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Quantitative elucidation of the molecular mechanisms of hydroxyl radical quenching reactivity of phenolic compounds

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Abstract

Reported discrepancies have confused the understanding of the molecular mechanisms of antioxidant reactivity somewhat. The consequent problems necessitate systematic investigations on the molecular orbital features of antioxidants and their correlation with antioxidant potentials. In the present work, phenolic compounds as typical antioxidants were selected to investigate their hydroxyl radical-scavenging properties, and the related mechanisms of action were studied theoretically by computational chemistry. A good correlation was observed between antioxidant activity and theoretical parameters, such as O–H bond dissociation energy (BDE), ionization potential (IP), enthalpy of electron transfer (E_a), chemical hardness (HOMO–LUMO gap), and spin delocalization of the phenoxyl radicals (D_s^r). The results demonstrate that the molecular mechanisms regulating the antioxidant action were more complex than hydrogen or electron-transfer processes and explain previous contradictions. Meanwhile, a satisfactory quantitative structure–activity relationship (QSAR) model was established which should be of predictive value in evaluating or screening hydroxyl radical-scavenging antioxidants.

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1. Introduction

The hydroxyl radical is the most reactive species derived from molecular oxygen, and reacts with organic compounds at near diffusion-controlled rates. This results in oxidative damage to DNA, amino acids, proteins, and membrane lipids. In previous works [1–7], the hydroxyl radical-scavenging potentials of phenolic compounds have been extensively investigated, but the exact molecular mechanisms of hydroxyl radical-quenching reaction have not been explored.

Some authors tried to identify the intermediates and products in order to determine the reaction pathway of hydroxyl radical with phenolics, but controversy arose [8,9]. A recent study performed by Khopde et al. [10] showed that one-electron oxidation of ferulic acid by hydroxyl radical first proceeded by an addition reaction, and then the radical addition products underwent water elimination to yield phenoxyl radicals. Similar results were reported by Dwibedy et al. [11], who proposed that phenolics undergo a one-electron oxidation to give phenoxyl type radical species. In fact, descriptors representing O–H bond dissociation energy (BDE) [12–15] and electron-donating properties [12,13, 16–19] have been obtained to prove at least in part the oxidation of phenolic acid.

However, some discrepancies have confused the explanation of the molecular mechanisms of hydroxyl radical-scavenging reactivity of antioxidants. The energy level of the highest occupied molecular orbital (E_{HOMO}), which determines the electron-donating ability according to Koopmans' theory, exhibited good correlation with the activity of tocopherolic antioxidant in some studies [12,16], but suffered serious fallibility in predicting the antioxidant activity of flavonoids that have intramolecular hydrogen bonds [17,18]. Further, E_{HOMO} can give a correct prediction of the activity difference between molecules with or without a meta-methyl group, but O–H BDE cannot [12]. On the other hand, some authors [14,15] confirmed the effectiveness of O–H BDE in predicting the activities of several series of antioxidants despite only one mechanism considered, and even concluded that the effectiveness of E_{HOMO} to explain antioxidant activity difference is a superficial phenomenon. These conflicting reports implied that the molecular mechanisms must be more complex in nature than hydrogen-abstraction or electron-transfer alone in governing the hydroxyl radical-quenching reactivity. This raises the questions of how and to what extent hydrogen- and electron-transfer processes regulate the reactivity of antioxidants. Moreover, how do they influence each other? Furthermore, what mechanisms may underlie the free radical reactions and how are they related to electron- and hydrogen-transfer processes? These questions prompted us to perform the present work so that new light could be shed on the mechanisms and resolve the controversy.

2. Materials and methods

2.1. Chemicals

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), caffeic acid (3,4-dihydroxycinnamic acid), and protocatechuic acid (3,4-dihydroxybenzoic acid) were purchased from Sigma Chemical (St. Louis, MO, USA). 2-Thiolbarbituic acid and 2-deoxy-D-ribose were obtained from Beijing Jingke Chemical (Beijing, China) and Fluka Chemie GmbH (Steinheim, Switzerland), respectively. All other chemicals were of analytical grade and were obtained from Beijing Chemical (Beijing, China). Oxygen-free de-ionized water was used to prepare all solutions.

