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The relationship between antioxidant activity, first electrochemical oxidation potential, and spin population of flavonoid radicals

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I have shown that by averaging antioxidant activity (AA) values measured by different methods it is possible to obtain an excellent correlation (R^2=0.960) between the first electrochemical oxidation potential, E_{p1}, and AA. Separate correlations using the AA values obtained with each of the four methods [R^2 were 0.561 for diphenyl-1-picrylhydrazyl (DPPH), 0.849 for Folin Ciocalteu reagent (FCR), 0.848 for the ferric-reducing ability of plasma (FRAP), and 0.668 for the Trolox equivalent antioxidant capacity (TEAC)] were all worse, and in some cases not useful at all, such as the one for DPPH. Also, the sum of atomic orbital spin populations on the carbon atoms in the skeleton of radicals ($\sum_{s(C)} AOSP_{Rad}$), calculated with the semi-empirical parameterisation method 6 (PM6) in water, was used to correlate both E_{p1} and AA, yielding R^2=0.926 and 0.950, respectively. This showed to be a much better variable for the estimation of E_{p1} and AA than the bond dissociation energy (BDE), R^2=0.854 and 0.901 for E_{p1} and AA, respectively, and especially the ionisation potential (IP), R^2=0.445 and 0.435 for E_{p1} and AA, respectively.

KEY WORDS: DFT; oxidation potential; PM6; polyphenols; radical scavenging

Antioxidant activity (AA) of flavonoids, a large group of polyphenolic secondary plant metabolites, has been exhaustively studied over the past few decades. Since the French paradox (1), vast amounts of studies were published on their protective effect regarding many diseases caused by oxidative stress such as neurodegenerative diseases (2), diabetes (3), cardiovascular diseases (4), cancer (5), and allergies (6). Recent research has also revealed other modes of flavonoid action (7, 8), but their protective effect has mostly been associated with direct radical (oxygen and nitrogen species) scavenging ability stemming from the ability of the resulting phenoxyl radicals to remain stable. This stability depends on the number and spatial relations between phenolic hydroxyl groups in the molecule, i.e. their electronic structure (9–18).

Several authors have suggested that AA should correlate with the first electrochemical oxidation potential (E_{p1}) (9, 10, 19, 20), but Tabart et al. (21) were the first to propose averaging of AA values obtained by different methods (whose different mechanisms may yield different results) to make correlations informative.

Zhang et al. (22) measured AAs for a set of 14 flavonoids using four methods: diphenyl-1-picrylhydrazyl (DPPH), Folin Ciocalteu reagent (FCR), ferric reducing ability of plasma (FRAP), and Trolox equivalent antioxidant capacity (TEAC). To show how good the correlation between flavonoid E_{p1} and AA actually is, I took their measurements (Table 1). I also wanted to check the correlation between flavonoid AAs and atomic orbital spin populations over the skeleton atoms of a radical molecule ($\sum_{s(C)} AOSP_{Rad}$), as $\sum_{s(C)} AOSP_{Rad}$ showed excellent correlation with E_{p1} (15). For that purpose I performed PM6 calculations in water to optimise the geometries of the 14 flavonoids and their cations and radicals.

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THEORETICAL METHODS

MOPAC calculations

The geometries of the 14 flavonoids and their cations and radicals in water were optimised using the MOPAC2016™ PM6 method (23). All of the initial structures were taken as planar. The eigenvector following (EF) optimisation procedure was carried out with a final gradient norm under 0.01 kcal/mol/Å. This approach was used for all of the studied structures.

Regression calculations

Regression calculations, including the leave-one-out procedure (LOO) of cross validation were calculated with the CROMRsel program (24). The standard error of the cross-validation estimate was defined as:

$$SE_{cv} = \sqrt{\sum_i \frac{\Delta X_i^2}{N}} \quad (1)$$

where ΔX and N denote cv residuals and the number of reference points, respectively.

