The effect of substituents on aromatic ring on antioxidant capacity of phenolic substances

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Abstract: This study comprises thirteen selected phenolic antioxidants as follows: vanillin, acetosyringone, eugenol, isoeugenol, 4-ethylguaiacol, guaiacol and acids: gentisic, 4-hydroxybenzoic, protocatechuic, ferulic, syringic, coffee, chlorogenic. For each of the phenolic substances, total antioxidant capacity (TAC) was measured using the FRAP and DPPH method expressed as TEAC (Trolox Equivalent Antioxidant Capacity); antioxidants with the concentration of 20 mg/l were compared with each other as well as against Trolox. It was found based on the results of measurements that the presence and position of some substitution groups on fenolic ring significantly affect the antioxidant capacity of these substances. Larger antioxidant capacity was measured in multiple hydroxylated substances. An effect was also observed of the type of the functional group where substances possessing a hydroxyl group instead of the methoxyl group featured significant antioxidant capacity. Similar was seen for substances possessing a carboxyl group instead of acetyl group. An effect was also observed immediately between the *ortho* and *para* positions of hydroxyl groups in aromatic ring. Examples include gentisic and protocatechuic acid, where while either of the acids has the identical molecular formula, i.e., $C_7H_6O_4$, for the hydroxyl group the antioxidant capacity is 1.2 to 1.7 times higher in case of the *ortho* position in gentisic acid than in the *para* position in protocatechuic acid.

Key Words: phenols, antioxidant capacity, functional group, FRAP, DPPH

INTRODUCTION

Phenolic substances form a large group of antioxidants. The purpose of antioxidants is to bind excess free radicals and thereby protect food products and substances that they contain. Foodstuffs are constantly subjected to tests for antioxidant potential. Determining the antioxidant activity is carried out in many different ways that feature different advantages (Bunaciu et al. 2016). Strong antioxidant activity was found for cherries, citrus fruit, plums, berry fruit and olives (Lobo et al. 2010). The chief sources of polyphenolic antioxidants include leguminous grains, fruit, vegetables and technologically prepared or produced foodstuffs such as red wine, chocolate, olive oil or green tea (Dolas and Gotmare 2015). Green/black tea is subjected to an extensive study because it contains up to 30% phenolic compounds in dry matter (Lobo et al. 2010). Contents of phenolic substances may also change during the period of storage (Snurkovic 2015). Phenolic antioxidants can have a synergetic effect (Sochor et al. 2010) and play an important role in the prevention and treatment of certain diseases caused by free radicals (Dacic and Gojak-Salimovic 2016), such as cancer, cardiovascular disease or neurodegenerative diseases (Parkinson/Alzheimer disease) (Costa et al. 2017). Individuals who regularly drink alcohol or are regularly exposed to tobacco smoke may find themselves to be short of antioxidants (Dolas and Gotmare 2015). The importance of phenolic substances thus plays an important role in the framework of nourishment and protection of the body. The background of the overall antioxidant capacity involves the influence of the individual substances, including phenolic compounds when individual phenols affect the total antioxidant capacity through varying degrees of intensity. Differences in terms of antioxidant capacity can also be very important between phenols, even in cases of similar

structures – substitution of a single functional group in the aromatic nucleus or even a changed position of the same group can make the difference. There are even multiple-fold differences appearing between such isomers of phenols.

MATERIALS AND METHODS

Phenols used to measure the antioxidant capacity were as follows: vanillin, acetosyringone, eugenol, isoeugenol, gentisic acid, 4-hydroxybenzoic acid, protocatechuic acid, 4-ethylguaiacol, guaiacol, ferulic acid, syringic acid, coffee acid, and chlorogenic acid (all with analytic purities ≥ 97%).

Other used substances were as follows: Methanol, Ethanol, Iron(III) chloride, Hydrochloric acid 35%, DPPH (2,2-diphenyl-1-picrylhydrazyl), buffer (Sodium acetate trihydrate & Acetic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tris(2-pyridyl)-s-triazine).

