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Study on the Multiple Mechanisms Underlying the Reaction Between Hydroxyl Radical and Phenolic Compounds by Qualitative Structure and Activity Relationship

Zhiyong Cheng, Jie Ren, Yuanzong Li,* Wenbao Chang and Zhida Chen

The Key Laboratory of Bioorganic and Molecular Engineering and Department of Chemical Biology, College of Chemistry and Molecular Engineering, Peking University, Beijing, China 100871

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Abstract—The activity–structure relationships (ASR) of phenolic compounds as hydroxyl-radical scavengers have mostly been studied and discussed with regard to their iron-chelating and hydrogen-donation properties in Fenton-type system, but extensive elucidation of multiple mechanisms underlying the hydroxyl radical scavenging reaction is out of obtaining up to now. In the present paper, a series of phenolic compounds was studied for their reactivity with hydroxyl radical by computed chemistry and deoxyribose degradation assay. The rate constant (K_S), an index dependent markedly on the reaction mechanism and intrinsic reactivity of antioxidants, was found to have good correlation with hydroxyl O–H bond strength (ΔH_f), electron-donating ability (ionization potential approximated by HOMO energy level), enthalpy of single electron transfer (E_a), and spin distribution of phenoxyl radicals (Ds^r) after H-abstraction. Moreover, the theoretical parameters were highly intercorrelated, suggesting that multiple mechanisms co-exist in the hydroxyl-radical-scavenging reaction and interact with each other. Multi-linear regression analysis indicated that, in addition to H-atom transfer, electron transfer process and stability of the resulted phenoxyl radicals also significantly influence the reactivity of quenching hydroxyl radicals. The QSAR model so established here was based on the elucidation of the complex molecular mechanisms, and may reasonably predict the antioxidant activity using simple experimental and calculated parameters.

Introduction

Free radical reactions, especially with participation of oxidative radicals, have been known to be involved in many biological processes that cause damage to lipids, proteins, membranes and nucleic acid, thus give rise to a variety of diseases.[1,2] Among the reactive oxygen species produced in living cells, hydroxyl radical is the most active and strongest oxidant agent, and can react with almost any substance at diffusion rate.[3,4] Phenolic compounds, which can scavenge free radicals derived from molecular oxygen and attenuate the oxidative stress, have attracted increasing interest in food and medicine.[5–7]

Quenching of hydroxyl radical by phenolic compounds is generally considered to undergo H-abstraction reaction, and indices representing the O–H bond dissociation energy (BDE), such as difference of heat of formation between antioxidant and its free radicals after H-abstraction (ΔH_f), the relative O–H BDE (ΔH_{abs}), were found to be correlated well with free radical scavenging activities.[8–11] Another consideration commonly occurring is iron-chelating capacity of phenolic antioxidants in assessing the inhibitory effects on hydroxyl radical formation in Fenton-type reactions, and factors favorable for iron-chelation always promote their antioxidant activity.[12–14] Most recently, Bossmann et al.[15] compared the results of oxidative degradation of 2, 4-dimethylaniline by means of H_2O_2/UV method with that of the thermal or photo-chemically enhanced Fenton reactions in the presence of 2,4-dimethylaniline, and presented the evidence for an electron transfer mechanism

*Corresponding author. Tel.: +86-10-6275-7954; fax: +86-10-6275-1708; e-mail: yli@chem.pku.edu.cn

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involved in the reactions. Khopde et al.[16] also confirmed the electron transfer equilibrium during the hydroxyl radical induced oxidation of 3-methoxy-4-hydroxy cinnamic acid. The energy level of the highest occupied molecular orbital (E-$_{HOMO}$), a theoretical parameter reflecting the electron-donation ability, was reported to show good correlation with the free radical scavenging activity,[10,17] which also inferred an electron transfer process involved in the H-abstraction reaction. However, Zhang and coworkers[18,19] suggested that effectiveness of E_{HOMO} to characterize antioxidant activity is doubtful and maybe only a superficial phenomenon.