2.2. Luminol-enhanced chemiluminescence assay

The chemiluminescent method has been widely used in assessing the antioxidant potential [20–24] based on the quenching of the chemiluminescence (CL) signal. Herein, we optimized the conditions of the CL reaction between Fenton's reagent and luminol by a LKB Wallac 1251 luminometer (Wallac Oy, Finland) with two dispensers. The reaction mixture contained, in a final volume of 1.0 mL, the following reagents at the final concentration stated: 6.0×10^{-4} mol/L Fe^{2+}–EDTA, 4.4×10^{-4} mol/L H_2O_2, and 2.0×10^{-4} mol/L luminol. The reaction was started by dispensing Fe^{2+}–EDTA and H_2O_2 into the mixture, and carried out in a KH_2PO_4–NaOH buffered solution (pH 7.4, 0.050 mol/L at final concentration) at room temperature (25 ± 1 °C). The dynamic curves were recorded immediately after initiation of the reaction.

2.3. Thiobarbituric acid reactive species (TBARS) assay on hydroxyl radical-scavenging activity

The deoxyribose method for antioxidant activity determination of the selected phenolic compounds was performed as described elsewhere [25,26] with slight modifications according to Jiang et al. [27] and Lopes et al [28]. Briefly, the reaction mixture contained, in a final volume of 1.0 mL, the following reagents at the final concentration stated: 2.8 mM 2-deoxy-D-ribose, 1.4 mM H_2O_2, 20 μM $FeCl_2$, and 100 μM EDTA without or with phenolic compounds in 10 mM KH_2PO_4–NaOH buffer (pH 7.4). EDTA and $FeCl_2$ were premixed and dispensed into the reaction mixture to trigger the Fenton reaction by a LKB 1291 dispenser (LKB Wallac, Finland). The mixture was fully mixed immediately after reaction initiation using a pulse mixer (LKB Wallac, Finland), and incubated at room temperature (25 ± 1 °C) for 5 s. The reaction was stopped by dispensing 1 mL of 10% (w/v) trichloroacetic acid [29], and reaction mixture was mixed with 1 mL of 1% (w/v) 2-thiobarbituric acid (TBA, in 50 mM NaOH containing 0.02% BHA), then further heated at 80 °C for 15 min to develop the chromophore. For spectrophotometric determinations, the above-developed chromophore was measured directly by reading the absorbance at 532 nm. For fluorescent determinations, the chromophore was extracted with 3 mL of 1-butanol, and

the fluorescence intensity of the organic phase was recorded at 553 nm with excitation at 532 nm, with a Perkin–Elemer LS50B luminescence spectrometer. All the solutions were made up immediately before use in de-aerated water except $FeCl_2$, which was dissolved in 1 mM of oxygen-free HCl for the preparation of Fe(II) solution.

2.4. Evaluating the activities of phenolic compounds to scavenge hydroxyl radical

In the Fe^{2+}–EDTA/H_2O_2/luminol system, the capacity of compounds to scavenge hydroxyl radicals was assessed according to their quenching effects on the CL signal. The LKB Wallac 1251 luminometer was employed to measure the CL signal in the absence (S_0) and presence (S_i) of phenolic compounds. The inhibitory rate was calculated as $I_R(\%) = (1 - S_i/S_0) \times 100\%$.

In the 2-deoxy-D-ribose degradation assay, the decay effect of phenolic compounds on the fluorescence signal was used as an index to evaluate their activities. The fluorescence intensity in the absence (I_0) or presence (I_i) of phenolic compounds was measured by a Perkin–Elmer LS50B luminescence spectrometer. Similarly, the absorbance at 532 nm in the absence (A_0) or presence (A_i) of the tested compounds was recorded using a HP-8453 UV–Vis spectrophotometer (Hewlett–Packard, USA). The inhibitory rate was calculated as $I_R(\%) = (1 - I_i/I_0) \times 100\%$ or $(1 - A_i/A_0) \times 100\%$.

2.5. Quantum chemical calculation

The semi-empirical quantum chemical method AM1 [30] was employed to calculate theoretical parameters, including the difference (ΔH_f) between the heat of formation of a parent phenolic molecule (H_p) and that of its phenoxyl radical (H_r), the enthalpy of single electron transfer (E_a, approximated by the difference between the heat of formation of the intermediate cation radical and that of the parent phenolic compounds), the spin distribution of the phenoxyl free radical (D_s^r) and the intermediate cation radical (D_s^c), and the HOMO and LUMO energy levels. Calculation of these parameters was performed at a restricted level for parent compounds and at an unrestricted level for neutral radicals and radical cations with AM1 method in the Gaussian 94 package [31]. On the basis of frequency analysis, the enthalpies of the title compounds were obtained, which include the zero-point energy correction. It should be noted that only the most stable conformation of antioxidants and their corresponding phenoxyl free radical were taken into consideration in discussing the possible hydroxyl radical scavenging mechanisms. For example, conformations possessing hydrogen bonds were selected in the calculation because hydrogen bonds stabilized both the molecules and the corresponding phenoxyl free radicals to a greater extent [32,33].

