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Structure–radical scavenging activity relationships of flavonoids

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Abstract

The objective of this work is to establish the structural requirements of flavonoids for appreciable radical-scavenging activity (RSA) and elucidate a comprehensive mechanism that can explain their activity. To this end, the RSA of 52 flavonoids against 2,2-diphenyl-1-picrylhydrazyl was determined. The relative change in energy (ΔH_f) associated with the formation of various flavonoidal and other phenolic radicals and also the spin distribution in these radicals were determined using computational programmes. By correlating experimental data with ΔH_f, structural features that affect activity have been identified and considered in perspective. It was shown with compelling evidences that the RSA of flavonoids could be mapped to one of their ring systems, making it possible to study their RSA by dissecting their structures and designing representative simpler models. Consequently, hydroxytoluene units were demonstrated to successfully account for the RSA of flavonoids due to ring B and also to satisfactorily do so for activities due to ring A. Further, a comprehensive model for the radical scavenging reactions of flavonoids (and in general, phenolic compounds), which could account for hydrogen atom donation and the termination of aroxyl radicals, was proposed. Finally, prediction of structural features that could endow flavonoids with appreciable radical scavenging capability was made by considering the stability data and the ease of termination. In conclusion, the underlying molecular phenomena of the RSA of flavonoids could be explained by the ease of hydrogen atom abstraction and the ease of the termination of the flavonoidal aroxyl radicals.

Keywords: SAR; Flavonoids; Free radical; DPPH; Heat of formation; Spin distribution

1. Introduction

Flavonoids are a large group of naturally occurring phenolic compounds ubiquitously distributed in the plant kingdom. The various classes of flavonoids differ in the level of oxidation of ring C of the basic benzo-γ-pyrone structure (Peterson and Dwyer, 1998; Cotelle, 2001; Amić et al., 2003). Flavonoids are important components of the human diet. The intake of flavonoids can range between 50 and 800 mg/day, depending on the consumption of vegetables and fruits (Hollman and Katan, 1999; Yang et al., 2001; Lugasi et al., 2003). Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, hepatoprotective, antioxidant, antithrombotic, vasodilating, antiviral and anticarcinogenic activities. However, most interest has been devoted to their antioxidant activity, which is due to their ability to reduce free radical formation and also to scavenge free radicals (Miller, 1996; Pietta, 2000; Mojžišová and Kuchta, 2001; Knekt et al., 2002). Comprehensive accounts on the medicinal significance of flavonoids are provided by Middleton et al. (2000), Narayana et al. (2001) and Havsteen (2002).

In very recent years, flavonoids as potent free radical scavengers have attracted a tremendous interest as possible therapeutics against free radical mediated diseases (Wall, 2000; Amić et al., 2003; Soobrattee et al., 2005). Flavonoids like many other polyphenols are excellent free radical scavengers (chain-breaking antioxidants) because they are highly reactive as hydrogen or electron donors (Cotelle, 2001; Kaur and Kapoor, 2001; Pannala et al., 2001; Yang et al., 2001; Blokhina et al., 2003). Various structure–activ-

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ity relationship (SAR) studies of flavonoids have pointed to the importance of the number and location of the phenolic OH groups present, for effective radical scavenging activity (van Acker et al., 1996; Yokozawa et al., 1998; Middleton et al., 2000; Pannala et al., 2001; Chen et al., 2002; Heim et al., 2002; Amić et al., 2003; Lugasi et al., 2003). Despite a number of consistent lines of evidence supporting the roles of few specific structural components in their radical-scavenging activity (RSA), the correlation between flavonoids' RSA and their chemical structures has stayed elusive (Cotelle, 2001; Amić et al., 2003). In addition, the explanation for the underlying molecular phenomena has not been adequate. One of the major reasons for these setbacks appears to be the strict adherence to classical concepts of SAR. The objective of this work is to establish the structural requirements of flavonoids for appreciable RSA. An attempt has been made to design a consistent and comprehensive model for the RSA of flavonoids. A prediction mechanism for the radical scavenging potentials of flavonoids was established by first designing prediction mechanisms for the hydrogen donating capacity and the ease of termination of flavonoid radicals. To this end experimental data, the theoretically calculated relative change in energy (ΔH_f) associated with the formation of various radicals from flavonoidal and other phenolic structures and also the spin density distribution in these radicals were considered. Ultimately, hydroxylation patterns anticipated to impart appreciable radical scavenging capability have been identified.

2. Results and discussion

DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The free radical DPPH, which shows absorption at 517 nm, is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH assay is considered a valid and easy assay to evaluate the RSA of antioxidants (Sánchez-Moreno, 2002). In agreement with the literature reports (Yang et al., 2001) excellent linear correlations for concentration vs. absorbance were obtained for all flavonoids tested and the concentrations giving 50% reduction in the absorbance of 0.004% DPPH solution (IC_{50}) were determined from the linear curves. The IC_{50} values of the tested flavonoids are given in Table 1. Some of the flavonoids tested were not completely soluble in MeOH and therefore a binary solvent of MeOH and DMSO (1:1) (MD) was used for them. In order to compare the activities of MeOH-soluble flavonoids and MeOH-insoluble ones, a relation coefficient was determined. The determination of this relation coefficient was done by comparing the IC_{50} values of rutin (positive control) in MeOH and in MD. The activity of rutin in MeOH was ca. 2.17 times stronger than in MD. Therefore, the RSA of flavonoids is apt to be ca. twice as stronger in MeOH as in MD. The theoretical IC_{50} values of MeOH-insoluble flavonoids in MeOH were thus approximated by Eq. (1). Dangles et al. (1999) have reported a very significant increase in the activity of quercetin, when going from non-protic to protic solvents. Protic solvents such as MeOH may form hydrogen bond with diphenylamino group of DPPH that would reduce electron delocalisation in DPPH and thus enhance its reactivity.

The most accepted reaction for the RSA of flavonoids is reaction (1) (van Acker et al., 1996; Cotelle, 2001; Amić et al., 2003):

$$\mathrm{Fl{-}OH} + \mathrm{DPPH^{\cdot}} \rightarrow \mathrm{Fl{-}O^{\cdot}} + \mathrm{DPPH_2} \quad (1)$$

Three different termination reactions could be envisaged for the aroxyl radical:

$$\mathrm{Fl{-}O^{\cdot}} + \mathrm{Fl{-}O^{\cdot}} \rightarrow \mathrm{Fl{-}O{-}O{-}Fl} \quad (2a)$$

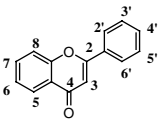
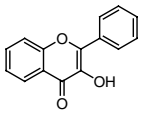
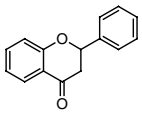
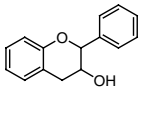
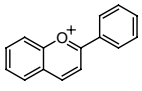
$$\mathrm{Fl{-}O^{\cdot}} + \mathrm{DPPH^{\cdot}} \rightarrow \mathrm{Fl{-}O} + \mathrm{DPPH} \quad (2b)$$

$$\mathrm{Fl{-}O^{\cdot}\ (semiquinone)} \xrightarrow{-\mathrm{H^{\cdot}}} \mathrm{Fl{=}O(quinone)} \quad (2c)$$