Then, the questions arise, that is, what role does the electron transfer process play in the antioxidant reaction? To what extent does this process contribute to hydroxyl radical scavenging ability? And is it possible that H-atom transfer and electron transfer processes govern the free radical reaction along with other molecular mechanism, for example, stability of phenoxy radicals, and activation energy of intermediate cation radical? In the present work, we examine a set of phenolic compounds (Fig. 1) for their antioxidant activity and structural properties, so that light is shed on the above-mentioned problems.

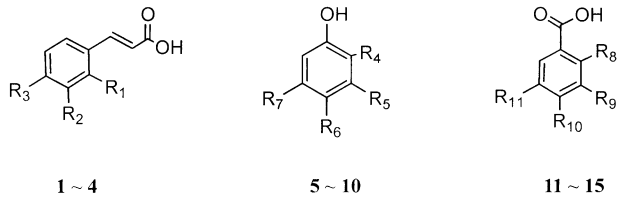


Figure 1. Chemical structure of the selected phenolic antioxidants. **1**,*o*-Coumaric acid, $R_1 = OH$; **2**, *p*-coumaric acid, $R_3 = OH$; **3**, ferulic acid, $R_2 = OCH_3$, $R_3 = OH$; **4**, caffeic acid, $R_2 = R_3 = OH$; **5**, catechol, $R_4 = OH$; **6**, pyrogallol, $R_4 = R_5 = OH$; **7**, phloroglucinal, $R_5 = R_7 = OH$; **8**, resorcinol, $R_5 = OH$; **9**, hydroquinone, $R_6 = OH$; **10**, *p*-aminophenol, $R_6 = NH_2$; **11**, protocatechuic acid, $R_9 = R_{10} = OH$; **12**, gallic acid, $R_9 = R_{10} = R_{11} = OH$; **13**, salicylic acid, $R_8 = OH$; **14**, m-hydroxyl-benzoic acid, $R_9 = OH$; **15**, *p*-hydroxyl-benzoic acid, $R_{10} = OH$. Other substituted groups for each molecule are hydrogen atoms unless specified.

Results and Discussion

Scavenging effects of phenolics on hydroxyl radicals

Hydroxyl radicals, generated by reaction of iron-EDTA complex with H_2O_2 in the presence of ascorbic acid, attack deoxyribose to form products that, upon heating with 2-thiobarbituric acid under acid conditions, yield a pink chromogen. Added hydroxyl radical scavengers compete with deoxyribose for the resulted hydroxyl radicals and diminish chromogen formation. Halliwell et al.[20] established a simple 'test-tube' assay method based on the inhibition of color formation and determined the rate constant for the reaction of a wide range of compounds (scavengers) with hydroxyl radical.

$$\frac{1}{A} = \frac{1}{A^0}\left(1 + \frac{k_s[S]}{K_{DR}[DR]}\right) \quad (1)$$

where A is the absorbance in the presence of a scavenger S at concentration $[S]$ and A^0 is the absorbance in the absence of a scavenger; Ks and K_{DR} are the rate constants of reaction of hydroxyl radicals with scavengers and with deoxyribose, respectively; $[DR]$ is the concentration of deoxyribose used in the experiment. From the slope of the plot of $1/A$ against $[S]$, the rate constant can be obtained with the K_{DR} value of $3.1 \times 10^9\ M^{-1}\ s^{-1}$.

Since they are derived directly from kinetic/dynamic scavenging reaction, the second-order rate constants of scavengers depend markedly on the reaction mechanism, and the test-tube is widely used to determine Ks, equally accurate but more simply and cheaper in investigations of mechanisms of free radical reactions.[21–23] In the present work, we employed Ks to assess the reactivity of the selected compounds in scavenging hydroxyl radicals. The detailed results were listed in Table 1.