Table 1 First oxidation potential, E_{p1}, for 14 flavonoids at pH=7, active sites (A), antioxidant activities measured by four methods (TEAC, FCR, FRAP, and DPPH) and their relative mean values, bond dissociation energies (BDE) and ionisation potentials (IP) calculated using PM6 and DFT, and the sum of atomic orbital spin populations on skeleton atoms ($\sum_{s(C)} AOSP_{Rad}$) calculated using PM6

Flavonoid	E_{p1}/V (pH 7)[a]	A site[b]	AAs measured by spectrophotometric assays[a]				Relative AA mean[b]	BDE(DFT)[a] kJ/mol	IP(DFT)[a] kJ/mol	BDE(PM6)[b] kJ/mol	IP(PM6)[b] kJ/mol	$\sum_{s(C)} AOSP_{Rad}$[b]
			TEAC	FCR	FRAP	DPPH						
Quercetin	0.202	4'	5.72	1.24	5.57	2.25	1	299.3	553.8	296.9	357.4	0.519
Myricetin	0.119	4'	4.54	1.02	4.15	1.89	0.76	308	555.8	290.0	361.1	0.364
Isorhamnetin	0.116	4'	5.06	1.04	3.81	1.40	0.72	310.1	554.8	297.2	351.2	0.488
Kaempferol	0.194	3	4.19	1.08	3.15	0.83	0.58	334.4	557.7	309.8	362.0	0.659
Luteolin	0.261	4'	3.03	0.91	2.42	1.65	0.54	308	592.4	312.2	376.3	0.631
Apigenin	0.623	4'	2.83	0.39	-	-	0.21	342.2	603.0	342.0	402.9	0.792
Quercitrin	0.326	3'	3.28	0.81	2.92	1.65	0.55	309.1	579.9	313.5	325.0	0.638
Taxifolin	0.238	4'	4.23	0.99	3.54	1.35	0.64	313.6	606.9	302.5	363.6	0.605
Hesperetin	0.524	3'	2.01	0.53	0.83	0.48	0.16	348.2	586.6	306.8	356.0	0.642
Naringenin	0.710	4'	1.29	0.55	0.08	0.27	0.06	346.9	612.7	332.8	388.0	0.776
Daidzein	0.601	4'	2.43	0.40	0.20	-	0.12	339	586.6	321.6	368.3	0.793
Genistein	0.614	4'	3.50	0.33	0.27	-	0.18	337.8	576.0	322.6	370.8	0.793
Catechin	0.266	4'	3.75	0.77	3.12	1.78	0.59	306.5	575.1	305.4	360.2	0.608
Epicatechin	0.208	4'	3.84	1.05	3.38	1.64	0.66	303.1	575.1	305.4	360.2	0.608

[a] – values taken from Zhang et al. (22); [b] – values obtained in this study; DPPH – diphenyl-1-picrylhydrazyl; FCR – Folin Ciocalteu reagent; FRAP – ferric reducing ability of plasma; TEAC – Trolox equivalent antioxidant capacity

RESULTS AND DISCUSSION

As concluded by Zhang et al. (22), the methods for measuring AA are inconsistent and, their mutual correlations vary from r=0.694 to 0.941 for the given set of flavonoids (Table 2). Also, linear correlation between E_{p1} and AAs yielded correlation coefficients from r=0.749 (DPPH *vs.* E_{p1}) and 0.818 (TEAC *vs.* E_{p1}) to 0.921 (FCR or FRAP *vs.* E_{p1}).

I therefore tried averaging antioxidant activity values (21) using a somewhat different averaging method and expressed the AA values for every method by their relative values in the range from 0 to 1 with the formula:

$$\text{relative AA} = \frac{AA_i - AA_{min}}{AA_{max} - AA_i} \qquad (2)$$

where AA_i is the AA value of flavonoid i, and AA_{max} and AA_{min} are the maximal and minimal AA values obtained by the method. Then I calculated the relative mean of the AA value for every flavonoid using relative values of the four methods (Table 1). For illustration, quercetin had a relative AA mean value equal to 1 because its AA was the highest by all four methods.