Sample pretreatment:

For each of the analysed phenols, approximately 100 mg was weighed out on an analytical scale, the precision being 0.1 mg. Each weighed phenol was quantitatively transferred into a 50 ml volumetric flask of 50 ml; 15 ml of ethanol was added into the flask and deionized water was supplemented to obtain a volume of 50 ml. The phenol solutions so mixed continued to be diluted with 0.1 ml solution and 9.9 ml distilled water to obtain a resulting concentration of solutions of 20 mg/l.

Total antioxidant capacity (TAC)

TAC was measured using two methods: the ferring reducing antioxidant power (FRAP) method, and the DPPH method. In both methods the quantitative determination (of the calibration curve) was making use of Trolox solutions of 0.1 to 0.5 mM.

For the FRAP method, the reaction mixture contained TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) dissolved in HCl, $FeCl_3$ and acetate buffer, the ratio being 1:1:10. The buffer solution (pH 3.6) was prepared by mixing 4 ml of concentrated CH_3COOH with 0.775 g of $CH_3COONa.3H_2O$ in a 250 ml volumetric flask and adding deionized water up to the punch mark. The solution of $FeCl_3$ was prepared by weighing out and dissolving 0.081 g of $FeCl_3 \cdot 6H_2O$ in a 25 ml volumetric flask adding deionized water up to the punch mark. The TPTZ complex in HCl was prepared from 0.078 g TPTZ dissolved in a 25 ml flask with water acidified with 0.088 ml of 35% HCl. To do the measurement, 2,000 µl of the reaction mixture was pipetted into the cuvette along with 25 µl of the sample diluted with deionized water. The filled up, 1.00 cm thick cuvette was shaken for 10 seconds using a mechanical shaker; the resulting solution was left in cuvettes for 10 minutes (reaction time). After this time the samples were analysed at a wavelength of 593 nm using a spectrometer, when absorbance of the sample was measured.

For the DPPH method 1,900 µl of the methanol solution of DPPH (the concentration of 0.1 mM) was pipetted into the cuvette along with 100 µl of the sample diluted with deionized water. The cuvette with the resulting solution was then shaken for 10 seconds on an orbital shaker. The mixed up solution was measured after 30 minutes using a spectrophotometer at the wavelength of 515 nm. The procedure was therefore the same as in our previous study (Hic et al. 2017).

RESULTS

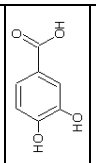
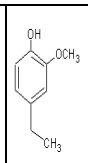
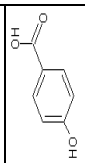
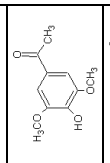
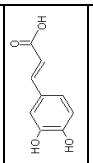
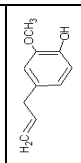
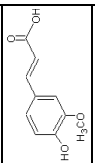
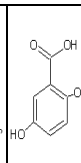
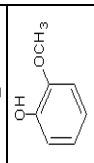
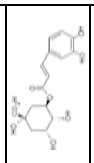
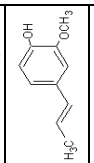
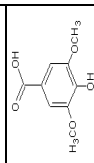
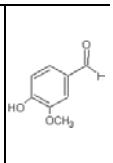
13 phenolic substances were measured for antioxidant capacity using the FRAP and DPPH methods. All of the results are expressed in the values of TEAC (Trolox Equivalent Antioxidant Capacity) when the values for each of the phenols obtained by measurement were converted to the exact concentration of 20.0 mg/l including the standard Trolox solution, which thus represented the TEAC value of 79.9 µmol/l for both FRAP and DPPH method. The solutions of the individual phenolic substances were subsequently matched against this value that represents 100%.

DPPH method

TEAC values higher than Trolox were measured for 10 phenolic substances (the same as in the case of the FRAP method, in Figure 1 and Table 1): gentisic acid 269.2 µmol/l (336.9%), protocatechuic acid 222.0 µmol/l (277.8%), syringic acid 193.1 µmol/l (241.6%), coffee acid 183.0 µmol/l (229.0%), 4-ethylguaiacol 177.3 µmol/l (221.9%), eugenol 165.0 µmol/l (206.5%), ferulic acid 125.7 µmol/l

(157.3%), guaiacol 120.8 µmol/l (151.2%), chlorogenic acid 119.2 µmol/l (149.2%) and Isoeugenol 100.3 µmol/l (125.5%). Conversely, TEAC values lower than the solution of Trolox alone, 79.91 µmol/l (100%), were found for: vanillin 58.3 µmol/l (73.0%), 4-hydroxybenzoic acid 57.9 µmol/l (72.5%) and acetosyringone 56.9 µmol/l (71.2%).