2.6. Statistical analysis

The analysis of variance (ANOVA) was used to assess the significance in the quantitative structure–activity relationship and the correlation between computed

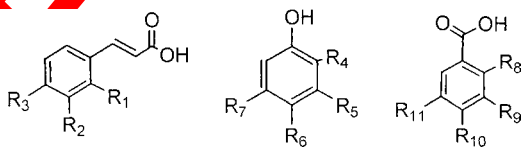
theoretical parameters. When the *F*-ratio of ANOVA was significant, the correlation or the contribution of theoretical parameters to established models was assessed by the least significant difference method ($P < 0.05$). All statistical analyses were performed by the statistical package SAS for windows, version 6.12 (SAS Institute, USA).

3. Results and discussion

Although a significant body of literature concerning the structure–activity relationships (SAR) of phenolic antioxidants has been published (see Refs. [34–36] and the references therein), few studies have focused on the multiple molecular mechanisms, and even fewer on the quantitative elucidation of the correlations between antioxidant activity and possible mechanistic determinants [35,36]. Phenolic compounds belonging to three subclasses (polyphenol, hydroxylated benzoic and cinnamic acids, see Fig. 1 for chemical structures), were investigated herein and their hydroxyl radical-quenching properties were determined by luminol-enhanced chemiluminescence (CL), fluorescence (FL), and a spectrophotometric assay. The AM1 method (Austin model 1), which was better than other semi-empirical methods [37] and even as good as *ab initio* methods [12], was employed to perform a theoretical study because it is such a powerful technique to elucidate reaction mechanisms [30,38,39]. Regression analyses on the experimentally measured activities and calculated theoretical parameters were performed to determine the interdependences of molecular mechanisms, and the correlation between antioxidant potentials and mechanistic determinants, so that an ideal QSAR model could be defined for the overall evaluation and prediction of antioxidant activities.

3.1. Optimization of the CL reaction system

To optimize the CL reaction conditions, we investigated the effects of (1) mixing order of reagents, (2) buffer concentration, (3) reactant concentrations, and (4) pH



1-4 **5-10** **11-15**

Fig. 1. Chemical structures of the selected phenolic antioxidant compounds. **1**, *o*-Coumaric acid, $R_1 = OH$, $R_2 = R_3 = H$. **2**, *p*-Coumaric acid, $R_3 = OH$, $R_1 = R_2 = H$. **3**, Ferulic acid, $R_2 = OCH_3$, $R_3 = OH$, $R_1 = H$. **4**, Caffeic acid, $R_2 = R_3 = OH$, $R_1 = H$. **5**, Catechol, $R_4 = OH$, $R_5 = R_6 = R_7 = H$. **6**, Pyrogallol, $R_4 = R_5 = OH$, $R_6 = R_7 = H$. **7**, Phloroglucinal, $R_5 = R_7 = OH$, $R_4 = R_6 = H$. **8**, Resorcinol, $R_5 = OH$, $R_4 = R_6 = R_7 = H$. **9**, Hydroquinone, $R_6 = OH$, $R_4 = R_5 = R_7 = H$. **10**, *p*-Aminophenol, $R_6 = NH_2$, $R_4 = R_5 = R_7 = H$. **11**, Protocatechuic acid, $R_9 = R_{10} = OH$, $R_8 = R_{11} = H$. **12**, Gallic acid, $R_9 = R_{10} = R_{11} = OH$, $R_8 = H$.**13**, Salicylic acid, $R_8 = OH$, $R_9 = R_{10} = R_{11} = H$. **14**, *m*-Hydroxy-benzoic acid, $R_9 = OH$, $R_8 = R_{10} = R_{11} = H$. **15**, *p*-Hydroxy-benzoic acid, $R_{10} = OH$, $R_8 = R_9 = R_{11} = H$.

value on the CL signal. Mixing order of reactants played a key role in the CL reaction, with the most sensitive signals and relatively fast reaction observed when H_2O_2 was dispensed last to initiate the CL reaction. Fig. 2 revealed the effects of reactant concentrations on the CL intensity. A H_2O_2 concentration of 4.4×10^{-4} mol/L was chosen for other measurements since it produces a strong CL signal, while higher concentrations cause substantial background levels. As an essential catalyst, Fe^{2+} serves a major function in the CL reaction, particularly in controlling the reaction speed. The CL intensity increased with increasing Fe(II) or luminol concentration. When luminol was at a concentration of 2.0×10^{-4} and Fe^{2+}–EDTA at 6.0×10^{-4} mol/L, the CL signal passed through a maximum level and then decreased.