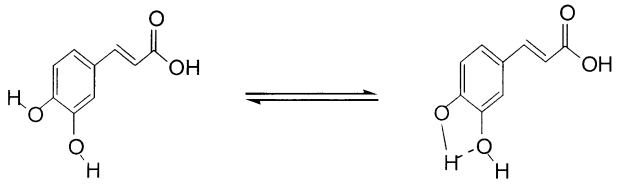
Inter-correlation between theoretical parameters

Because of its reliable accuracy,[24] AM1 method was chosen to study a series of 15 compounds, and the calculation results are listed in Table 1. Phenoxyl free radical of antioxidants containing catechol moiety may

Table 1. In vitro hydroxyl radical scavenging reactivity (log Ks) and computed theoretical parameters (H_r, H_p, H_c, ΔH_f, Ea, Ds^c, Ds^r, E_{HOMO})

Compounds	H_p kcal/mol	H_r kcal/mol	H_c kcal/mol	ΔH_f kcal/mol	Ea kcal/mol	E_{HOMO} kcal/mol	Ds^r	Ds^c	log Ks
1	−96.71	−73.58	97.34	23.13	194.0	−212.8	0.3345	0.1522	9.16
2	−98.71	−73.64	92.54	25.07	191.3	−209.3	0.3394	0.1204	9.12
3	−136.0	−112.2	49.51	23.88	185.54	−204.6	0.3106	0.1529	9.37
4	−142.2	−118.1	46.24	24.13	188.46	−206.7	0.3150	0.0794	9.86
5	−66.21	−44.03	122.4	22.18	188.6	−204.1	0.3539	0.1219	9.79
6	−110.5	−88.30	78.64	22.15	189.1	−205.9	0.3483	0.0429	n.d*
7	−111.3	−83.00	83.22	28.29	194.5	−212.1	0.3781	0.0778	8.01
8	−66.34	−38.92	127.3	27.43	193.6	−209.7	0.3825	0.0742	8.33
9	−65.54	−41.45	120.7	24.09	186.2	−200.6	0.3539	0.1503	9.68
10	−21.67	−0.7680	146.3	20.90	167.9	−182.8	0.3322	0.1132	9.83
11	−142.2	−119.9	46.24	22.38	188.5	−206.7	0.3151	0.0794	9.75
12	−199.1	−175.8	−2.005	23.34	197.1	−215.3	0.3346	0.0435	n.d*
13	−97.20	−73.75	96.61	23.45	193.8	−212.6	0.3350	0.1541	9.01
14	−111.3	−83.12	90.79	28.14	202.1	−218.8	0.3394	0.1997	8.09
15	−98.72	−73.65	92.54	25.07	191.3	−209.3	0.3926	0.1204	9.00

*n.d.: Not determined.



Scheme 1. Two conformations of phenolics containing catecholic hydroxyls.

be in two conformations, forming intra-molecular hydrogen bonds or not (Scheme 1). Evidently, the latter is more stable in energy, so their ΔH_f values were calculated by this conformation.

Among the calculated parameters, ΔH_f value was widely used to characterize O–H bond strength and as index of the activity to scavenge free radicals.[8,11,25] Given that antioxidant action can also involve single electron transfer (SET) followed by proton transfer as an alternative or in addition to H-abstraction, we calculated *Ea* and E_{HOMO}, which indicate enthalpy of single electron transfer and ability of phenolics to donate electrons, respectively. As expected, the ionization potential (approximated by the HOMO energy level) was well correlated with the enthalpy of electron transfer (*Ea*) from a phenolic compound. We assumed that the facility of hydrogen transfer is related not only to hydroxyl O–H bond strength (bond dissociation energy) but also to the stability of the corresponding phenoxyl radicals after H-abstraction. From this point of view, ΔH_f should show some correlation to the spin distribution (Ds^r) which represents the degree of hyper-conjugation of the p-type lone pair and aromatic ring. As shown in Figure 2, in addition to the correlation between Ds^r and ΔH_f, a correlation between *Ea* and ΔH_f was observed. This suggested that factors facilitating single electron transfer in these compounds also facilitate their H-abstraction, which is in agreement with the good correlation existing between the second-order rate constant of H-abstraction and the peak oxidation potential reported by Mukie et al.[26,27] However, the correlation between Ds^r and Ds^c was poor ($r^2 = 0.0651$), indicating factors stabilizing radical cation are independent from those stabilizing neutral phenoxyl radicals, as demonstrated in ref. 28.