Table 1 Tukey's honest significance test for phenols measured by DPPH in µmol/l. (One-way ANOVA: p < 0.05 (*); p < 0.01 (**); not significant p > 0.05 (n.s.), calculated from three measurements.

	 {1} Protocatechuic acid	 {2} 4-ethylguaiacol	 {3} 4-hydroxybenzoic acid	 {4} Acetosyringone	 {5} Coffee acid	 {6} Eugenol	 {7} Ferulic acid	 {8} Gentisic acid	 {9} Guaiacol	 {10} Chlorogenic acid	 {11} Isoeugenol	 {12} Syringic acid	 {13} Vanillin
{1}		n.s.	**	**	**	**	**	**	**	**	**	**	**
{2}	0.9914		**	**	**	**	**	**	**	**	**	**	**
{3}	0.0001	0.0001		n.s.	**	**	**	**	**	**	**	**	n.s.
{4}	0.0001	0.0001	1.0000		**	**	**	**	**	**	**	**	n.s.
{5}	0.0002	0.0002	0.0001	0.0001		n.s.	**	**	**	**	**	n.s.	**
{6}	0.0067	0.0005	0.0001	0.0001	0.8256		**	**	**	**	**	n.s.	**
{7}	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001		**	**	n.s.	n.s.	**	**
{8}	0.0001	0.0002	0.0001	0.0001	0.0001	0.0001	0.0001		**	**	**	**	**
{9}	0.0001	0.0001	0.0001	0.0001	0.0039	0.0002	0.0011	0.0001		**	**	**	**
{10}	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.9774	0.0001	0.0002		n.s.	**	**
{11}	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	1.0000	0.0001	0.0045	0.7758		**	**
{12}	0.0045	0.0003	0.0001	0.0001	0.8983	1.0000	0.0001	0.0001	0.0002	0.0001	0.0001		**
{13}	0.0001	0.0001	1.0000	1.0000	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	