The CL intensity shows strong pH-dependency from pH 5.8 to 11.0, increasing continuously with the increase of pH, whereas an increase in buffer concentration results in a lower CL intensity. We presumed that alkaline conditions favored the excitation of luminol, while high ionic strength increased the chances of chemiluminescence-quenching collision between excited luminol molecules and other particles [40]. In view of the physiological pH value and proper buffer capacity, the investigation on antioxidant activities was performed at pH 7.4 [41,42], and 50 mM of KH_2PO_4–NaOH buffer.

3.2. Scavenging effects of phenolic compounds on hydroxyl radicals

The compounds were tested for their potency to scavenge hydroxyl radicals produced from Fenton's reagent in two experimental models. In the first test, luminol was attacked and excited by hydroxyl radical. A quick CL reaction occurred (less than 10 s) and yielded strong chemiluminescence when luminol was mixed with Fenton's reagent. Chemiluminescence-quenching effects can discriminate between selected compounds for their hydroxyl radical-scavenging ability. The second test was based on the hydroxyl radical-induced degradation of deoxyribose, where aldehydes were generated, which then reacted with thiobarbituric acid to form a pink chromophore under acid conditions. Given that the FL assay has higher sensitivity

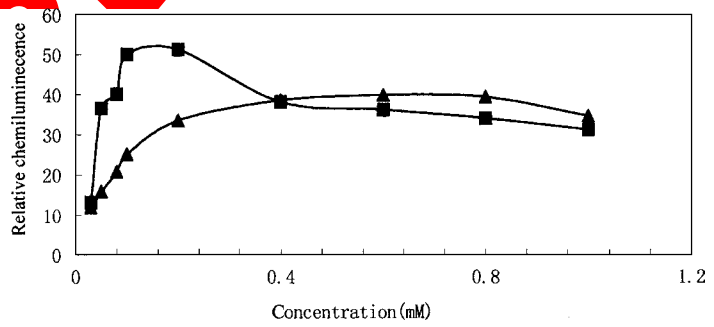


Fig. 2. Effects on the chemiluminescence signal of concentrations of Fe(II) (▲) and luminol (■). The experimental conditions are specified in Section 2.

and a lower detection limit but is more sensitive to experimental conditions, the chromophore was quantified by fluorescence as well as the spectrophotometric assay to ensure the accuracy of the results and to avoid the bias described by Visioli et al. [43]. The antioxidant potentials were quantified by the concentration causing 50%-inhibition (IC_{50}) of the FL and CL signals, and the absorbance at 532 nm. The detailed results are listed in Table 1.

Fig. 3 compares the results from the three analytical methods. Obviously, the results of the spectrophotometric method agree well with that of the FL assay

Table 1
In vitro hydroxyl radical scavenging activities (IC_{50}/μM, mean±SD)

Compounds	CL assay	FL assay	A_{532} assay	Compounds	CL assay	FL assay	A_{532} assay
1	60.8 ± 2.7	52.1 ± 4.2	53.9 ± 3.0	**9**	31.0 ± 0.9	35.3 ± 2.6	36.2 ± 1.4
2	61.5 ± 3.9	56.4 ± 2.9	58.9 ± 1.9	**10**	17.9 ± 0.4	20.9 ± 1.1	18.8 ± 0.8
3	58.0 ± 3.2	53.7 ± 3.8	51.2 ± 1.7	**11**	47.3 ± 2.0	52.5 ± 2.7	50.3 ± 1.0
4	39.7 ± 1.5	41.0 ± 3.4	42.3 ± 2.2	**12**	43.1 ± 1.5	47.6 ± 4.1	49.0 ± 2.6
5	32.1 ± 0.7	34.2 ± 1.2	31.6 ± 1.5	**13**	91.0 ± 4.7	77.1 ± 4.9	75.4 ± 3.5
6	28.9 ± 1.8	31.8 ± 2.4	28.0 ± 1.0	**14**	92.7 ± 2.5	83.6 ± 3.6	79.8 ± 3.2
7	64.7 ± 4.2	70.4 ± 6.3	68.1 ± 2.7	**15**	94.1 ± 4.4	86.2 ± 5.9	82.2 ± 2.6
8	66.4 ± 1.3	68.8 ± 3.6	65.5 ± 3.1				

