MODELING AND STATISTICAL ANALYSIS OF DPPH SCAVENGING ACTIVITY OF PHENOLICS

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Investigations addressing correlation between antioxidant activity and theoretical descriptors are plentiful in the literature. This task is quite ambitious, bearing in mind the rather complicated interactions in living cells. In this study we have tried to simplify the problem by looking for direct correlations between calculated characteristics and scavenging activity, neglecting the specificity of cellular environment. To address the problem of antioxidant activity, a set of 20 phenolic compounds and their phenoxyl radicals were investigated at the unrestricted B3LYP level of theory using the 6-31+G(d,p) basis set. Three important descriptors of the considered compounds were related to the results of the DPPH scavenging activity. Significant linear correlations were obtained in several cases.

Keywords: Phenolic antioxidants; DFT/B3LYP; DPPH scavenging activity; QSAR; Regression analysis; Radical scavengers; Ab initio calculations; Polyphenols.

The function of antioxidants consists in scavenging active radicals ($R^{\bullet}$) generated in different ways in higher organisms. Thus they prevent undesired chemical decays in cells and the development of diseases like cancer, atherosclerosis, inflammations, etc.[1,2].

The scavenging reaction of phenolic antioxidants can be illustrated by Scheme 1.

$$Ar\text{–}OH + R^{\bullet} \longrightarrow Ar\text{–}O^{\bullet} + R\text{–}H$$

SCHEME 1
Reaction between phenolic antioxidants and active radicals

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The parent antioxidant molecule transforms into a radical which has no capacity for initiating unwanted chain radical processes. Consequently, this radical takes part in trapping active radicals called "second radical scavenging"[3] (Scheme 2).

Scheme 2
Second radical scavenging reaction

The reaction mechanism of the first step is arguable; hence, various alternatives are discussed in the literature[4–7]: direct H-atom transfer, electron transfer followed by proton transfer, sequential proton loss and electron transfer, etc.

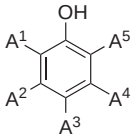
In fact, the antioxidant efficiency is determined by the rate and the conversion degree of these reactions and all experimentally determinable and theoretically computable descriptors of antioxidant/scavenging activity originate from kinetics and conversion data. They can be divided into the following groups: (i) indices estimating O–H bond strength – bond dissociation enthalpy[8] (BDE) or structural parameters – bond length, charge distributions etc.; (ii) indices presenting molecular electron-donation capacity – ionization potential or its theoretical analogue – HOMO energy and (iii) indices showing the stability of the obtained phenoxyl radicals (Ar–O•) – spin distribution, C–O bond length in the radicals, etc.[9].

Correlations between antioxidant activity and theoretically or experimentally derived descriptors belonging to the types mentioned above are usually obscured[10] by the dependence of the antioxidant activity on additional factors such as their permeability into the cell (lipophilicity), coordination ability, and resistance to enzymatic degradation[11]. Therefore, a preceding analysis of correlation between the main descriptors and scavenging activity is more meaningful.

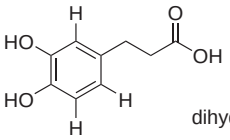
The establishment of relations between basic descriptors and antioxidant/scavenging activity is also hindered due to the different reaction mechanisms in operation. In polar solvents a more probable reaction mechanism is the two-step electron–proton transfer rather than one-step hydrogen atom abstraction. In such a case the proper descriptor is not the O–H BDE but the ionization potential (or HOMO energy) of the molecule[4–7].

The present work has the following objectives: to create sufficiently reliable quantum-chemical models for a series of natural antioxidants and their phenoxyl radicals; to compare their ability to react with DPPH

(1,1-diphenyl-2-(2,4,6-trinitrophenyl)hydrazyl) according to the calculated structural indices of antioxidant/scavenging activity, and finally, to summarize the most general features of the effective DPPH scavengers. Structural formulae of the investigated compounds are given in Figs 1–4.