Figure 1 clearly shows that E_{p1} has an excellent correlation with the relative AA mean, yielding r=0.980, SE=0.049, and SE_{cv}=0.057. Quercetin was dropped from the calculation because it showed an unusually high relative AA and lay far from the regression line (Figure 1). The same goes for separate AA linear correlations with E_{p1}; by dropping quercetin for the same reasons, all E_{p1} vs. AA dependences yielded better correlations (Table 2).

These results are even more important considering that the correlation between E_{p1} and relative AA mean is better than any separate AA correlation (Table 2) or any combination of two AA methods. Even the pair of the best separate AA correlations, i.e. average of FCR and FRAP values, yielded a slightly worse correlation, r=0.974.

The reason for quercetin to have such a high AA value measured by DPPH was explained by Foti (25), who has suggested that quercetin reacts with DPPH• radical so fast that it is consumed long before any conventional spectrophotometer can measure it. Moreover, quercetin quinone, a product of quercetin oxidation, also absorbs radiation at 519 nm, same as DPPH•. However, quercetin AA measured by all four methods was much greater than expected from its first oxidation potential. I therefore left

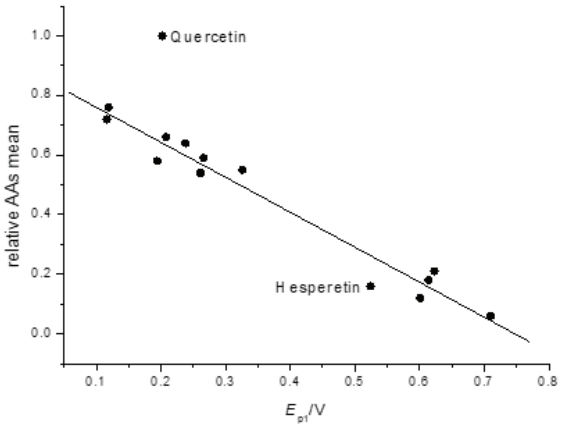

Figure 1 The dependence of relative AA mean on E_{p1} (pH=7) for the set of 14 flavonoids. Linear regression yielded R^2=0.960, SE=0.049, and SE_{cv}=0.057 after exclusion of quercetin

it out from the AA vs. E_{p1} regression for the set of flavonoids (Figure 1).

Furthermore, I correlated the $\sum_{s(C)} AOSP_{Rad}$ from our earlier study (15) to the first oxidation potential and relative AA mean of flavonoids. Namely, flavonoids more prone to oxidation have lower $\sum_{s(C)} AOSP_{Rad}$. This means they have lower quantity of unpaired electrons in the rings of their radicals, resulting with more balanced electronic structure (15). In order to optimise the radical molecule from which I further calculated the $\sum_{s(C)} AOSP_{Rad}$ of each flavonoid, I determined generally accepted (26, 27) active sites (A site, Table 1) using our earlier method (15).

When $\sum_{s(C)} AOSP_{Rad}$ was correlated to E_{p1}, the regression yielded SE=0.080 (Model 1, Table 3). As can be seen from Figure 2, hesperetin showed the highest residual from the fit line (0.201 V) and, compared to regressions using $\sum_{s(C)} AOSP_{Rad}$, it is by far the largest residual obtained of all the flavonoids we have studied (15). By leaving hesperetin out of the set, the regression yielded significantly better statistics, SE=0.055 (Model 2, Table 3). A similar result was obtained when I correlated $\sum_{s(C)} AOSP_{Rad}$ to the relative mean AAs. The regression yielded SE=0.054 (Model 7, Table 3, Figure 3) with hesperetin excluded from the calculation (in addition to quercetin).