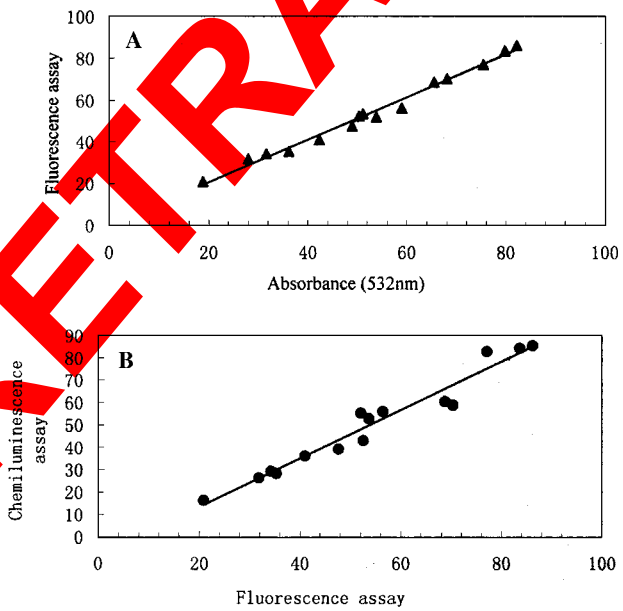


Fig. 3. The correlation between the results determined by fluorescent (FL), chemiluminescent (CL), and spectrophotometric asaay (IC_{50}: in μM). (▲) $IC_{50}(FL) = 1.024(\pm0.032)IC_{50}(A_{532}) + 0.082(\pm0.008)$, $n = 15$, $r^2 = 0.9873$, $F = 1008.39$, $P \leqslant 0.0001$; (●) $IC_{50}(CL) = 1.082(\pm0.065)IC_{50}(FL) - 8.313(\pm3.741)$, $n = 15$, $r^2 = 0.9550$, $F = 276.62$, $P \leqslant 0.0001$.

($r^2 = 0.9873$, $P \leqslant 0.0001$) and that of CL assay ($r^2 = 0.9526$, $P \leqslant 0.0001$). Moreover, a good relationship exists between the result of FL assay and that of CL assay ($r^2 = 0.9550$, $P \leqslant 0.0001$). ANOVA strongly suggests that there is no significant difference among the three series of results.

In the hydroxyl radical-scavenging reaction, the compounds with two hydroxyl groups on the benzene ring (compounds **11**, **12**) are more reactive than monohydroxylated ones (compounds **13**, **14**, **15**). In addition, the catecholic or pyrogallolic structure (compounds **5**, **6**) contribute more to the antioxidant activity than does the resorcinol structure (compounds **7**, **8**). These differences are due to the fact that hydrogen-abstraction is facilitated by electron donating substituents and the overlap of the p-type lone pair orbital of the oxygen at the *p*- or *o*-position with the singly occupied molecular orbital of the radical [13,44,45]. Furthermore, a larger stabilization of the phenoxyl radical can be achieved due to the formation of a quinone or semi-quinone species after hydrogen-transfer [13]. This species also accounts for the activity difference to some extent.

3.3. Theoretical studies by computational chemistry

Theoretical parameters employed to characterize radical scavenging activity can be roughly grouped into three classes: (1) indices reflecting O–H bond dissociation enthalpy (BDE), such as ΔH_f [13,37] and relative BDE [12]; (2) parameters representing electron-donating ability, such as ionization potential (IP) or relative adiabatic ionization potential ΔH_{ox} [12,13,19], enthalpy of single electron transfer (also defined as activation energy of intermediate cation radical E_a or E_{set}) [12,19]; and (3) factors stabilizing the corresponding radical after hydrogen-abstraction [19,46]. In order to perform a theoretical investigation on the free radical-scavenging mechanism, we calculated the related physico-chemical parameters, including heat of formation of a parent molecule (H_p) and that of its phenoxyl radical (H_r), the heat of formation of cation radical H_c, the spin distribution of phenoxyl free radical (D_s^r) and the intermediate cation radical (D_s^c), and the HOMO and LUMO energy levels. The ΔH_f value was calculated from the difference between H_p and H_r, and the activation energy of intermediate cation radical (E_a) [19], was approximated by the difference between H_c and H_p, and also defined as enthalpy of electron transfer [12]. The calculation results are presented in Table 2.