Reaction (2a) is coupling reaction between two flavonoid radicals (dimerisation) (Pannala et al., 2001; Amić et al., 2003), reaction (2b) is coupling of a flavonoid radical with a DPPH radical (Pannala et al., 2001; Amić et al., 2003) and reaction (2c) is an alternative and perhaps the predominant termination mechanism which occurs via a further loss of a hydrogen atom from the flavonoid radical to form a quinone (Cotelle, 2001; Pannala et al., 2001; Yang et al., 2001). These termination mechanisms were adopted in this study and they were considered to account reasonably for the experimental observations. Flavonoid radicals seem to follow one or more of these termination mechanisms depending on their structures. Termination reactions do not necessarily lead to termination of radical scavenging reactions since oxidation products (dimers or quinones) and their degradation products may further be reactive towards DPPH radicals (Dangles et al., 1999). Termination of aroxyl radicals is primarily dictated by their spin distribution as suggested by van Acker et al. (1996). A prerequisite for the termination of a flavonoid radical by further loss of hydrogen atom is the presence of a hydroxyl attached to a carbon with a positive spin density (Fig 1). Moreover, for a successful radical scavenging reaction, not only the ease of hydrogen abstraction but also the ease of the termination of the newly formed radical appears to be crucial (van Acker et al., 1996).

A theoretical, quantum chemically determined, suitable parameter for describing the abstraction of a hydrogen atom from an O–H bond is the difference in heat of formation between the flavonoid and its corresponding radical ($\Delta\Delta H_f$ or in the present study, ΔH_f). The delocalisation possibilities within the flavonoid radicals will largely contribute to the corresponding ΔH_f value (van Acker et al., 1996). ΔH_f enables a comparison to be made between the stabilization achieved by hydrogen abstraction (towards radical formation) from alternative positions within an individual molecule, as well as between molecules (Vaya et al., 2003).

Table 1
Experimentally determined DPPH radical scavenging activity of flavonoids

Flavonoid		IC_{50} (μM)
Flavone		
	Luteolin-5-*O*-glucoside (5-*O*-glucoside,7,3′,4′-OH)	5.73 ± 0.13
	Luteolin (5,7,3′,4′-OH)	11.04 ± 0.38
	7,8-Dihydroxyflavone (7,8-OH)	15.50 ± 0.12
	8-Hydroxyacacetin (5,7,8-OH,4′-OMe)	20.28 ± 0.20
	Luteolin 7-*O*-glucoside[a] (7-*O*-glu,5,3′,4′-OH)	28.17 ± 0.69
	Cosmosiin[a] (7-*O*-glucoside-5,4′-OH)	85.67 ± 6.64
	8-Hydroxyflavone (8-OH)	166.43 ± 3.61
	Vicenin-2 (6,8-*C*-glucoside,5,7,4′-OH)	171.28 ± 5.26
	4′-Methoxy-3,6,8-trichloro-5,7-dihydroxyflavone[a] (3,6,8-Cl,5,7-OH,4′-OMe)	201.52 ± 9.34
	5,7-Dihydroxy-3′,4′-dimethoxyflavone[a] (5,7-OH,3′,4′-OMe)	313.18 ± 19.89
	Diosmin[a] (7-*O*-rutinoside,5,3′-OH,4′-OMe)	442.26 ± 26.21
	Apigenin (5,7,4′-OH)	463.40 ± 22.28
	Diosmetin (5,7,3′-OH,4′-OMe)	465.13 ± 15.32
	Chrysin[a] (5,7-OH)	492.57 ± 23.94
	Acacetin[a] (5,7-OH,4′-OMe)	529.80 ± 29.55
	5-Hydroxy-3′,4′,7-trimethoxyflavone[a] (5-OH,7,3′,4′-OMe)	539.84 ± 27.78
	7-Hydroxy-5-methyl-4′-methoxyflavone[a] (5-Me,7-OH,4′-OMe)	808.71 ± 25.45
	3′,4′-Dimethoxy-5-hydroxy-7-methylflavone (5-OH,7-Me,3′,4′-OH)	>1000
	Tectochrysin[a] (5-OH,7-OMe)	>1000
	6-Hydroxyflavone (6-OH)	>1000
	7-Hydroxyflavone (7-OH)	>1000
	5-Hydroxy-7-methylflavone (5-OH,7-Me)	>1000
	Flavone	>1000
Flavon-3-ol		
	Quercetagetin (3,5,6,7,3′,4′-OH)	9.02 ± 0.16
	Rutin (3-rutinoside,5,7,3′,4′-OH)	9.40 ± 0.31
	Isoquercitrin (3-*O*-glucoside,5,7,3′,4′-OH)	9.45 ± 0.06
	Hyperoside (3-*O*-galactoside,5,7, 3′,4′-OH)	10.01 ± 0.00
	Quercetin (3,5,7,3′,4′-OH)	10.89 ± 0.03
	Robinetin (3,7,3′,4′,5′-OH)	11.02 ± 0.56
	Rhamnetin (3,5,3′,4′-OH,7-OMe)	13.50 ± 0.79
	Fisetin (3,7,3′,4′-OH)	14.06 ± 0.21
	Quercetin 3,5-di-*O*-glucoside (3,5-*O*-glucoside,7,3′,4′-OH)	14.41 ± 0.93
	Morin (3,5,7,2′,4′-OH)	17.27 ± 0.13
	Kaempferol (3,5,7,4′-OH)	28.05 ± 0.28
	Galangin (3,5,7-OH)	71.64 ± 1.07
	Isorhamnetin 3-*O*-apiosylrutinoside (3-*O*-apiosylrutinoside, 5,7,4′-OH,3′-OMe)	155.49 ± 4.61
	Quercetin 3,7,3′,4′–tertarmethylether[a] (5,-OH,3, 7,3′,4′-OMe)	261.40 ± 17.93
	Kaempferol 3-*O*-[2″-(4‴-acetylrhamnosyl)(1 → 2)- 6″-glucosyl] glucoside	377.11 ± 16.28
	Kaempferol 3,5-di-*O*-glucoside (3,5-*O*-glucoside,7,4′-OH)	528.37 ± 21.26
	3-Hydroxyflavone (3-OH)	695.93 ± 11.67
	Pentamethoxymorin (3,5,7,2′,4′-OH)	>1000
	Pentamethoxyquercetin (3,5,7,3′,4′-OMe)	>1000
Flavanone		
	Taxifolin (3,5,7,3′,4′-OH)	9.27 ± 0.26
	Hesperetin (5,7,3′-OH,4′-OMe)	236.63 ± 0.86
	Hesperidin (7-*O*-rutinoside, 5,3′-OH,4′-OMe)	281.41 ± 2.62
	Naringenin (5,7,4′-OH)	>1000
	Naringin (5,4′-OH,7-*O*-neohesperidoside)	>1000
	4′-Methoxy-3,6,8-trichloro-5,7-dihydroxyflavanone (3,6,8-Cl,5,7-OH,4′-OMe)	>1000
Flavan-3-ol		
	(−)-Epicatechin (3,5,7,3′,4′-OH)	16.09 ± 0.41
	(+)-Catechin (3,5,7,3′,4′-OH)	18.19 ± 0.93
Anthocyanidin		
	Cyanidin chloride (3,5,7,3′,4′-OH)	11.62 ± 0.11
	Malvin (3,5-O-glu,7,4′-OH,3′,5′-OMe)	20.20 ± 0.73

[a] IC_{50} values estimated from MD system.