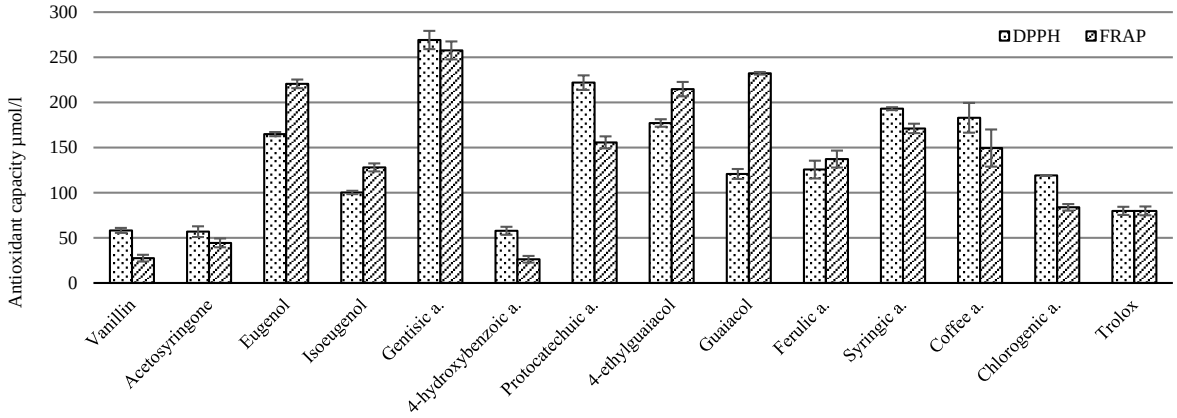
FRAP method

TEAC values higher than Trolox were measured for 10 phenolic substances (in Figure 1 and Table 2): gentisic acid 257.7 µmol/l (322.5%), guaiacol 232.3 µmol/l (290.7%), 4-ethylguaiacol 214.8 µmol/l (268.8%), eugenol 220.6 µmol/l (276.1%), syringic acid 171.2 µmol/l (214.2%), protocatechuic acid 155.8 µmol/l (195.0%), coffee acid 149.6 µmol/l (187.2%), ferulic acid 137.3 µmol/l (171.8%), Isoeugenol 128.0 µmol/l (160.2%) and chlorogenic acid 89.2 µmol/l (104.9%). Conversely, TEAC values lower than the solution of Trolox alone, 79.91 µmol/l (100%), were recorded in three phenolic substances: acetosyringone 44.5 µmol/l (55.7%), vanillin 27.6 µmol/l (34.5%) and 4-hydroxybenzoic acid 26.3 µmol/l (32.9%).

Table 2 Tukey's honest significance test for phenols measured by FRAP in µmol/l. One-way ANOVA: p < 0.05 (*); p < 0.01 (**); not significant p > 0.05 (n.s.), calculated from three measurements.

	{1} Protocatechuic acid	{2} 4-ethylguaiacol	{3} 4-hydroxybenzoic acid	{4} Acetosyringone	{5} Coffee acid	{6} Eugenol	{7} Ferulic acid	{8} Gentisic acid	{9} Guaiacol	{10} Chlorogenic acid	{11} Isoeugenol	{12} Syringic acid	{13} Vanillin
{1}		**	**	**	n.s.	**	n.s.	**	**	**	n.s.	n.s.	**
{2}	0.0002		**	**	**	n.s.	**	n.s.	*	**	**	**	**
{3}	0.0002	0.0002		n.s.	**	**	**	**	**	**	**	**	n.s.
{4}	0.0002	0.0002	0.2731		**	**	**	**	**	**	**	**	n.s.
{5}	1.0000	0.0002	0.0002	0.0002		**	n.s.	**	**	**	n.s.	n.s.	**
{6}	0.0002	0.5179	0.0002	0.0002	0.0002		**	n.s.	**	**	**	**	**
{7}	0.3514	0.0002	0.0002	0.0002	0.7657	0.0002		**	**	**	*	**	**
{8}	0.0002	0.3296	0.0002	0.0002	0.0002	1.0000	0.0002		**	**	**	**	**
{9}	0.0002	0.0328	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002		**	**	**	**
{10}	0.0002	0.0002	0.0002	0.0004	0.0002	0.0002	0.0002	0.0002	0.0002		**	**	**
{11}	0.9292	0.0002	0.0002	0.0002	0.5761	0.0002	0.0148	0.0002	0.0002	0.0002		n.s.	**
{12}	0.4020	0.0002	0.0002	0.0002	0.1234	0.0002	0.0014	0.0002	0.0002	0.0002	0.9984		**
{13}	0.0002	0.0002	1.0000	0.3694	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	

Figure 1 Antioxidant capacity of 20.0 mg/l



DISCUSSION

The measurement showed that the antioxidant capacity is influenced by a number of hydroxyl groups, their sites of binding in the aromatic ring, and also the number and position of other substituents bound to the aromatic ring. A similar argument is given in the 2016 article by Khan et al., where it is argued that methoxyl (-O-CH_3) and hydroxyl (-OH) substitution groups in the aromatic nucleus may affect the antioxidant potential as well as the capacity of phenolic substances. The next similar opinion is given in the 2004 article by Leopoldini et al., where it is argued that antioxidant activity of phenolic compounds seems to be related to their molecular structure and number of hydroxyl groups, and to their conjugation and resonance effects.