The correlation between ΔH_f and the E_{HOMO} ($r^2 = 0.5695$) is less satisfactory than the one between ΔH_f and enthalpy of single electron transfer ($r^2 = 0.6193$), even though a high degree of correlation between HOMO energy level and *Ea* exists ($r^2 = 0.9812$). That is, electron re-organization plays a non-negligible role in the antioxidant process.

Relation between theoretical parameters and experimental antioxidant activity

In the present work, we determined the reaction rate constant of selected phenolic compounds with hydroxyl radicals. Since it was derived from the kinetic process of hydroxyl radical scavenging where the second order was maintained, the rate constant could discriminate the antioxidants according to their intrinsic reactivity. That is, this experimentally measured parameter is markedly dependent on the reaction mechanism and displays the relative activity to scavenge hydroxyl radical. It appears that the mono-hydroxylated compounds were less active than those having two hydroxyl groups on the benzene ring, for example, compounds **1** and **2** < compounds **4** and **11**. The high reactivity of compounds **5, 9** and **10** in contrast to compound **8** can be explained by a large stabilization of the phenoxyl radical due to the oxygen (or nitrogen) at *para*- or *ortho*-position as already proposed by Ingold et al.[10,29] While the high reactivity of compound **3** compared to compounds **1** and **2** might be explained by electron effects of ortho oxygen atoms, and it can also result from a subtle equilibrium between intra- or inter-molecular hydrogen-bond.[30,31] The same mechanism may also account for the reactivity of compounds with catocholic or pyrogallolic structure.

Although compounds **6** and **12** possess beneficial factors contributing to their activity in the reaction with hydroxyl radicals, their rate constants could not be measured accurately in the present experiment because they stimulate the chromogen formation significantly. The major reason might be that compounds **6** and **12** would produce marked quantities of hydroxyl radicals during the incubation period by, (1) re-reducing Fe (III) to Fe (II) through redox cycling, which will participate in Fenton to generate more $^{\bullet}OH$;[32] (2) by auto-oxidation to produce H_2O_2 or $O_2^{\bullet-}$ which enhance $^{\bullet}OH$ formation through Fenton reaction or Harber-Weiss reaction,[33] or (3) by yielding quinone-type pro-oxidant.[34]

A significant correlation was established between the logarithm of rate constant and ΔH_f described as:

$$\log Ks = -0.300(\pm 0.019)\Delta H_f + 16.568(\pm 0.470)$$

$$n = 13,\quad r^2 = 0.8416,\quad F = 260.79,\quad P \leq 0.0001 \qquad (2)$$

From eq 2 one can see that ΔH_f accounted most for the reactivity of these phenolic compounds, and the increase of log *Ks* paralleled a decrease of ΔH_f value, the difference of heat of formation between a mother molecule and its phenoxyl free radical. Since ΔH_f value is a measure of bond strength, the smaller the ΔH_f value, the weaker the O–H bond. It is not difficult to understand weaker O–H bond will facilitate hydrogen-abstraction, promising more active antioxidants.