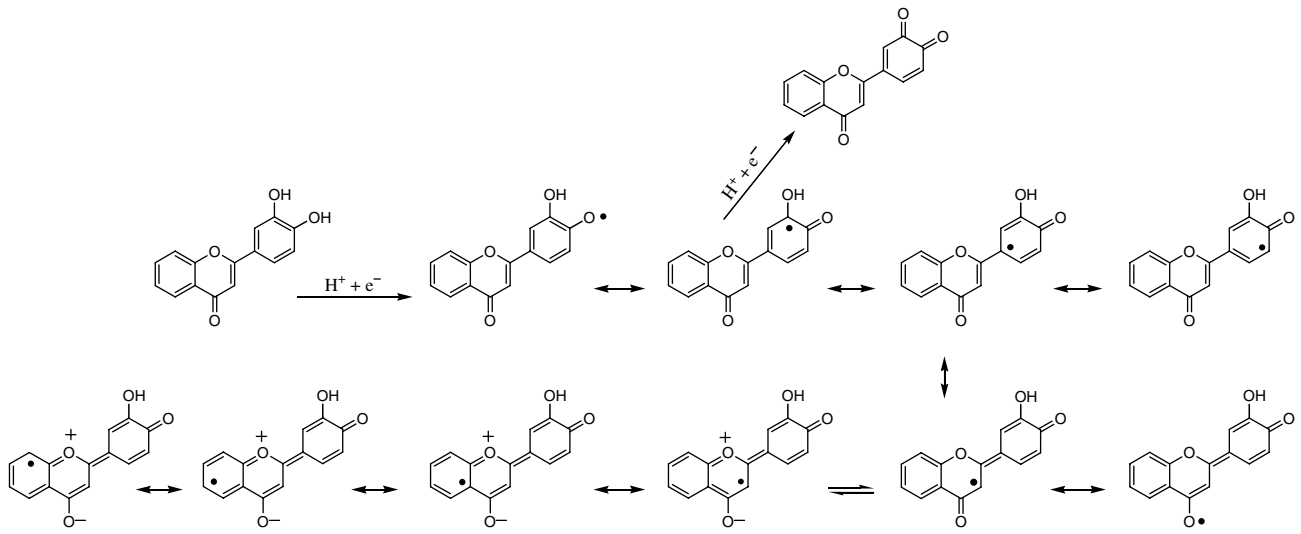


Fig. 1. An illustration for the termination of flavonoid radicals by further loss of a hydrogen atom from a hydroxyl attached to a carbon with a positive spin density.

As could be seen from Table 1, hydroxylation of the inactive flavone structure generally increased RSA. Hydroxyl substituents have long been established as the essential components for flavonoids' RSA (Cotelle, 2001; Yang et al., 2001; Heim et al., 2002; Amić et al., 2003). However, the increase in activity was observed to depend mainly on the position and/or pattern of hydroxylation rather than the number of hydroxyl groups. The effect of hydroxyl substituents on RSA of flavonoids could be one or both of the following kinetic or thermodynamic phenomena: (1) as a source of hydrogen atom(s) in the neutralization of radical species; (2) altering the stability of a flavonoid radical formed by the abstraction of a hydrogen atom from another hydroxyl group.

In accordance with the literature reports, a glance at Table 1 would be quite enough to develop the impression that *ortho*-dihydroxy (catechol), pyrogallol or 3-OH substitutions impart high activity while isolated monohydroxyl or *meta*-dihydroxyl substitutions in most cases do not so. Another observation is that catechol groups could impart appreciable activity when they were found on either ring A (e.g. 7,8-dihydroxyflavone and 8-hydroxyacacetin) or ring B (e.g. luteolin and hyperoside). Also, it can be clearly seen that in flavonoid glycosides, RSA is due to the aglycon parts and the presence of sugars (both qualitatively and quantitatively) does not seem to be essential for appreciable RSA. This is illustrated by, for example, the relatively high and close IC_{50} values of rutin, isoquercitrin, hyperoside and quercetin 3,5-di-*O*-glucoside, and also the significantly different RSAs of kaempferol 3,5-di-*O*-glucoside and quercetin 3,5-di-*O*-glucoside.

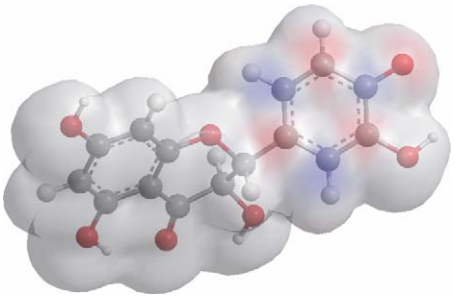
The relatively high and close RSAs of different flavonoids with certain similar features, such as 3-OH, 3′,4′-diOH, 3′,4′,5′-triOH or 7,8-diOH groups, was taken as an indication that the major site of activity could be mapped to some part of the flavonoid structure. In other words, flavonoids with a similar active site could exhibit at least a certain range of variation that does not affect their activity significantly. This could be clarified by a number of examples. For instance, rutin, hyperoside, isoquercitrin, luteolin, luteolin 5-*O*-glucoside, luteolin 7-*O*-glucoside, (+)-catechin, (−)-epicatechin, taxifolin and quercetin 3,5-di-*O*-glucoside are all highly active and have one thing in common, which is 3′,4′-diOH. However, their ring A and/or ring C show considerable variations. Again, morin, quercetin, kaempferol, fisetin, robinetin, rhamnetin, quercetagetin, and also galangin show relatively high activity; once again all of them have 3-OH/4-oxo/Δ_{2-3} in common, yet noticeable differences exit among their A and B rings. Another similar observation is the relatively high and close IC_{50} values of 7,8-dihydroxyflavone and 8-hydroxyacacetin, which possess 7,8-diOH group in common. Once more, these two flavonoids, besides their difference with respect to hydroxylation at C5, their B rings are also noticeably different from one another. These observations gave an indication to the possibility of investigating the RSA of flavonoids by identifying important features, dissecting their structures and ultimately designing representative simpler models.

First, the role of ring B in RSA was investigated and in line with what has just been discussed and also reported (van Acker et al., 1996; Pannala et al., 2001), the substitution pattern of ring A and ring C could successfully be disregarded. For this purpose, luteolin, taxifolin and (+)-catechin, which owe their RSA to their 3′,4′-diOH substituted ring B were selected. The different ΔH_fs associated with the abstraction of hydrogen atoms (Table 2) and also the spin distribution in the most probable radicals of these flavonoids were determined (Fig. 2).