According to Koopmans' theory [47] and molecular orbital theory, the HOMO energy determines the ionization potential (IP) and so is correlated with the ability of the phenolic compounds to donate electrons. This principle was demonstrated by the excellent correlation between the HOMO energy and the activation energy of intermediate cation radicals as shown in the following equation:

$$E_{HOMO} = -1.055(\pm 0.040)E_a - 6.547(\pm 0.755)\ n = 15,$$
$$r^2 = 0.9813,\ F = 683.19,\ P \leqslant 0.0001. \quad (1)$$

Interestingly, a correlation was observed between E_a and ΔH_f (Eq. (2)), suggesting an electron-transfer mechanism in the hydrogen-abstraction process, or that these two

Table 2
Computed theoretical parameters by AM1 method[a]

Compounds	H_p	H_r	H_c	ΔH_f	E_a	HOMO	LUMO	H–L_{gap}	D_s^r	D_s^c
1	−96.71	−73.58	97.34	23.13	194.1	−212.8	43.22	−256.0	0.3345	0.1522
2	−98.71	−73.64	92.54	25.07	191.3	−209.3	50.74	−260.1	0.3394	0.1204
3	−136.0	−112.2	49.51	23.88	185.5	−204.6	49.49	−254.1	0.3106	0.1529
4	−142.2	−118.1	46.24	24.13	188.5	−206.7	47.81	−254.5	0.3150	0.0794
5	−66.21	−44.03	122.4	22.18	188.6	−204.1	93.62	−297.7	0.3539	0.1219
6	−110.5	−88.30	78.64	22.13	189.1	−205.9	101.1	−307.0	0.3483	0.0429
7	−111.3	−83.00	83.22	28.29	194.5	−212.1	105.9	−318.0	0.3781	0.0778
8	−66.34	−38.92	127.3	27.43	193.6	−209.7	93.36	−303.0	0.3825	0.0742
9	−65.54	−41.45	120.7	24.09	186.2	−200.7	85.14	−285.8	0.3539	0.1503
10	−21.67	−0.7680	146.3	20.90	167.9	−182.8	91.85	−274.7	0.3322	0.1132
11	−142.2	−119.9	46.24	22.38	188.5	−206.7	65.04	−278.8	0.3151	0.0794
12	−199.1	−175.8	−2.005	23.34	197.1	−215.3	63.23	−278.5	0.3346	0.0435
13	−97.20	−73.75	96.61	23.45	193.8	−212.6	58.96	−277.6	0.3350	0.1541
14	−111.3	−83.12	90.79	28.14	202.1	−218.8	57.70	−276.5	0.3394	0.1997
15	−98.72	−73.65	92.54	25.07	191.3	−209.3	67.27	−288.0	0.3926	0.1204

[a] The calculated parameters are all in kcal/mol for H_r, H_p, H_c, ΔH_f, E_a, HOMO, LUMO energy levels, and hydrogen-L_{gap} except D_s^e and D_s^r.

processes coexist and influence each other. However, the relationship between ΔH_f and HOMO energy is less satisfactory in spite of its good correlation with E_a (see Eq. (3)). The difference arises presumably from the calculation process where electron reorganization after electron transfer was neglected when calculating the HOMO energy, but was taken into consideration when calculating the activation energy of intermediate cation radicals. Reasonably, electron reorganization accompanied the electron abstraction process, and played a significant role in hydroxyl radical-scavenging reaction [12]

$$E_a = 2.322(\pm 0.597)\Delta H_f + 134.342(\pm 0.032)$$
$$n = 15,\ r = 0.7331,\ F = 15.10,\ P \leqslant 0.0019. \quad (2)$$

$$E_{HOMO} = -2.347(\pm 0.671)\Delta H_f - 105.747(\pm 16.777)$$
$$n = 15,\ r = 0.7547,\ F = 12.22,\ P \leqslant 0.0039. \quad (3)$$

In addition to the facility of hydrogen transfer and electron transfer, the stability of the phenoxyl radical resulting from hydrogen-abstraction has been shown to be responsible for good antioxidant activity [46]. The relationship shown in Fig. 4(A) also confirmed this conclusion, that is, the facility of hydrogen-transfer was determined by the stability of the neutral radical (D_s^r) to some extent. Generally, electron-attracting substituents and hyper-conjugative interaction were considered as the main inducing factors of radical stabilization, and hyper-conjugation was more important for antioxidant compounds (expressed by spin distribution, D_s [19]). The regression analyses on ΔH_f, E_a, D_s^r, and D_s^c showed that the factors facilitating hydrogen-abstraction also contributed to single electron transfer (see Eq. (2)). However, factors stabilizing intermediate cation radicals appear independent from those stabilizing neutral phenoxyl radicals as demonstrated in Fig. 4(B) and on the basis of literature precedence [48].