Selecting independent variables using stepwise multilinear regression (with MAXR Model), a satisfactory QSAR model was found between hydroxyl radical scavenging activity and calculated theoretical parameters (enthalpy of homolytic O–H bond cleavage ΔH_f, enthalpy of single electron transfer *Ea*, ionization potential or ability to donate electron approximated by E_{HOMO}, and stability of the corresponding phenoxyl

radical after H-abstraction from phenolic compounds Ds^r).

$$\begin{aligned}\text{Log } Ks = {} & -0.227(\pm 0.028)\Delta H_f + 0.171(\pm 0.047)\\ & Ea + 0.169(\pm 0.043)E_{HOMO} - 9.807(\pm 3.600)\\ & Ds^r + 20.4949(\pm 1.091)\end{aligned}$$

$$n = 13,\quad r^2 = 0.9890,\quad F = 179.07,\quad P \le 0.0001 \qquad (3)$$

Statistical significance of the independent variables is, ΔH_f: $F = 64.65$, $p \le 0.0001$; Ea: $F = 13.19$, $p \le 0.0067$; E_{HOMO}: $F = 15.41$, $p \le 0.0044$, and Ds^r: $F = 7.42$, $p \le 0.0261$. These results strongly suggested all the retained independents contributed significantly to the QSAR model or the correlation between hydroxyl radical scavenging activity and calculated theoretical parameters.

Further standardizing eq 3 gave the following relative contribution of independent variables: 4.6% for E_{HOMO}, 6.2% for Ds^r, 4.1% for Ea and 85.1% forΔH_f. Ds^c was not retained in eq 3, indicating it was not important in the hydroxyl radical scavenging mechanism. The percentage analysis confirmed the importance of hydroxyl O–H bond strength (bond dissociation energy approximated by ΔH_f) and that it contributed most to the model.[8–11] On the other hand, it demonstrates that hydrogen abstraction is not the sole mechanism responsible for the reaction between phenolic antioxidants and the free radicals generated from the Fenton reaction. As far as E_{HOMO} is concerned, although its relative contribution is much smaller than that of ΔH_f, it significantly influences the reactivity in statistics. The statistical significance of other calculated physicochemical parameters as well as the improvement of the correlation coefficient further evidenced that multiple mechanisms other than H-abstraction underlie the free radical scavenging reaction, and their contributions could not be neglected. Otherwise, one-sidedness is inevitable. Taking this into account, it was not difficult to understand why HOMO energy level alone exhibits good relation with activity of tocopherolic antioxidant in some studies,[10,17] while it is invalid for predicting the antioxidant activity of flavonoids that possess intramolecular hydrogen bonds;[25,35] and why HOMO energy