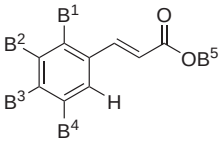


BHT, $A^1 = A^5 =$ *tert*-But; $A^3 =$ Me; $A^2 = A^4 = H$
BHA1, $A^2 =$ *tert*-But; $A^3 =$ OMe; $A^1 = A^4 = A^5 = H$
BHA2, $A^1 =$ *tert*-But; $A^3 =$ OMe; $A^2 = A^4 = A^5 = H$
TBHQ, $A^2 =$ *tert*-But; $A^3 =$ OH; $A^1 = A^4 = A^5 = H$

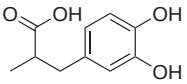


dihydrocaffeic acid

FIG. 1
Structural formulae of phenolic derivatives and dihydrocaffeic acid



cinnamic acid, $B^1 = B^2 = B^3 = B^4 = B^5 = H$
o-coumaric acid, $B^1 =$ OH, $B^2 = B^3 = B^4 = B^5 = H$
m-coumaric acid, $B^2 =$ OH, $B^1 = B^3 = B^4 = B^5 = H$
p-coumaric acid, $B^3 =$ OH, $B^1 = B^2 = B^4 = B^5 = H$
ferulic acid, $B^2 = OCH_3$, $B^3 =$ OH, $B^1 = B^4 = B^5 = H$
isoferulic acid, $B^2 =$ OH, $B^3 = OCH_3$, $B^1 = B^4 = B^5 = H$
cafeic acid, $B^2 = B^3 =$ OH, $B^1 = B^4 = B^5 = H$
sinapic acid, $B^2 = B^4 = OCH_3$, $B^3 =$ OH, $B^1 = B^5 = H$

rosmarinic acid, $B^3 = B^4 =$ OH, $B^1 = B^2 = H$, $B^5 =$ 

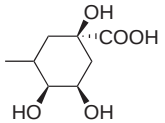
chlorogenic acid, $B^2 = B^3 =$ OH, $B^1 = B^4 = H$, $B^5 =$ 

FIG. 2
Structural formulae of cinnamic acid derivatives

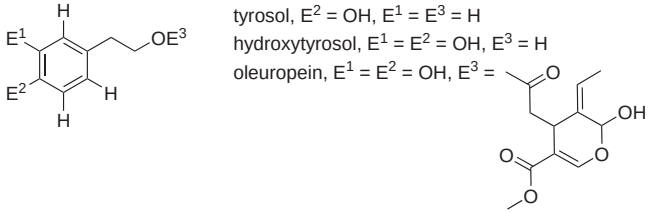


FIG. 3
Structural formulae of tyrosol derivatives

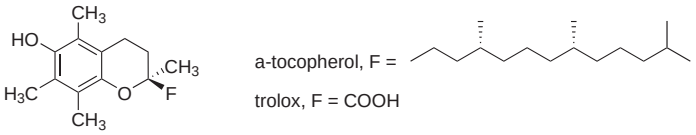


FIG. 4
Structural formulae of α-tocopherol and trolox

METHODS

A set of 20 phenolic antioxidants have been selected for the purpose of the study, their scavenging activity versus DPPH carefully explored[12] and expressed as the relative scavenging activity (% RSA) for 20-min reaction periods and antioxidant concentration of 0.25 relative to that of DPPH in mol/mol. The investigation of scavenging activity was made in ethanol (except in the cases explicitly specified).

Full geometry optimization was performed by the unrestricted B3LYP method[13] using Gaussian 03 program package[14] and 6-31+G(d,p) orbital basis set[15]. It was found that the DFT methods (and in particular unrestricted B3LYP functional) give more reliable results for the BDE of O–H [16]. The utilization of other post-HF methods is rather costly. Further extension of the orbital basis usually does not improve the results[17].

All available intramolecular hydrogen bonds have been taken into account in the initial geometry generation. In the case of two hydroxy groups only the dissociation of *para*-hydroxy group was considered in the formation of corresponding radicals.

The regression analysis was performed using the MS Excel program package.

RESULTS AND DISCUSSIONS

The values of three frequently used descriptors were obtained: (i) energy of the highest occupied molecular orbital (HOMO) of the compounds, (ii) C–O bond length and (iii) atomic spin density at the oxygen atoms in the radicals.

These descriptors can be treated as random variables. We have tested the hypotheses of linear correlation between the scavenging activity of the phenolic compounds (dependent variable) and each of the three parameters (independent variables). The obtained values of the descriptors (i)–(iii) and of the scavenging activity (% RSA) are presented in Table I.