In the event that the two substances are similar in terms of structure, but differ only in terms of the type of the bound functional group, larger antioxidant capacity was observed for substances possessing a hydroxyl group (-OH) than those featuring a methoxyl group (-O-CH_3) (Svestkova 2018). In this case it involves coffee acid (TEAC: 149.6 µmol/l for FRAP and 183.0 µmol/l for DPPH) with a hydroxyl group that has larger capacity than ferulic acid (TEAC: 137.3 µmol/l for FRAP and 125.7 µmol/l for DPPH) with a methoxyl group; in the case of the FRAP method, however, no statistically significant difference was observed. The second example is protocatechuic acid (TEAC: 155.8 µmol/l for FRAP and 222.0 µmol/l for DPPH) with a hydroxyl group (-OH), which has larger capacity than vanillin (TEAC: 155.8 µmol/l for FRAP and 222.0 µmol/l for DPPH) with a methoxyl group (-O-CH_3); here, while there is a difference between more substituents (carboxylic acid vs. carbaldehyde), the theory is supported coined by Dacic and Gojak-Salimovic (2016) who indicate in their article that the larger the hydroxylation, the larger the antioxidant activity of phenolic substances. The substances measured by us possessed one or two hydroxyl groups in their aromatic ring; all of the substances possessing two groups featured larger antioxidant capacity than Trolox; this included the weakest substance, chlorogenic acid (TEAC: 89.2 µmol/l for FRAP and 119.2 µmol/l for DPPH); this acid would increase the larger antioxidant activity at the same concentrations in µM than in mg/l. This is because of its molecular weight of 354.31 g/mol – one roughly twice compared with the other antioxidants.

Similarly to the hydroxyl group showing increased antioxidant capacity compared to the methoxyl group, the carboxyl group (-COOH) of syringic acid (TEAC: 171.2 µmol/l for FRAP and 193.1 µmol/l for DPPH) showed some increased levels than it was seen for the acetyl group (-CO-CH_3) of acetosyringone (TEAC: 44.5 µmol/l for FRAP and 56.9 µmol/l for DPPH); between these two substances there were almost fourfold differences in either of the methods. In the case of the DPPH method, the ethyl group (-CH_2-CH_3) in the aromatic nucleus too increased the antioxidant capacity compared with simple hydrogen (-H) as was seen on the example of 4-ethylguaiacol (TEAC: 214.8 µmol/l for FRAP and 177.3 µmol/l for DPPH) with an ethyl group compared with guaiacol (TEAC: 232.3 µmol/l for FRAP and 120.8 µmol/l for DPPH) with a hydrogen atom, when values increased by about half; for the FRAP method, however, values were actually unchanged.

Dacic and Gojak-Salimovic (2016) also found that in phenolic acids the antioxidant activity is influenced by the position of hydroxyl groups (-OH) within the framework of the carboxyl functional

group (-COOH), providing the example of hydroxybenzoic acid with the OH group in the *ortho* or *para* position toward the carboxyl group as a substance that does not show any strong antioxidant activity unlike *meta*-hydroxybenzoic acid which does show the activity (Dacic and Gojak-Salimovic, 2016). In the case of gentisic acid and protocatechuic acid measured by us, the substances that possess the same molecular formulas (C_7H_{64}) and one hydroxyl group in the *meta* position, gentisic acid is the one that has larger antioxidant capacity (TEAC: 257.7 µmol/l for FRAP and 269.2 µmol/l for DPPH) than protocatechuic acid (TEAC: 155.8 µmol/l for FRAP and 222.0 µmol/l for DPPH). In these two acids, the second hydroxyl group is in the *ortho* position for gentisic acid, or, in the *para* position for protocatechuic acid. It follows from this that antioxidant capacity is influenced by not only the *ortho* vs. *meta* factor, i.e., different positions in terms of the reaction aspect (Gulcin 2012); differences are even seen between the *ortho* and *para* positions, i.e. those that are similar in terms of the standard reaction aspect. The same is argued also in the 2013 article by Bendary et al., where it was discovered that the ortho position was more active on ethen para and meta positions of compounds.