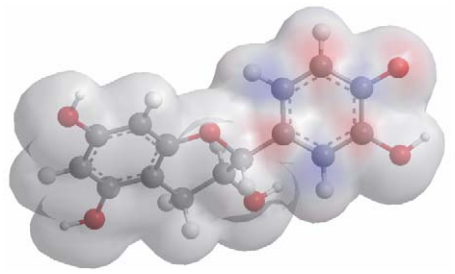
As can be seen from Table 2, in taxifolin, (+)-catechin and luteolin the most likely site that donates hydrogen is 4′-OH. Accordingly, the spin distribution in the aroxyl

Table 2
Calculated differences in heat of formation (ΔH_f) associated with the production of the various possible radicals of selected flavonoids

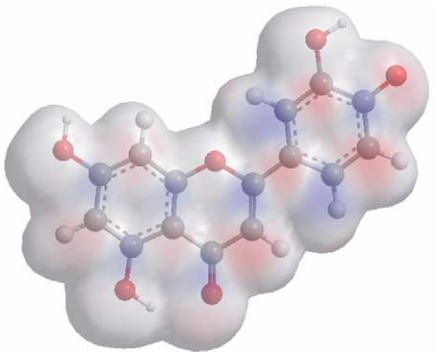
Flavonoid	ΔH_f (kcal/mol)					
	3r	5r	7r	2′r	3′r	4′r
Taxifolin	61.08	46.76	45.23	–	32.20	31.98
(+)-Catechin	58.61	38.30	38.87	–	32.59	32.30
Luteolin	–	47.83	45.01	–	33.48	33.39
Galangin	30.63	47.05	45.84	–	–	–
Kaempferol	30.91	47.92	46.58	–	–	38.22
Morin	27.94	47.51	46.24	37.95		37.52
Quercetin	31.17	46.97	45.67	–	33.49	33.27
Apigenin	–	47.72	44.59	–	–	39.35



a. Taxifolin radical$_{4'\text{-OH}}$



b. (+)-Catechin radical$_{4'\text{-OH}}$



c. Luteolin radical$_{4'\text{-OH}}$

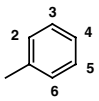
Fig. 2. 3D Maps of the spin density of selected flavonoid radicals. The red region is where positive spin density is expected, which represents better probability of finding the unpaired electron. The blue region, on the hand, represents areas of negative spin density where the spin density is expected to be below the normal.

radicals of these flavonoids formed by the abstraction of a hydrogen atom from their 4′-OH was determined. Fig. 2 depicts the 3D maps of the spin distribution in the respective flavonoid radicals. Also, the spin density at each atom of these flavonoid radicals was estimated (data not shown). In the 3D map of the spin distribution, the red region is where high spin density is expected, which represents better probability of finding the unpaired electron (positive spin density). The blue region, on the contrary, represents the area where the spin density is predicted to be below the normal (negative spin density). Fig. 2c indicates that luteolin radical$_{4'\text{-OH}}$ is stabilized by delocalisation of electrons involving essentially all the ring components of the flavone. On the other hand, it is clear from the spin density of taxifolin radical$_{4'\text{-OH}}$ and (+)-catechin radical$_{4'\text{-OH}}$ that the respective flavonoid radicals are stabilized by electron delocalisation essentially confined to ring B. These observations are in good agreement with the claim that *ortho*-dihydroxy structure in ring B, is the radical target site for all flavonoids with a saturated 2–3 double bond, including flavan-3-ols, flavanones and also cyanidin chloride (Middleton et al., 2000). In all the three cases, termination step could be ascribed to further loss of a hydrogen atom most likely from 3′-OH (Dangles et al., 1999; Cotelle, 2001; Pannala et al., 2001) or possible dimerisation (Pannala et al., 2001). Thus, it appears that ring B has the capacity to impart significant activity by acting as a hydrogen atom donor and also by enabling the formation of a relatively stable flavonoid radical through electron delocalisation, in a more or less similar way to the whole flavone nucleus. In light of this, the 2–3 double bond and/or 4-oxo groups may not be essential for appreciable RSA, which is mainly due to ring B substitution pattern.

It has been suggested that, conjugation between rings A and B permits stability through a resonance effect (Cotelle, 2001; Heim et al., 2002). However, as can be deduced from the above examples, the involvement of rings A and C in the delocalisation of an unpaired electron of ring B-origin may entail the disturbance of their relatively stable structures. It is rational to assume that ring B of taxifolin radical$_{4'\text{-OH}}$ and (+)-catechin radical$_{4'\text{-OH}}$ will be more strained than that of luteolin radical$_{4'\text{-OH}}$, but energy wise this may not be compared to the disturbance of the other stable parts of luteolin. Altogether, this could mean that in flavonoids, if their RSA is due to ring B then not only that the 2–3 double bond, 4-oxo or 3-OH group, or any of their combinations are not essential, but also the rest of the molecule can be disregarded for, at least, theoretical purposes. This fact may enable to analyse and predict possible activity due to ring B by considering simpler models.

For simplicity purpose, therefore, hydroxyl-substituted toluenes were considered. The ΔH_fs of their aroxyl radicals formed by the abstraction of a hydrogen atom from their hydroxyl groups were determined. The distribution of spin density in all these aroxyl radicals was also analysed. Table 3 summarizes the ΔH_fs of all the possible hydroxytoluene

Table 3
Calculated differences in heat of formation (ΔH_f) associated with the production of hydroxytoluene-derived radicals

	ΔH_f (kcal/mol)				
	2r	3r	4r	5r	6r
2-OH	36.54				
3-OH		37.66			
4-OH			36.16		
2,3-OH	31.30	32.12			
2,4-OH	36.63		36.91		
2,5-OH	32.66			33.51	
3,5-OH		38.27		38.27	
3,4-OH		31.34	30.77		
2,6-OH	36.89				36.89
2,3,4-OH	32.79	29.91	32.70		
2,3,5-OH	28.87	30.96		33.67	
2,3,6-OH	29.75	29.13			31.92
2,4,6-OH	38.07		38.23		38.07
2,4,5-OH	32.14		30.03	29.38	
3,4,5-OH		32.79	28.65	32.79	
2,3,4,5-OH	29.21	30.34	29.39	30.09	
2,3,4,6-OH	31.77	27.50	32.36		33.76
2,3,5,6-OH	27.66	29.13		29.13	27.66
2,3,4,5,6-OH	30.22	27.92	31.03	27.92	30.22
2-OH, 3-OMe	33.68				
3-OH, 4-OMe		33.98			
3-OMe, 4-OH			33.52		
3,5-OMe, 4-OH			31.91		

radicals and the radicals of their four methoxyl derivatives. In radicals formed by the abstraction of a hydrogen atom from either 2-OH, 4-OH or 6-OH, C1, C3 and C5 are centres of positive spin density while C2, C4 and C6 are centres of negative spin density. In the case of radicals formed from either 3-OH or 5-OH, the reverse is the case i.e. C1, C3 and C5 have negative spin density while C2, C4 and C6 have positive spin density.