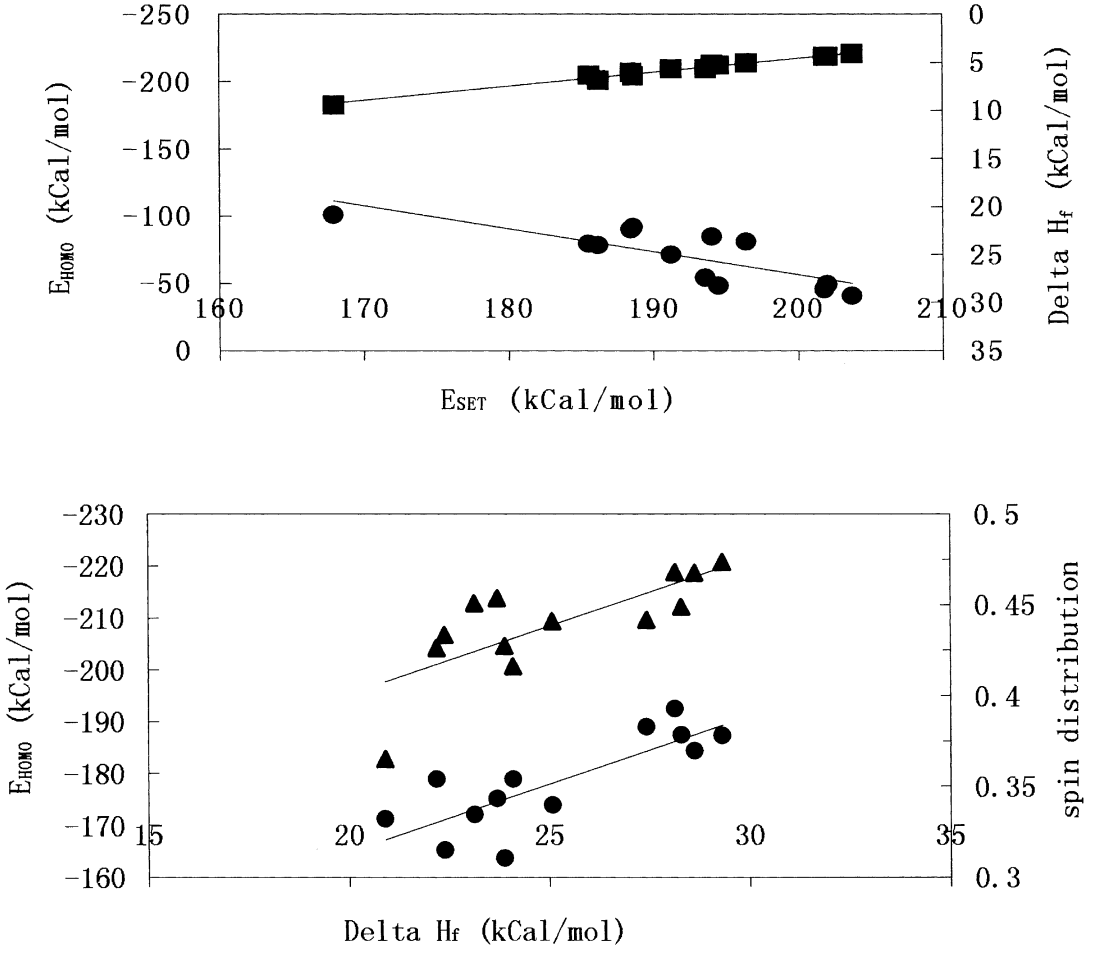


Figure 2. The inter-correlation between one computed molecular orbital parameters.

level can give a correct prediction for the activity difference between molecules with or without a meta-methyl group, but O–H BDE can not.[10] These discrepancies in assessing and predicting antioxidant activity revealed the one-sidedness in terms of only one mechanism involved, and also explained the superficial phenomenon as described by Zhang et al.[18,19]

Comparing eq 2 to eq 3, one can see inclusion of other mechanical parameters such as E_{HOMO}, *Ea* and Ds^r markedly improves the statistical quality and predictive value. In practical terms, increase of hydroxyl-radical quenching reactivity of phenolic compounds (log *Ks*) paralleled the increase of ionization potential or ability to give electron (E_{HOMO}) and enthalpy of single electron transfer (*Ea*), but the decrease of O–H bond dissociation energy (ΔH_f value) and unpaired spin distribution of phenoxyl radicals (Ds^r). It is known that free radicals are stabilized by electron de-localization in a conjugated system. We calculated the spin distribution of free radicals and the corresponding cation radicals as a sum of the square of coefficients of single occupied molecular orbital (SOMO) divided by the number of atoms bearing the unpaired electron (*Ds*).[36] This parameter is of predictive value in estimating the radical stabilization. The lower the *Ds*, the more stable the radical. Based on this principle and the above results, it can be concluded that factors stabilizing phenoxyl radical contribute positively to the reactivity of phenolic antioxidants or, more accurately, facilitate H-abstraction, as further demonstrated by the relation between ΔH_f and Ds^r in Figure 2. However, Ds^c was eliminated from the QSAR model, indicating its contribution to the reactivity is not significant. We ascribed this difference to the possibility that electron reorganization occurs after one-electron transfer.