Kanski et al. (2002) found in his measurement that ferulic acid (with a larger conjugated system of double bonds) has much stronger antiradical properties against DPPH radicals than observed for vanillin acid. Through our measurements, similar results were achieved, i.e., that ferulic acid (TEAC: 137.3 µmol/l for FRAP and 125.7 µmol/l for DPPH) has much larger antioxidant capacity than vanillin – a derivative close to vanillic acid (TEAC: 27.6 µmol/l for FRAP and 58.3 µmol/l for DPPH) that featured similarly low TEAC values as did 4-hydroxybenzoic acid (TEAC: 26.3 µmol/l for FRAP and 57.9 µmol/l for DPPH). Results for coffee acid (TEAC: 149.6 µmol/l for FRAP and 183.0 µmol/l for DPPH) instead show, compared to those achieved for protocatechuic acid (TEAC: 155.8 µmol/l for FRAP and 222.0 µmol/l for DPPH) that while the lesser antioxidant conjugated system has slightly higher TEAC values, a statistically insignificant difference was observed in the case of the FRAP method. Zhang et al. (2017) who compared eugenol and isoeugenol using the DPPH and FRAP methods reports that isoeugenol with a larger conjugated system had a larger efficiency (activity) than eugenol. On the contrary, Bortolomeazzi et al. (2010) found in his measurements that eugenol exhibited larger capacity of capturing DPPH radicals than was true for isoeugenol. Ito et al. (2005), describing the capturing of the DPPH radical at various concentrations, came to the conclusion that there is a very little difference between eugenol and isoeugenol in terms of activity (while isoeugenol was a more efficient substance at a concentration of 50 µM, eugenol took its place from the concentration of 70 µM onwards). The solutions of the concentration of 20 mg/l that we measured correspond to 122 µM of eugenol (TEAC: 220.6 µmol/l for FRAP and 165.0 µmol/l for DPPH) and 122 µM of isoeugenol (TEAC: 128.0 µmol/l for FRAP and 100.3 µmol/l for DPPH), correlating with their antioxidant capacity levels with the observation performed by Ito et al. (2005) where eugenol showed larger antioxidant capacity than isoeugenol.

CONCLUSION

The antioxidant capacity of the phenols, their concentration being 20 mg/l for each, was calculated as a percentage of the effect compared with Trolox (which accounted for 100%) using the FRAP and DPPH method. The differences in measurements between the FRAP and DPPH methods were caused due to the various principles of the methods used. Antioxidants that had larger antioxidant capacity when measured by the FRAP method rather than by the DPPH technique were based on the structure of guaiacol and its derivatives in position 4: eugenol, isoeugenol, 4-ethylguaiacol and ferulic acid, except vanillin. Instead, most acids (gentisic acid, 4-hydroxybenzoic acid, protocatechuic acid, syringic acid, coffee acid and chlorogenic acid) had, along with acetosyringone and vanillin, a lower FRAP value than the DPPH value.

On average, the antioxidant capacity exhibited by substances with 2 hydroxyl groups (gentisic acid, protocatechuic acid, coffee acid and chlorogenic acid), with the average effect of 248% Trolox when using the DPPH method and 202% Trolox when using the FRAP method, was larger than that showed by substances possessing just one such group (vanillin, acetosyringone, eugenol, isoeugenol, 4-ethylguaiacol, guaiacol, ferulic acid, syringic acid and 4-hydroxybenzoic acid), with the average effect of 147% Trolox when using the DPPH method and 167% Trolox when using the FRAP method. Furthermore, a difference was observed between *ortho* and *para* positions of hydroxyl groups where gentisic acid with an *ortho* position was found to possess larger antioxidant capacity, whatever

of the two methods was used (323% or 337%), than protocatechuic acid with a *para* position (195% and 279% observed using both of the methods). Using the DPPH method, hydroxyl groups were observed to possess larger antioxidant capacity than methoxyl groups when coffee acid had a greater effect than Trolox (229%) with its hydroxyl group than ferulic acid with a methoxyl group (157%). A similar effect was also observed for a carboxyl group over an acetyl group using either of the two methods where syringic acid (214% and 242%) had roughly a fourfold effect than acetosyringone (56% and 71%) compared with Trolox. The effect of a larger conjugated system on the antioxidant capacity was not clear, as pointed out by the literature as well; for any comparison to be exhaustive, various concentrations would be necessary for measuring while our measurement was focused only on antioxidant solutions of 20 mg/l.

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