3.4. Relationship between experimental activity and computed theoretical descriptors

Since the antioxidant activities measured by the three analytical methods produce significantly interdependent results ($r^2 = 0.9873,\ 0.9550,\ 0.9526$, respectively), we

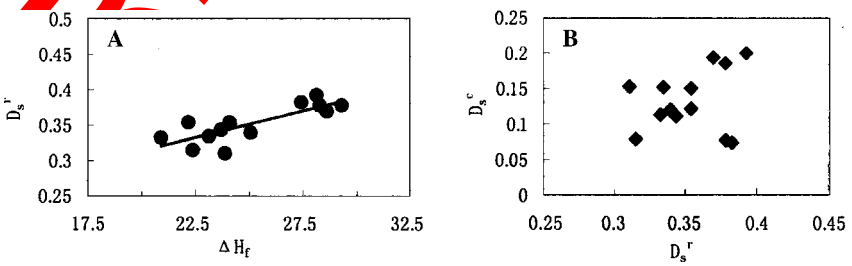


Fig. 4. The correlation between D_s^r and ΔH_f and D_s^c. (A) ●: $D_s^r = 0.007(\pm 0.002)\Delta H_f + 0.176(\pm 0.038)$, $n = 15$, $r^2 = 0.6515$, $F = 21.82$, $P \leqslant 0.0004$; (B) ◆: $D_s^c = 0.609(\pm 0.548)D_s^r - 0.092(\pm 0.193)$, $n = 15$, $r^2 = 0.0866$, $F = 1.23$, $P \leqslant 0.2869$.

selected one of them (IC_{50}-A_{532}) to investigate the correlation between experimentally measured activities and computed theoretical parameters. A linear relationship was derived between the IC_{50} and ΔH_f, ionization potential (E_{HOMO}), enthalpy of single electron transfer (E_a), spin distribution of phenoxyl radicals (D_s^r), and the chemical hardness (H–L_{gap}). The relationship can be described by the following stepwise equations:

$$IC_{50} = 6.462(\pm 0.685)\ \Delta H_f - 107.790(\pm 17.127)$$
$$n = 15,\ r^2 = 0.8724,\ F = 88.88,\ P \leqslant 0.0001, \quad (4)$$

$$IC_{50} = 7.030(\pm 0.512)\ \Delta H_f + 0.266(\pm 0.072)\text{H–L}_{gap} - 47.323(\pm 20.422)$$
$$n = 15,\ r^2 = 0.9403,\ F = 94.43,\ P \leqslant 0.0001, \quad (5)$$

$$IC_{50} = 5.558(\pm 0.439)\ \Delta H_f - 0.585(\pm 0.124)E_a + 0.220(\pm 0.044)\text{H–L}_{gap} - 146.007(\pm 24.309)$$
$$n = 15,\ r^2 = 0.9802,\ F = 181.23,\ P \leqslant 0.0001, \quad (6)$$

$$IC_{50} = 5.780(\pm 0.426)\ \Delta H_f - 0.585(\pm 0.124)E_a - 1.381(\pm 0.812)E_{HOMO} + 0.179(\pm 0.048)\text{H–L}_{gap} - 155.874(\pm 23.188)$$
$$n = 15,\ r^2 = 0.9846,\ F = 160.06,\ P \leqslant 0.0001, \quad (7)$$

$$IC_{50} = 4.628(\pm 0.448)\ \Delta H_f - 3.618(\pm 0.732)E_a - 3.377(\pm 0.810)E_{HOMO} + 0.261(\pm 0.041)\text{H–L}_{gap} + 246.377(\pm 71.869)D_s^r - 183.610(\pm 18.016)$$
$$n = 15,\ r^2 = 0.9933,\ F = 268.08,\ P \leqslant 0.0001. \quad (8)$$

As a measure of bond strength [13,37], the ΔH_f value determines the facility of hydrogen-transfer from phenolic compounds to free radicals. The smaller the ΔH_f values, the weaker the O–H bonds. As shown by Eq. (2), weaker O–H bonds will facilitate hydrogen-abstraction, and the resulting phenoxyl radicals will be more stable. Thus, ΔH_f makes a positive contribution to IC_{50} values. When ΔH_f increases, the transfer of hydrogen from the parent compounds becomes more unfavorable, rendering the phenolic compounds poorer antioxidants (Eq. (4)).