The effect of a substituent is determined by comparing the ΔH_f of the unsubstituted radical with that of the substituted radical. If the data on the ΔH_f of the radicals formed from the simplified models in Table 3 is analysed together with the corresponding spin distributions, it reveals some important relationships between the patterns of hydroxyl substitution and the stability levels of the aroxyl radicals. These relationships are summarized as follows:

- By and large, a hydroxyl group increases the stability of a radical (decrease ΔH_f) if it is substituted on a carbon with a positive spin density and has the opposite effect when it is substituted on a carbon with negative spin density. Magnitude wise, however, stabilizing effect appears to be greater.
- A hydroxyl *ortho* to the hydrogen-donating hydroxyl provides an extra radical stabilizing effect. If there are two such hydroxyls (as in pyrogallol) this extra radical stabilizing effect appears to be provided by only one of them.
- *ortho*-Dihydroxyl groups separated from the hydrogen-donating hydroxyl, by at least one carbon, provide further stabilization.

A substituent effect may be electrostatic or inductive (Havsteen, 2002). An attempt has been made to approximate the quantitative effects of the aforementioned substitution patterns, on the stability of a radical. Careful selection of corresponding pairs is essential for the analysis of both qualitative and quantitative effects of a substitution pattern. The stabilizing effect of a single hydroxyl substituent separated by two carbon atoms from the radical-forming hydroxyl (or similarly, one of the hydroxyl groups of a pyrogallol) is usually in the order of 1.43–4.15 kcal/mole with a mean value of 2.50 ± 0.80 kcal/mole ($n = 14$), while its destabilizing effect is in the order of 0.08–2.02 kcal/mole with a mean value of 1.20 ± 0.58 kcal/mole ($n = 15$). A hydroxyl that is substituted *ortho* to the radical-forming hydroxyl, can bring about a decrease in ΔH_f in the order of 5.30–6.80 kcal/mole, with a mean value of 5.24 ± 1.47 kcal/mole ($n = 18$), which could be attributed to the possibility of hydrogen bonding (van Acker et al., 1996; Amić et al., 2003). Moreover, an *ortho*-dihydroxyl separated from the radical-forming hydroxyl by at least one carbon was observed to decrease the ΔH_f of the corresponding unsubstituted radical by about 1.25–4.62 kcal/mole, with a mean value of 3.04 ± 1.11 kcal/mole ($n = 10$).

In order to describe the level of stability of radicals, ΔH_f needs to be associated with a certain scale. Activity was used as a guide to design a scale for stability since stability is invariably a precondition for activity (van Acker et al., 1996; Amić et al., 2003), although, stability does not necessarily imply activity as it has been shown in this study. Comparison of the RSAs of selected flavonoids with the ΔH_fs of the corresponding simplified models has yielded a striking correlation (Table 4), and from this correlation the following scale was arbitrarily set for the prediction of the stability of hydroxytoluene radicals: $\Delta H_f < 33.0$ kcal/mole = stable and $\Delta H_f > 33.0$ kcal/mole = unstable.

Table 3 was used to predict the ease of hydrogen atom abstraction from ring B and also could help to identify the active parts of flavonoids (Fig. 3). Ultimately, by combining this stability data with the anticipated ease of termination, which is mainly dictated by the spin distribution in a radical, the hydroxylation patterns that could impart appreciable radical scavenging capability were established. Accordingly, the following hydroxyl substitution patterns of ring B of flavonoids including flavones, flavanones, flavonols, dihydroflavonols, catechins and anthocyanidins are expected to be associated with appreciable radical scavenging property: 2′,3′-diOH, 2′,5′-diOH, 3′,4′-diOH, 2′,3′,4′-triOH, 2′,3′,5′-triOH, 2′,3′,6′-triOH, 3′,4′,5′-triOH, 2′,4′,5′-triOH, 2′,3′,4′,5′-tetraOH, 2′,3′,4′,6′-tetraOH, 2′,3′,5′,6′-tetraOH and 2′,3′,4′,5′,6′-pentaOH. Furthermore, 3′,5′-diOMe,4′-OH substitution produced a stable radical and appears to be the main functionality responsible for the excellent RSA of malvin.

Table 4
Correlation between the radical scavenging activity of selected flavonoids and the ΔH_f values of their corresponding representative hydroxytoluene and flavone model radicals

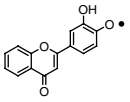
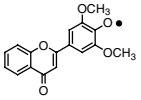
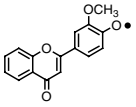
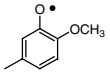
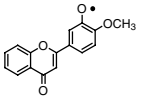
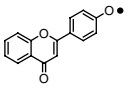
Flavonoid	IC_{50}	Corresponding representative model radicals and heat of formation (ΔH_f) associated with their production			
		Hydroxytoluene		Flavone	
		Model radical	ΔH_f (kcal/mol)	Model radical	ΔH_f (kcal/mol)
Taxifolin	9.27		30.77		33.17
Rutin	9.40				
Isoquercitrin	9.45				
Hyperoside	10.01				
Luteolin	11.04				
Quercetin 3,5-di-*O*-glucoside	14.41				
(−)-Epicatechin	16.09				
Malvin	20.20		31.91		33.92
Isorhamnetin 3-*O*-apiosylrutinoside	155.49		33.52		35.75
Hesperetin	236.63		33.98		35.52
Hesperidin	281.41				
Diosmin	442.26				
Diosmetin	465.13				
Kaempferol 3-*O*-[2″-(4‴-acetylrhamnosyl)-(1 → 2)- 6″-glucosyl] glucoside	377.11		36.16		39.07
Apigenin	463.40				
Kaempferol 3,5-di-*O*-glucoside	528.37				
Naringenin	>1000				
Naringin	>1000				

Table 3 could also be used for the prediction of the stability levels of ring A derived radicals (Fig. 3). However, the nature of the heterocyclic oxygen atom and the 5-OH should be considered for more reliable and consistent prediction. Therefore, the flavone nucleus was used and the effect of the pattern of ring A hydroxylation was studied. The study was also extended to repeat the analysis of ring B hydroxylation pattern using the flavone nucleus. Table 5 summarizes the ΔH_f associated with the radicals of hydroxyl derivatives of flavone produced by the abstraction of a hydrogen atom. These data enabled a more consistent prediction of the stability of flavonoid radicals. The stability scale for the flavonoidal models is $\Delta H_f < 35.40$ kcal/mole = stable and $\Delta H_f > 35.40$ kcal/mole = unstable. This scale is discussed in a subsequent section. Table 6 summarizes the qualitative spin distribution in these flavonoid radicals.

The hydroxylation patterns of ring B that were predicted from the improved flavone models to be associated with appreciable RSA were the same as the ones from the simplified hydroxytoluene models. In case of ring A, major RSA resides mainly on 8-OH, the plausible reason behind being the catechol-like arrangement that it forms together with the heterocyclic oxygen atom. A number of 8-OH containing ring A hydroxyl substitution patterns are predicted to be appreciably active, among which 7,8 dihydroxyflavone and 5,7,8-triOH in 8-hydroxyacacetin have been proven experimentally to be so. On the other hand, 5-OH appears to be the least likely position to donate hydrogen in most cases. The following ring A substitutions are expected to impart appreciable RSA in flavones, flavanones, flavonols, dihydroflavonols, catechins, and anthocyanidins: 5,6-diOH, 5,8-diOH, 6,7-diOH, 7,8-diOH, 5,6,7-triOH, 5,6,8-triOH, 5,7,8-triOH, 6,7,8-triOH, and 5,6,7,8-tetraOH.