Bond dissociation energy (BDE) and ionisation potential (IP), which I calculated using PM6 [BDE/IP(PM6)] (Table 1) as variables to correlate E_{p1} and AAs, showed worse statistics. Regressions for BDE yielded SE=0.102 (Model 3, Table 3). After the exclusion of

Table 2 Correlation coefficients, r, between the four methods and E_{p1}

Assay	TEAC	FCR	FRAP	DPPH	E_{p1}	E_{p1} (without Q)
TEAC	1	0.771	0.885	0.750	0.818	0.848
FCR		1	0.941	0.694	0.921	0.940
FRAP			1	0.859	0.921	0.976
DPPH				1	0.749	0.774
E_{p1}					1	1

Q – quercetin; DPPH – diphenyl-1-picrylhydrazyl; FCR – Folin Ciocalteu reagent; FRAP – ferric reducing ability of plasma; TEAC – Trolox equivalent antioxidant capacity

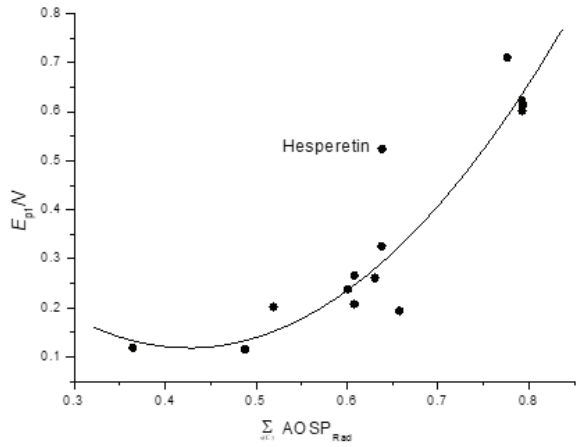

Figure 2 The dependence of experimental E_{p1} (pH=7) on $\sum_{s(C)}$ AOSP$_{Rad}$, calculated using the PM6 method for the set of 14 flavonoids. Quadratic regression yielded R^2=0.926, SE=0.055, and SE$_{cv}$=0.072 after exclusion of hesperetin (Model 2, Table 3)

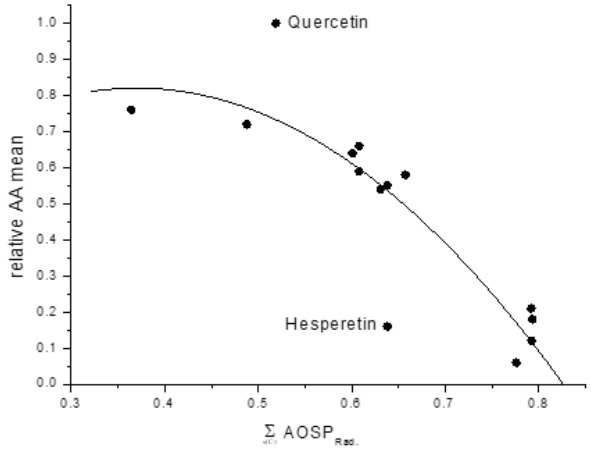

Figure 3 The dependence of experimental relative AA mean on $\sum_{s(C)}$ AOSP$_{Rad}$, calculated using the PM6 method for the set of 14 flavonoids. Quadratic regression yielded R^2=0.950, SE=0.054, and SE$_{cv}$=0.073 after exclusion of quercetin and hesperetin (Model 7, Table 3)

hesperetin – because it has the highest residual (0.224 V) and did not fit into regression – the statistics was much better, SE=0.083 (Model 4, Table 3, Figure 4). When BDE(PM6) was correlated with AAs, SE was 0.107 (after exclusion of hesperetin beside quercetin, Model 8, Table 3). IP(PM6) yielded SE=0.169 and 0.241 for the correlation with E_{p1} and AAs, respectively, which is much worse than BDE and especially $\sum_{s(C)}$ AOSP$_{Rad}$.

Linear regressions yielded results similar to the statistics obtained with BDE and IP calculated with density functional theory [BDE/IP(DFT)] (22) (Table 1). Regressions for BDE yielded SE=0.110 (Model 5, Table 3) when correlating E_{p1}. After the exclusion of kaempferol, because in this case kaempferol had the highest residual (0.284 V), the statistics was much better, SE=0.078 (Model 6, Table 3, Figure 5). When BDE(DFT) was correlated with AAs, the SE was 0.079 (after exclusion of kaempferol beside quercetin, Model 9, Table 3), which is much better than with BDE(PM6). IP(DFT) yielded SE=0.15 and 0.207 for correlation with E_{p1} and AAs, respectively, which was similar to IP(PM6).