TABLE I
Scavenging activities of phenolic antioxidants and their parameters

No.	Compound[a]	RSA, % (SA)	HOMO energy, eV (*E*)	C–O length, Å (*BL*)	Spin density at O atom (*SD*)
1	dihydrocaffeic acid	93.9	–0.2221	1.2601	0.3461
2	rosmarinic acid	88.4	–0.2212	1.2523	0.2856
3	caffeic acid	76.6	–0.2291	1.2843	0.3801
4	chlorogenic acid	52.0	–0.2270	1.2528	0.2869
5	sinapic acid	56.1	–0.2238	1.2453	0.3151
6	ferulic acid	30.9	–0.2226	1.2515	0.3159
7	isoferulic acid	3.5	–0.2243	1.2510	0.3754
8	*p*-coumaric acid	3.6	–0.2336	1.2497	0.3350
9	hydroxytyrosol	57.0	–0.2195	1.2603	0.3446
10	oleuropein	41.3	–0.2218	1.2599	0.3405
11	tyrosol	2.7	–0.2256	1.2594	0.4049
12	α-tocopherol	54.0	–0.1927	1.2603	0.3511
13	trolox	53.4	–0.1975	1.2597	0.3552
14	TBHQ	58.7	–0.2099	1.2588	0.3621
15	BHA1	22.3	–0.2064	1.2598	0.3794
16	BHA2	22.3	–0.2058	1.2592	0.3577
17	BHT	8.0	–0.2294	1.2575	0.3476
18	*o*-coumaric acid	3.5	–0.2376	1.2518	0.3698
19	*m*-coumaric acid	2.6	–0.2421	1.2597	0.4234
20	cinnamic acid	0.5	–0.2495	1.2706	0.5507

[a]For structures, see Figs 1–4.

The regression equations of the variable % RSA (*SA*) on the variables HOMO energy (*E*), C–O bond length (*BL*) and spin density (*SD*), respectively, are

$$SA = 896.6E + 235.7, \quad r_{SA,E} = 0.4146, \quad s = 28.65, \tag{1}$$

$$SA = 653BL - 786.2, \quad r_{SA,BL} = 0.1766, \quad s = 30.99, \tag{2}$$

$$SA = -277.4SD + 136.8, \quad r_{SA,SD} = 0.5088, \quad s = 27.10. \tag{3}$$

Here *s* denotes the standard deviation of regression.

The values of the sample correlation coefficients *r* in Eqs (*1*) and (*2*) are lower than the critical value of $r_{0.95}^{20} = 0.444$ for the set size 20 at 95% confidence level. Therefore, there is not sufficient evidence to support the hypotheses of significant linear correlation between the scavenging activity *SA* and the independent variables *E* and *BL*. The correlation coefficient in Eq. (*3*) is higher than $r_{0.95}^{20}$. Therefore, a significant linear correlation exists only between the scavenging activity and the spin density. The spin density describes the radical stability and hence the reaction conversion degree. Therefore, the linear correlation suggests that better spin density delocalization in the radicals is a necessary condition for the efficacy of the scavengers.

In addition, a significant linear correlation between the C–O length and spin density has been found: $BL = 0.0784SD + 1.23$, $r_{BL,SD} = 0.05326$, $s = 0.01$, which eliminates the C–O bond length as independent variable. The details of the regression analysis are given in the Appendix.

On the other hand, the low correlation with the HOMO energy suggests that there probably exist features important for the scavenging reaction which have not been taken into account or mechanisms other than electron–proton transfer are operative.

In compounds with two O–H groups the one situated in *para*- or *ortho*-position to the main substituent in the aromatic rings is considered to be an easier dissociable hydroxy group (DHG). All values for the C–O bond lengths and spin densities on oxygen presented in Table I are for the radicals obtained from DHG. The first reason for choosing this group as an easier DHG is the difference in the activities and in the calculated spin densities of ferulic and isoferulic acid (see Table I). In the case of *para*-hydroxy group dissociation (in ferulic acid), the spin density is more favorable as the activity is almost ten times higher (see Table I). The second

reason follows from the comparison of the spin densities of BHA1 (3-*tert*-butyl-4-methoxyphenol) and BHA2 (2-*tert*-butyl-4-methoxyphenol) phenoxyl radicals. The radical stabilizing effect of the *tert*-butyl group is more favorable when it is placed in *ortho*- than in *meta*-position.

The second hydroxy group in the aromatic ring can facilitate the dissociation of DHG by forming H-bonds with it or can be involved in the reaction with active radicals although at lower rate than DHG.