According to the predictions based on Tables 3 and 5, kaempferol and morin (Fig. 3) are not expected to have RSA that is mainly due to either ring A or ring B. The fact that these two flavonoids have appreciably low IC_{50} values indicates that ring C may be responsible for their RSA.

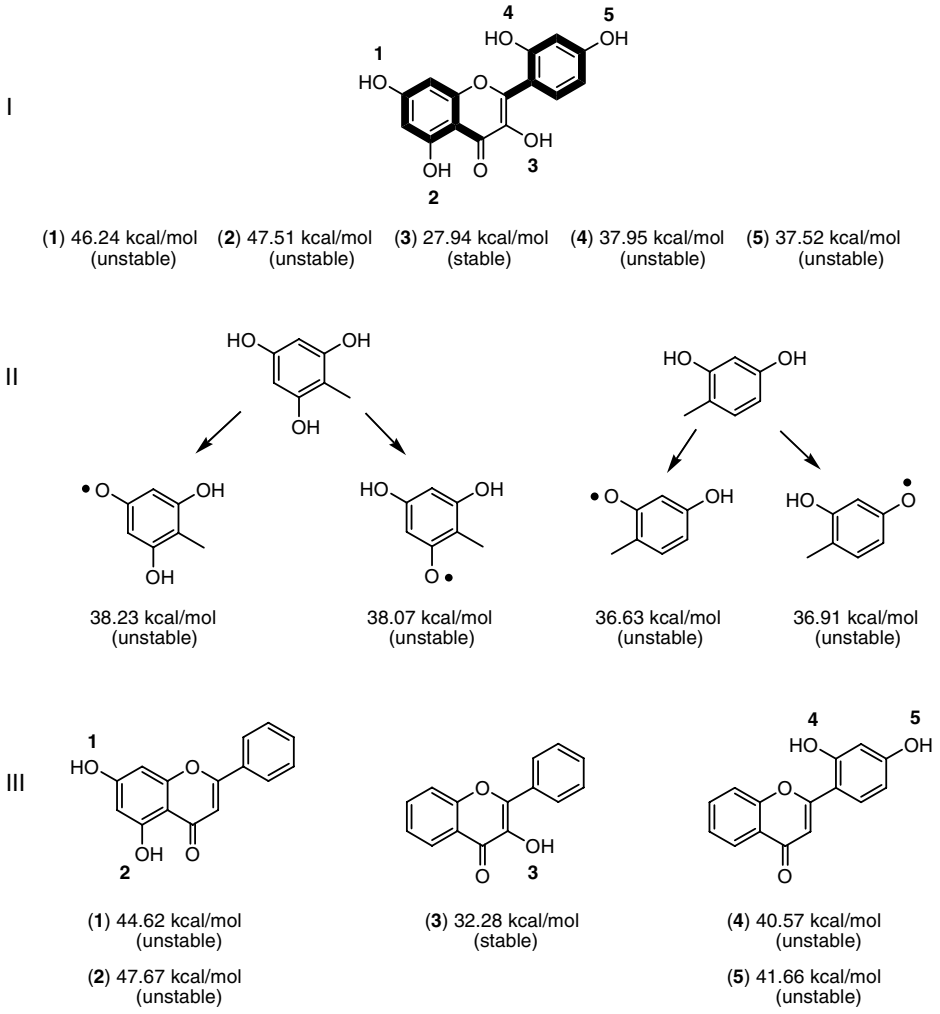
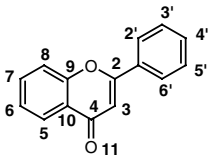


Fig. 3. Identification of the hydrogen-donating hydroxyl of morin, using (I) morin's structure, (II) hydroxytoluene models and (III) flavone models. All the three analyses lead to the conclusion that the first hydrogen is abstracted form 3-OH.

This was ascertained by comparing the ΔH_f associated with the formation of the respective aroxyl radicals. As can be seen from Table 2, in kaempferol and morin, the first hydrogen atom is apparently abstracted from 3-OH. Similarly, the moderate RSA of galangin maps to its 3-OH. Reports also show that flavonoids which lack catechol OHs on ring B, but possess a 3-OH next to the 4-keto group, including galangin have a high RSA (Amić et al., 2003).

Since quercetin and luteolin had almost identical IC_{50} values, maximum effectiveness for radical scavenging cannot be said to require a 3-OH. Nonetheless, the 3-OH, as shown in the preceding discussion, may be responsible for the actual RSA of some flavonoids, and its activity seems to require the presence of 2–3 double bond. The ΔH_f of 3-hydroxyflavone radical is 32.28 kcal/mole, strongly suggesting that hydrogen abstraction from 3-OH is highly favoured, however 3-hydroxyflavone was among the least active flavonoids. It appears that the termination of 3-hydroxyflavone radical could be impaired which ultimately might have resulted in the low RSA of the flavonoid. According to our unpublished hypothesis, in protic media such as MeOH, a very small proportion of flavonoid molecules may undergo homolytic dissociation at their hydroxyl groups giving rise to flavonoidal aroxyl radicals and hydrogen atoms that would exist in equilibrium with the undissociated molecules. The addition of an external radical such as DPPH depletes the hydrogen atoms shifting the equilibrium towards the dissociation of the flavonoid molecules. Yet, the rate of depletion of hydrogen atoms should be paralleled by the termination of the aroxyl radicals if further appreciable dissociation is to occur. Amić et al. (2003) have suggested a termination of the aroxyl radicals formed on the 3-OH of flavonols such as galangin, by the addition of another radical (another aroxyl radical or DPPH) on C2. This was based on the assumption that C2 is more susceptible to radical attack since calculations have shown that it is one of the most suitable centres for the unpaired electron. In the present study, the spin density on C2 of galangin $\text{radical}_{3\text{-OH}}$ was determined to be 1.02

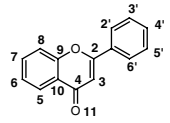
Table 5
Calculated differences in heat of formation (ΔH_f) associated with the production of hydroxyflavone-derived radicals