Using together all experimental values for E_{p1} from Table 1 and those reported in reference 15, at pH=7, in regression with $\sum_{s(C)}$ AOSP$_{Rad}$ I obtained R^2=0.910, SE=0.074, and SE$_{cv}$=0.080 (N=28), and after the exclusion of hesperetin R^2=0.937, SE=0.062, and SE$_{cv}$=0.068 (Figure 6, N=27), which speaks in favour of the stability of $\sum_{s(C)}$ AOSP$_{Rad}$ as a variable for the estimation of the first oxidation potential. The results obtained are even more valuable since the average difference in E_{p1} for quercetin, myricetin, luteolin, apigenin, and epicatechin in Table 1 and those reported in reference 15 was 0.032 V, with maximal difference for epicatechin (0.045 V).

I can conclude that $\sum_{s(C)}$ AOSP$_{Rad}$ again proved as a promising variable for the estimation of the first oxidation potential (15) and consequently antioxidant activity. It is considerably better than BDE(PM6) or BDE(DFT) (Models

Table 3 Models for the estimation of the first oxidation potential, E_{p1}, and antioxidant activity, AA

	Model	Method	N	Slope (SE) Independent variable			Intercept (SE)	R²	SE	SE$_{cv}$
				$\sum_{s(C)}$ AOSP$_{Rad}$	($\sum_{s(C)}$ AOSP$_{Rad}$)²	BDE				
E_{p1}	1	PM6	14	–2.7(16)	3.4(13)	-	0.65(48)	0.844	0.080	0.092
	2	PM6	13	–3.6(12)	4.10(94)	-	0.89(35)	0.926	0.055	0.072
	3	PM6	14	-	-	0.0126(21)	–3.56(66)	0.746	0.102	0.121
	4	PM6	13	-	-	0.0130(18)	–3.71(55)	0.833	0.083	0.105
	5	DFT	14	-	-	0.0097(18)	–2.75(58)	0.703	0.110	0.127
	6	DFT	13	-	-	0.0106(13)	–3.03(42)	0.854	0.078	0.094
AA	7	PM6	12	4.1(11)	–4.80(93)	-	–0.11(33)	0.950	0.054	0.073
	8	PM6	12	-	-	–0.0151(24)	5.21(75)	0.802	0.107	0.142
	9	DFT	12	-	-	–0.0138(14)	4.87(47)	0.901	0.079	0.094

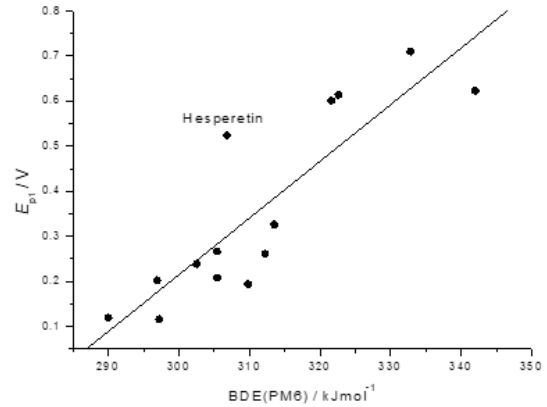

Figure 4 The dependence of experimental E_{p1} (pH=7) on BDE, calculated using the PM6 method for the set of 14 flavonoids. Linear regression yielded R^2=0.833, SE=0.083, and SE_{cv}=0.105 after exclusion of hesperetin (Model 4, Table 3)