The comparison of % RSA of *p*-coumaric and caffeic acid shows that the latter is by an order of magnitude more active than the former. This can be due to the intramolecular hydrogen bond. The spin density at the O-atom in the radical of the *p*-coumaric acid is lower (0.335) than that of the caffeic acid (0.380). According to this index, the former should be more active but the experimental values of the activities are reverse. More illustrative for the role of the second hydroxy group is a comparison of activities of tyrosol (2.7% RSA) and hydroxytyrosol (57.0% RSA). This difference is too big to be explained by insignificant differences in HOMO energies (0.006 eV) or in spin densities (0.060).

A general view on the scavenging activities of the compounds shows that the four most active compounds have two hydroxy groups located at adjacent (*ortho*) positions. The next two compounds, namely sinapic and ferulic acids, have one hydroxy and one methoxy groups placed in adjacent positions.

These findings together with the comparisons of *p*-coumaric and caffeic acid, and tyrosol and hydroxytyrosol show that the participation of DHG in hydrogen bonds is of crucial importance for the potential of scavengers. Besides, more efficient should be the H-bond between oxygen from DHG and hydrogen from the neighbouring hydroxy group. The other pattern of H-bonding, between the hydrogen of DHG and the oxygen of the neighbouring methoxy group (which is the only possible in sinapic and ferulic acids), is not so effective.

The conclusion that the H-bond involving the oxygen of DHG is more important in the reaction process is supported by the comparison of % RSA values obtained in different solvents. The activities of the investigated compounds are higher in ethanol than in acetonitrile[12]. Ethanol can form both types of H-bonds, whereas acetonitrile produces H-bonds only with the hydrogen of DHG.

Therefore, a possible reason for the lack of linear correlation between the scavenging activity of the investigated compounds and the HOMO energy is the presence of two types of compounds (with one hydroxy group and with two adjacent hydroxy groups) in the set of compounds under study.

In an attempt to find a significant linear correlation between the scavenging activity and HOMO energy, a new subset (Subset 1) has been formed in which the compounds with two *ortho*-positioned hydroxy groups were not included. A similar approach has been suggested in ref.[10].

Subset 1 consists of the compounds numbered 5–8, 11–15, 17–19 in Table I. The regression analysis gives the following correlation between the scavenging activity and the energy of HOMO

$$SA = 1174E + 283.7, \quad r_{SA,E} = 0.7567, \quad s = 16.62. \qquad (4)$$

The correlation coefficient is 0.7567, which is higher than the critical value $r^{12}_{0.95} = 0.576$. This result allows us to make the conclusion that there is a significant linear correlation between these two variables. The smaller the absolute value of the HOMO energy, the higher the scavenging activity. The correlation suggests the electron-transfer mechanism in the rate-determining stage of the reaction between the investigated scavengers and DPPH in Subset 1. In addition, it underlines the importance of reaction kinetics for the efficiency of scavengers and suggests that electron–proton transfer is the preferred mechanism in Subset 1.

The presence of alkyl (methyl or *tert*-butyl) and methoxy substituents in aromatic rings usually increases their ability to donate electrons. The compounds which possess such substituents have the lowest HOMO energy (see Table I). On the other hand, the compounds with the lowest HOMO energies are not the most active against DPPH; the reason is probably not only the absence of H-bonds but also in the steric reasons (Fig. 5). Probably the steric requirements of DPPH could not be satisfied by antioxidants with

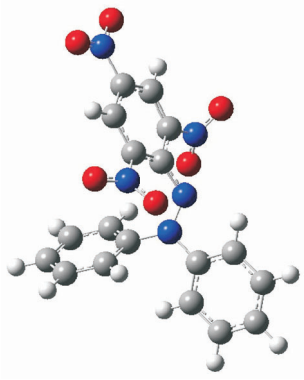


FIG. 5
Optimized structure of DPPH radical (C, dark grey; H, light grey; N, blue; O, red)

bulky substituents around the DHG. The spin density in DPPH is concentrated at one of the nitrogen atoms, which is sterically hindered and probably this is the reason for the discrepancy between the HOMO energy and scavenging activity.

In order to evaluate the influence of the neighbouring bulky substituents (if any) in the next subset (Subset 2), only the compounds without methyl and *tert*-butyl groups in the *ortho*-position to DHG were included, namely the compounds 1–4, 8–11, 14, 15, 19, 20. In Subset 2 are included both compounds with one and with two neighbouring hydroxy groups.

