fe^{2+} by the examined substance.



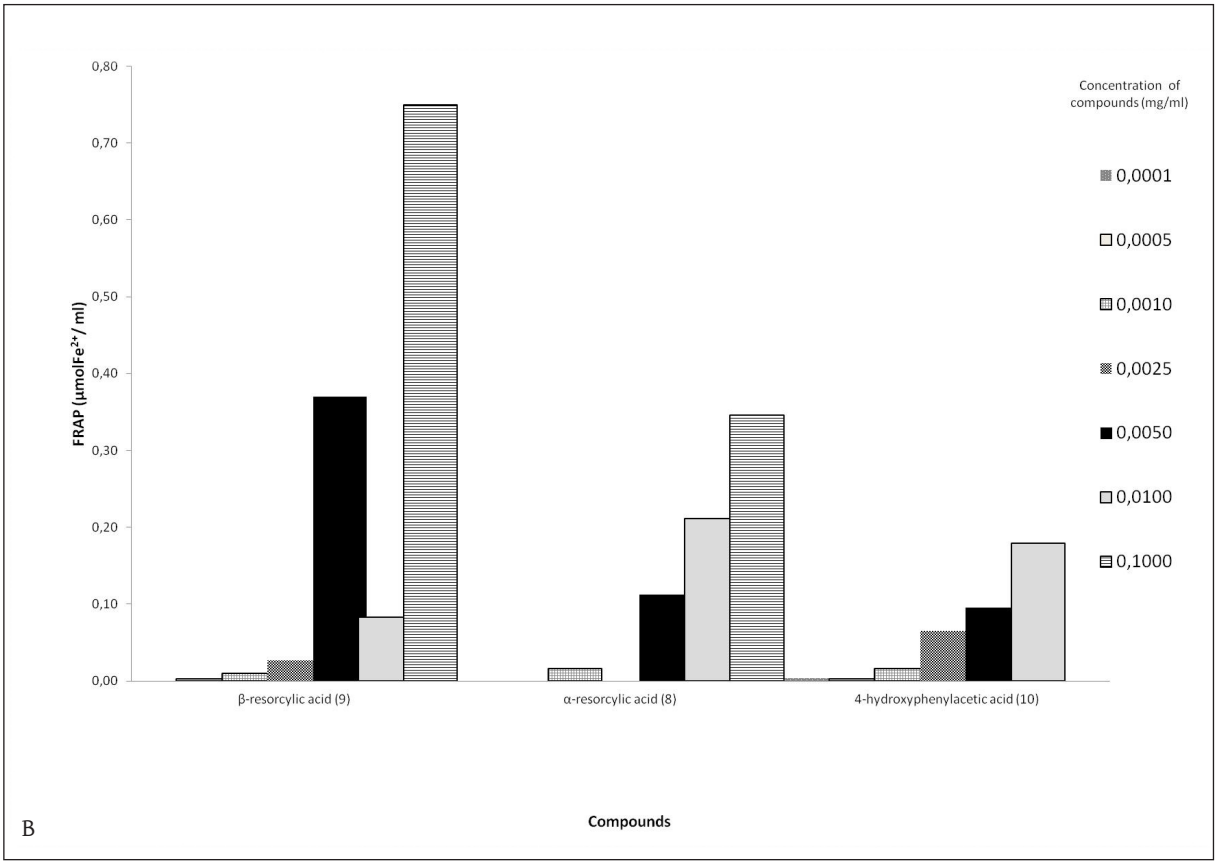


Fig. 2. Antioxidant activity (FRAP) of the various phenols; a - For strong antioxidants, b - For weak antioxidants

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