It should be noted that too many independent variables in one equation may cause chance correlation as described by Topliss and Costello,[37] and incorporation of multiple independent variables correlating with each other may mislead QSAR equation. In order to overcome these problems, several regression approaches such as partial least square analysis (PLSA)[38] and principal component regression (PCR)[39] were developed which involve the formation of orthogonal linear combinations of the original independent variables. However, the new derived variables may be obscure in meaning, and be of disadvantage for definite elucidation of free radical reaction mechanism.[40,41] More seriously, these methods, in which correlated variables are systematically eliminated, can lead to loss of information when applied severely.[41] Therefore, Randic et al.,[40,42–44] who have reported satisfactory QSAR models in their studies, addressed in details the principles for multiple linear regression methods. Following Randic et al.,[42–45] we herein made 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 variables model.[45,46]

Conclusion

The QSAR analyses reported here demonstrated the complexity of the molecular mechanism governing the reactivity of phenolic compounds in scavenging hydroxyl radicals. Among the theoretical parameters investigated, some have been found to be useful to identify active antioxidants in a series of compounds, such as ΔH_f, enthalpy of single electron transfer, HOMO energy level and unpaired spin distribution of phenoxyl radicals. Although H-abstraction contributes most to the hydroxyl-radical-scavenging reactivity, electron transfer and the stability of the corresponding phenoxyl radicals resulting from H-abstraction also play an important role in the radical reaction, as can be seen from the QSAR model derived from the stepwise multi-linear regression. In addition to their significant correlation with experimental reactivity, the molecular theoretical parameters computed by AM1 were also inter-correlated, which also suggested that these compound acted as antioxidants by multiple mechanisms.

In the present work, rate constants of gallic acid and pyrogallol were not determined because of their significant pro-oxidant activity, but it does not affect the regression analysis on activity and structure. Allowing for the same phenolic compounds can act as both antioxidants and pro-oxidants depending on the concentration of compounds and free radical source,[47] a systematic study on pro-oxidant property need to be carried out and to find its relation with concentrations, oxidation potentials and structural features. This work is under progress in our laboratory.

Experimental

Chemicals

Caffeic acid (3,4- dihydroxycinnamic acid) and protocatechuic acid (3, 4- dihydroxybenzoic acid) were purchased from Sigma chemical Co. (St. Louis, MO, USA). 2-thiobarbituric acid and 2-deoxy-D-ribose are from Beijing Jingke Chemical CO. (Beijing, China) and Fluka (AG, Chem. Fabrif), respectively. All other chemicals were of analytical grade and from Beijing Chemical Co. (Beijing, China). Ultra-pure de-ionized water was used to prepare solution.

Second-order rate constant determination by TBARS assay

Scavenging of the hydroxyl radical and the corresponding rate constants were determined as described by Halliwell et al.[20,48] with a slight modification according to Jiang[49] and Lopes et al.[13] Briefly, 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 10 s. The reaction was stopped by dispensing 1 mL of 10% (w/v) trichloroacetic acid,[50] 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. The developed chromogen was determined by reading the absorbance at 532 nm. All solutions were made up immediately before use in de-aerated water except that $FeCl_2$ was dissolved in 1 mM of oxygen-free HCl for preparation of Fe (II) solution.

Theoretical studies

Theoretical parameters, including the difference (ΔH_f) between heat of formation of a mother phenolic molecule (H_p) and that of its phenoxyl radical (H_r), activation energy of intermediate cation radical[36] (*Ea*, also defined as enthalpy of electron transfer[10] and approximated by the difference between heat of cation radical H_c and that of neutral phenolics H_p), spin de-localization of phenoxyl free radical (Ds^r) and intermediate cation radical (Ds^c), HOMO energy levels, were calculated with AM1 method in Gaussian94 package.[51] On the basis of frequency analysis, 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 stronger extent.[52,53]

Statistical analysis

The analysis of variance (ANOVA) was used to assess significance in the quantitative structure–activity relationship and the correlation between computed theoretical parameters. When the *F*-ratio of ANOVA was significant, their relation 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 Inc., USA).

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