Correlation with HOMO energy is still not significant. The correlation coefficient 0.4672 has been found and it is again lower than the critical value needed to support the significance of the linear correlation. This allows us to reject the idea of the influence of bulky substituents on the rate-determining step of the reaction. Such a statement is valid for compounds with methyl groups in *ortho*-position to the DHG, but has not been checked for compounds with *tert*-butyl groups because of the insufficient number of such compounds.

The new equation for the dependence of scavenging activity on spin density has almost the same significance as before the exclusion of the compounds with bulky substituents. For these compounds the regression analysis gives the following correlation

$$SA = -293.6SD + 150.2, \quad r_{SA,SD} = 0.5916, \quad s = 29.41. \qquad (5)$$

The correlation coefficient 0.5927 is higher than the critical value $r^{12}_{0.95}$. This result does not increase the degree of significance of the correlation for Subset 2 in comparison with all the compounds. Hence, the absence of methyl groups near DHG does not change the radical stability and its influence on the reaction conversion degree is negligible.

CONCLUSIONS

This study presents results of a statistical approach to the relation between the DPPH scavenging activity and some of the most popular descriptors of the antioxidant/scavenging activity for a series of natural antioxidants. Analysis of the obtained results shows that there exists a significant linear correlation between the scavenging activity and the spin density.

Similar significant relations between the scavenging activity and HOMO energy have been found after appropriate classification of the compounds according to the presence of a second hydroxy group.

The following requirements for prediction of scavenging activity towards DPPH can be formulated from the obtained results:

1) The availability of O–H group included in the aromatic system. This requirement comes from the strong dependence of the scavenging activity on spin density delocalization. Unquestionable evidence that the extension of the conjugated system leads to higher scavenging activity has not been found for the considered group of compounds. It can be assumed that the presence of a benzene ring is a sufficient condition for the activity.

2) The occurrence of substituents with positive mesomeric and inductive electronic effects. These substituents increase reductive properties of the compounds. Alkyl and mesomeric methoxy groups possessing positive inductive effects have such function. The role of the carboxyvinyl substituent in derivatives of cinnamic acid, coumaric acids, etc., is probably negative from this point of view but it should be positive according to the first requirement. The role of the second ring in the molecules containing chromane rings like α-tocopherol and trolox can be interpreted in a similar way.

3) The presence of hydrogen bonds involving DHG and adjacent functional groups. Particularly effective is the hydrogen bond of the DHG oxygen and the hydrogen from a neighbouring hydroxy group.

APPENDIX

The modified χ^2 criterion has shown that the random variables *SA*, *E*, *BL* and *SD* are normally distributed. The population correlation coefficients ρ are defined as usual[18]: for instance, for variables *E* and *SA* by equation $\rho_{SA,E} = M((E-\mu_E)(SA-\mu_{SA}))/\sigma_E\sigma_{SA}$. Here *M* denotes the expected value operator, σ the standard deviation and μ the mean of the respective variables. We have tested the null hypothesis H_0:|ρ| = 0 against the alternative hypothesis H_1:|ρ| ≠ 0. The critical value for a set of 20 members at 95% confidence level is $r_{0.95}^{20}$ = 0.444 (cf. ref.[18]). The values of the sample correlation coefficients $r_{SA,E}$ and $r_{SA,BL}$ are lower than $r_{0.95}^{20}$ (cf. Eqs (*1*) and (*2*)). Therefore, the test has failed to reject the null hypothesis and there is no sufficient evidence of a significant linear correlation between *SA* and *E* (or *SA* and *BL*).

The value of $r_{SA,SD}$ is higher than $r_{0.95}^{20}$, which allows to reject H_0 and to conclude that there is a significant linear correlation between *SA* and *SD* (cf. Eq. (*3*)).

The considered subsets of compounds, possessing one hydroxy group (Subset 1) or without bulky substituents (Subset 2), consist of 12 members each. The critical value for the set size 12 at 95% confidence level is $r_{0.95}^{12}$ = 0.576 (cf. ref.[18]). The values of the sample correlation coefficients in Eqs (*4*)

and (*5*) are higher than $r^{12}_{0.95}$ and therefore the null hypotheses are rejected for these two subsets. These results support the conclusion that there is a significant linear correlation in these cases.

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