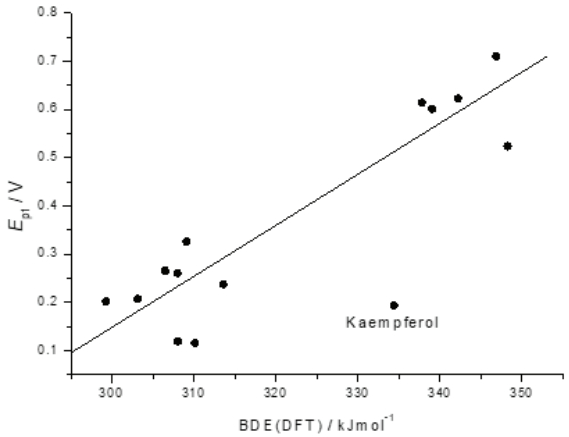

Figure 5 The dependence of experimental E_{p1} (pH=7) on BDE, calculated using the DFT method for the set of 14 flavonoids. Linear regression yielded R^2=0.854, SE=0.078, and SE_{cv}=0.94, after exclusion of kaempferol (Model 6, Table 3)

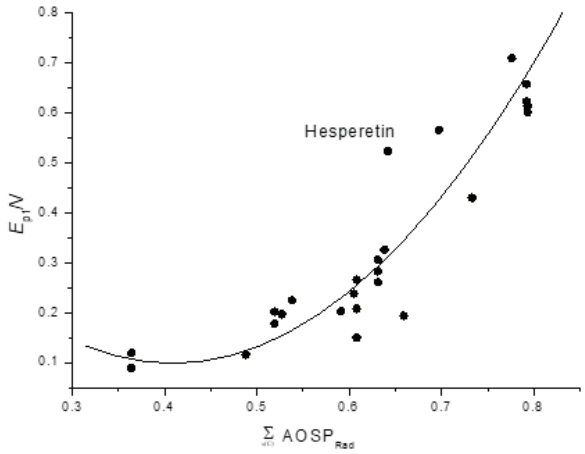

Figure 6 The dependence of experimental E_{p1} (pH=7) on $\sum_{s(C)}$ $AOSP_{Rad}$, calculated using the PM6 method for the set of 28 experimental E_{p1} values (Table 1 and ref. 15). Quadratic regression yielded R^2=0.937, SE=0.062, and SE_{cv}=0.068 after exclusion of hesperetin

1–6, Table 3), while IP should not be used as a variable for modelling either E_{p1} or AA.

Acknowledgments

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Odnos između antioksidacijskih aktivnosti, prvog elektrokemijskog oksidacijskog potencijala i spinskih populacija atomskih orbitala radikala flavonoida

U radu je pokazano da je usrednjivanjem vrijednosti antioksidacijskih aktivnosti (AA) mjerenih različitim metodama moguće dobiti izvrsno slaganje (R^2=0,960) između prvog elektrokemijskog oksidacijskog potencijala (E_{p1}) i AA. Pojedinačne korelacije AA vrijednosti dobivene svakom od četiriju metoda [R^2 je 0,561 za difenil-1-pikrilhidrazil (DPPH), 0,848 za Folin-Ciocalteu reagens (FCR), 0,848 za sposobnost plazme da reducira željezo (FRAP) i 0,668 za Trolox ekvivalent antioksidacijske aktivnosti (TEAC)] bile su lošije, a u nekim slučajevima potpuno nekorisne, kao što je primjerice korelacija s DPPH. Također, zbroj spinskih populacija atomskih orbitala ugljikovih atoma u skeletu molekule radikala ($\sum_{s(C)} AOSP_{Rad}$), izračunana semi-empirijskom parametrizirajućom metodom 6 (PM6) u vodi, korelirana je s E_{p1} i AA, dajući R^2=0,926 i 0,950. Pokazano je da je to puno bolja varijabla za procjenu vrijednosti E_{p1} i AA od energije disocijacije veze (BDE), R^2=0,854 i 0,901 za E_{p1} i AA, a naročito od ionizacijskog potencijala (IP), R^2=0,445 i 0,435 za E_{p1} i AA.

KLJUČNE RIJEČI: DFT; oksidacijski potencijal; PM6; polifenoli; uklanjanje radikala