Ring A	ΔH_f (kcal/mol)				
	5r	6r	7r	8r	
5-OH	45.71				
6-OH		37.11			
7-OH			41.37		
8-OH				35.82	
5,6-OH	37.24	34.87			
5,7-OH	47.67		44.62		
5,8-OH	40.18			31.87	
6,7-OH		33.54	34.82		
6,8-OH		37.91		35.59	
7,8-OH			35.02	32.33	
5,6,7-OH	39.20	33.12	37.66		
5,6,8-OH	34.93	34.71		31.99	
5,7,8-OH	42.82		41.47	30.23	
6,7,8-OH		35.70	33.88	33.22	
5,6,7,8-OH	36.06	32.73	36.89	31.94	
Ring B	**2′r**	**3′r**	**4′r**	**5′r**	**6′r**
2′-OH	39.97				
3′-OH		38.85			
4′-OH			39.07		
2′,3′-OH	33.71	34.36			
2′,4′-OH	40.57		41.66		
2′,5′-OH	35.33			34.73	
3′,5′-OH		39.28		39.28	
3′,4′-OH		33.29	33.17		
2′,6′-OH	39.11				39.11
2′,3′,4′-OH	35.65	32.02	36.38		
2′,3′,5′-OH	30.79	33.23		35.84	
2′,3′,6′-OH	30.23	30.28			33.17
2′,4′,6′-OH	41.69		43.48		41.69
2′,4′,5′-OH	35.32		33.86	31.53	
3′,4′,5′-OH		34.48	30.90	34.48	
2′,3′,4′,5′-OH	31.68	32.60	33.47	33.45	
2′,3′,4′,6′-OH	32.29	28.98	36.10		35.63
2′,3′,5′,6′-OH	27.97	30.06		30.06	27.97
2′,3′,4′,5′,6′-OH	32.00	31.02	36.04	31.02	32.00
2′-OH, 3′-OMe	34.71				
3′-OH, 4′-OMe		35.52			
3′-OMe, 4′-OH			35.75		
3′,5′-OMe, 4′-OH			33.92		

Table 6
Qualitative spin distribution of hydroxyflavone-derived radicals

Radicals formed from



	Qualitative spin distribution at:															
	C2	C3	C4	C5	C6	C7	C8	C9	C10	O11	C1′	C2′	C3′	C4′	C5′	C6′
3-OH, 6-OH, 8-OH, 3′-OH	+	−	+	+	−	+	−	+	−	−	−	+	−	+	−	+
5-OH, 7-OH, 2′-OH[a], 4′-OH	−	+	−	−	+	−	+	−	+	+	+	−	+	−	+	−

+ Positive spin density.
− Negative spin density.
[a] Except in 2′-hydroxyflavone in which the spin distribution is exactly the opposite.

making it the most suitable centre for the unpaired electron. Similarly, the spin density on C2 of 3-hydroxyflavone radical was 1.01 making it once again the most suitable centre for the unpaired electron. However, the low RSA of 3-hydroxyflavone suggests that the actual or at least the predominant termination mechanism for aroxyl radi-

cals formed by the abstraction of a hydrogen atom from the 3-OH might be the further loss of a hydrogen atom to form stable quinones. Applying this termination mechanism to galangin radical$_{3'\text{-OH}}$ for instance, can satisfactorily explain why galangin exhibits appreciable RSA. Galangin forms a stable radical and this radical is terminated by a further loss of a hydrogen atom from 5-OH or 7-OH (since C5 and C7 are centres of positive spin density), the latter being a better choice. It might appear paradoxical to expect a fast abstraction of a hydrogen atom from either 5-OH or 7-OH of galangin and invoke their involvement in the termination of galangin radical$_{3'\text{-OH}}$. However, Dangles et al. (2000) have reported that deprotonation typically lowers the bond dissociation values of the remaining hydroxyl groups of the flavonoid structures they studied. In accordance with the forwarded discussion, it is thus suggested that the termination of 3-hydroxyflavone radical requires a hydroxyl group at either C5, C7, C2′ (C6′) or C4′. Moreover, the presence of 5-OH in particular may enhance the RSA due to 3-OH as it might lead to the formation of a catechol like arrangement in ring C (Heijnen et al., 2001).

Quercetin has both a catechol substituted ring B and 3-OH/4-oxo/Δ_{2-3}. From the forgoing discussion, it is rational to assume that the most likely hydroxyls to donate hydrogen atom would be 4′-OH and 3-OH. In order to determine which hydroxyl preferentially donates hydrogen atom, the ΔH_fs of the five possible quercetin radicals were compared (Table 2). The results strongly suggest that a hydrogen atom is donated from the 3-OH. This is in agreement with literature reports that in the case of quercetin, abstraction of 3-OH hydrogen leads to a more stable radical tautomer (Amić et al., 2003) and 3-OH is the site for hydrogen abstraction in a number of flavonols (Vaya et al., 2003). Nevertheless, in the absence of a free 3-OH, the ring B catechol group could take over, as it appears to be the case in rutin, isoquercitrin, hyperoside and quercetin 3,5-di-*O*-glucoside.

The difference in the active sites of quercetin and taxifolin indicates that RSA due to 3-OH requires the presence of 2–3 double bond. In order to establish the significance of the 4-oxo group in relation to 3-OH activity, the ΔH_f associated with the abstraction of a hydrogen atom from 3-hydroxy-2-phenyl-4*H*-1-benzopyran was determined. The ΔH_f obtained (22.01 kcal/mole) was much lower than that of 3-hydroxyflavone radical, which leads to the conclusion that 3-OH/Δ_{2-3} enables the formation of a very stable radical, even in the absence of 4-oxo group. However, as would be expected, the spin distribution in the radical of 3-hydroxy-2-phenyl-4*H*-1-benzopyran was restricted to rings B and C only, signifying that the 4-oxo group is essential for accessing ring A hydroxyl (5-OH or 7-OH) that may be critical for termination. Nevertheless, if a ring B hydroxyl (2′-OH, 4′-OH or 6′-OH) is available, the presence of 4-oxo group may not be a necessity for RSA associated with 3-OH. Furthermore, it was observed that in the case of 3-OH activity, stability of the 3-OH derived radical does not correlate with actual RSA, which might indicate that the actual RSA depends mainly on the ease of hydrogen abstraction from the second hydroxyl group in the termination step.

Important deviations have been observed in the case of 5-OH and also 8-OH derived radicals when using the simplified hydroxytoluene models and the associated ΔH_f scale. For instance, if 2,3,6-trihydroxytolune is used to predict the stability of 5-OH and 6-OH derived radicals in 5,6-dihydroxyflavone, it leads to the conclusion that both would be stable and pretty much comparable. However, analysis done on 5,6-dihydroxyflavone reveals that the stability levels of the two possible radicals are significantly different, the one derived from 5-OH being unstable. This and other similar observations indicated that 5-OH is associated with low hydrogen donating ability because of the hydrogen bond that it might form with 4-oxo group. Hydrogen bonding increases the stability of the parent flavonoidal structure, and its elimination, conversely, lowers the stability of radicals formed on 5-OH possibly due to the repulsion between the electron clouds of the oxygen on C5 and the carbonyl oxygen of ring C. Similarly, the fixed

Table 7
Approximated quantitative effects of various hydroxylation patterns on the stability of flavonoid radicals

A hydroxylation pattern and its effect on the stability of radicals	Differences between the ΔH_f values[a] of substituted and unsubstituted radicals (kcal/mol)	
	Ring A	Ring B
Stabilizing effect of a single hydroxyl group (separated by two carbon atoms from the radical forming hydroxyl, or one of the hydroxyl groups of a pyrogallol)	2.37 ± 1.60 ($n = 12$)	2.68 ± 1.03 ($n = 14$)
Destabilizing effect of a single hydroxyl group (separated by at least one carbon from the radical forming hydroxyl)	2.59 ± 1.68 ($n = 9$)	1.85 ± 0.89 ($n = 14$)
Stabilizing effect of a hydroxyl group *ortho* to the radical forming hydroxyl	4.71 ± 2.37 ($n = 15$)	5.38 ± 1.80 ($n = 19$)
Stabilizing effect of a catechol group separated from the radical forming hydroxyl by at least one carbon	2.07 ± 1.57 ($n = 4$)	4.06 ± 1.93 ($n = 8$)