According to the principle of maximum hardness [49,50], *chemical systems at equilibrium are as hard as possible*, i.e., the most stable structure of the molecule is the one with the maximum H–L_{gap} [51,52]. Thus, the H–L_{gap} reflects the reactivity of compounds, and the molecules with larger H–L_{gap} are less active in the radical reaction than those with smaller H–L_{gap}. The regression Eqs. (5)–(8) confirm this assumption and reveal that the negative contribution of H–L_{gap} to antioxidant activity is significant ($P \leqslant 0.0001$), indicating its value in predicting antioxidant properties.

With further standardization of the Eq. (8), ΔH_f and the H–L_{gap} were found to contribute most to the QSAR model, and were thus the best predictors of antioxidant activity. Although other parameters did not contribute to the model as much

as the former two descriptors, inclusion of them in the stepwise multi-linear regression markedly improved the correlation between antioxidant activity and molecular orbital theoretical parameters (i.e., r^2 increases from 0.8724 to 0.9933). The relative contribution and statistical significance of these parameters are: ΔH_f 87.8%, $P \leqslant 0.0001$ ($F = 106.96$); E_{HOMO} 0.5%, $P \leqslant 0.0024$ ($F = 17.37$); E_a 4.0%, $P \leqslant 0.0008$ ($F = 24.26$); D_s^r 0.9%, $P \leqslant 0.0075$ ($F = 11.75$); and H–L_{gap} 6.8%, $P \leqslant 0.0001$, ($F = 40.84$). The statistical significance of the physicochemical parameters confirmed the assumption that multiple mechanisms other than hydrogen-abstraction or electron-transfer alone governed the antioxidant activities of phenolic compounds. Stability of phenoxyl radical, electron rearrangement, and the chemical reactivity of parent molecules all played key roles in regulating the behavior of phenolic compounds in free radical reactions. In practical terms, the increase in antioxidant activity parallels the decrease in the O–H BDE, absolute chemical hardness and the spin distribution of phenoxyl radicals, but parallels the increase in the enthalpy of single electron transfer and the ionization potential.

It should be noted that although some variables correlated somewhat with each other, they were not eliminated from the QSAR models so that important mechanistical information was not missing [53–57]. Other regression approaches such as partial least square analysis (PLSA) and principal component regression (PCR) [58,59], which involve the formation of orthogonal linear combinations of the original independent variables, can yield new variables which are fewer in number than the original, but the new variables may be obscure in meaning and disadvantageous for definite elucidation of free radical reaction mechanisms [56,60]. More seriously, those methods in which correlated variables are systematically eliminated, can lead to loss of information when applied severely [60]. Therefore, Randic et al. [53–56], who have generated satisfactory QSAR models in their studies, addressed in detail the principles for multiple linear regression methods. Following Randic et al. [53–55,57], we carried out a systematical investigation on the multiple mechanisms underlying the hydroxyl radical-scavenging reactions. And with *MAXR* Model, *stepwise* regression can eliminate effectively the unrelated variables among the calculated physico-chemical parameters that were to be screened, and find the best multiple-variable model [57,61].

4. Conclusions

In this paper, we carried out a theoretical investigation on the possible mechanisms governing the hydroxyl radical scavenging reactivity of a series of phenolic antioxidants by computational chemistry, and explored the correlation between experimentally determined antioxidant activity and molecular orbital characteristics. Based on the interdependence of physico-chemical variables and their correlation with antioxidant activity, it is reasonable to conclude that multiple mechanisms regulate the antioxidant actions of phenolic compounds in a significant way although they contribute to the antioxidant activity to different degrees. If the hydroxyl radical-quenching reactivity was only ascribed to one mechanism, bias is inevitable

when assessing or predicting antioxidant activity. That is why some authors [12,14–18] arrived at contradictory conclusions when explaining the differences in antioxidant activity using only O–H bond dissociation energy or the HOMO energy level.

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