[a] Calculated differences in heat of formation associated with the production of radicals.

nature of the heterocyclic oxygen atom could not be accounted by the hydroxytoluene models. The flavone models were thus used to analyse ring A hydroxylation patterns and later on to repeat the analysis done on ring B. Readjustment of the ΔH_f scale has been found to be necessary since the ΔH_fs of corresponding radicals in Tables 3 and 5 differ by 2.40 ± 1.20 ($n = 46$) with the flavone radicals having higher ΔH_f values. Thus, the cutoff point for the stability level of the flavone models was made 35.40 kcal/mole. The scale for the flavone models is consistent with the experimental data on ring A or ring B related activity in flavonoids. The right-hand side in Table 5 shows the correlation between the RSAs of representative flavonoids with the ΔH_fs of the corresponding flavone model radicals.

The effects of hydroxyl substituents on the stability of a flavonoid radical are essentially similar to those observed in the case of hydroxytoluenes. However, a certain degree of variation was noticed for the quantitative effects of the various hydroxyl substitution patterns, in the case of ring A and ring B and thus the two ring systems were separately studied. Table 7 summarizes the results.

The effect of a hydroxyl substituent(s) on radicals formed on another ring system varied considerably. Particularly in flavones and flavonols, the mutual effect between ring A and ring B was observed to be negligible, while the hydroxylation patters of ring A and ring B noticeably affected the stability levels of 3-OH derived radicals (even though this did not correlate with the observed activity). 3-OH in turn could affect the stability levels of ring B derived radicals more than it affected those from ring A. These conclusions were reached at, after comparing the ΔH_fs of the various radicals formed from luteolin, galangin, kaempferol, morin, quercetin, chrysin, apigenin, 4′-hydroxyflavone, 2′,4′-dihydroxyflavone and 3′,4′-dihydroxyflavone. One possible reason for the negligible mutual influence between rings A and B could be the incomplete conjugation between them due to the absence of a perfect co-planarity. As was reported (Cotelle, 2001; Heim et al., 2002) and also observed in this study, in the case of flavones for instance there could be a 20–30° difference between the planes of the two rings.

The theoretical predictions made in the present work were flawlessly consistent with the experimental data. Perusal of the literature also revealed a promising agreement between our predictions and the reported activities of a number of flavonoids, which were not included in this work. Among these for instance, baicalein (5,6,7-trihydroxyflavone) and its glycoside, baicalin (baicalein 7-*O*-glucuronide) have demonstrated high RSA in DPPH assay (Yokozawa et al., 1998; Cai et al., 2005) while as would be expected, astragalin (kaempferol 3-*O*-glucoside) (Cai et al., 2005), afzelin (kaempferol 3-rhamnoside) and kaempferitrin (kaempferol 3,7-dirhamnoside) (Yokozawa et al., 1998) were not active. Yokozawa et al. (1998) have also shown that oxyayanin A (2′,5,5′-trihydroxy-3,4′,7-trimethoxyflavone) and scutellarein (5,6,7,4′-tetrahydroxyflavone) were highly active against DPPH whereas chrysoeriol (4′,5,7-trihydroxy-3′-methoxyflavone) was not.

In conclusion, the underlying molecular phenomena of the RSA of flavonoids could be explained by the ease of hydrogen atom abstraction and the ease of termination of the flavonoidal aroxyl radicals. It was shown with compelling evidences that hydroxytoluene units could successfully account for the RSA of flavonoids due to ring B and also appreciably could do so for activities due to ring A. This has indicated the possibility of synthesizing simplified phenolic structures that retain appreciable RSA. The results generated in this study are in agreement with literature reports. Even though, this work mainly focuses on the effects of hydroxylation patters on the RSA of flavonoids, its approach could be followed to investigate the effect of, literally any substitution pattern (including methoxylation, *C*-methylation and glycosylations) and also to study the SAR of other non-flavonoid aromatic radical-scavenging compounds. Moreover, to the authors' best knowledge, this is the first time that an isolated *para*-dihydroxyl group on either ring A or B is suggested as an active hydrogen donating functionality in flavonoids.

3. Materials and methods

3.1. Chemicals, reagents and solvents

The following authenticated flavonoids were kindly provided by Prof. Wayne Thomas Shier (College of Pharmacy, University of Minnesota, Minneapolis, MN, USA): acacetin, apigenin, (−)-epicatechin, (+)-catechin, chrysin, 5,7-dihydroxy-3′,4′-dimethoxyflavone, 7,8-dihydroxyflavone, 3′,4′-dimethoxy-5-hydroxy-7-methylflavone, cyanidin chloride, fisetin, flavone, hesperidin, 8-hydroxyacacetin, 3-hydroxyflavone, 7-hydroxyflavone, 8-hydroxyflavone, 5-hydroxy-7-methylflavone, 7-hydroxy-5-methyl-4′-methoxyflavone, 5-hydroxy-3′,4′,7-trimethoxyflavone, 4′-methoxy-3,6,8-trichloro-5,7-dihydroxyflavanone, 4′-methoxy-3,6,8-trichloro-5,7-dihydroxyflavone, malvin, morin, naringenin, pentamethoxymorin, pentamethoxyquercetin, taxifolin and tectochrysin. Diosmetin, diosmin, galangin, hesperetin, 6-hydroxyflavone, hyperoside, isoquercitrin, kaempferol, luteolin 5-*O*-glucoside, naringin, quercetagetin, quercetin, quercetin 3,7,3′,4′-tetramethylether, rhamnetin, robinetin, and rutin were kindly donated by Prof. Franz Bucar (Department of Pharmacognosy, Institute of Pharmaceutical Sciences, University of Graz, Austria). Cosmosiin, isorhamnetin 3-*O*-apiosylrutinoside, kaempferol 3,5-di-*O*-glucoside, kaempferol 3-*O*-[2″-(4‴-acetylrhamnosyl)(1 → 2)-6″-glucosyl] glucoside, luteolin, luteolin 7-*O*-glucoside, quercetin 3,5-di-*O*-glucoside and vicenin-2 were obtained from Dr. Abdel Nasser B. Singab (Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Abbassia, Cairo, Egypt). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (95% pure) was purchased from Sigma–Aldrich Chemicals Private Limited (Sitharamapalya,

Mahadevapura, India). MeOH (HiperSolv™ For HPLC) (BDH, England) and dimethylsulphoxide (DMSO) (GRP™) (BDH, England) were purchased from local market.

3.2. Free radical scavenging activity of flavonoids

The free radical scavenging activity of flavonoids was determined with 1,1-diphenyl-2-picrylhydrazyl (DPPH). The method adopted by Burits et al. (2001) was employed in this study with some modifications. 10–500 μl of 0.2–7 mg/ml MeOH solutions of the flavonoids were added to 3, 4 or 5 ml of 0.004% (w/v) DPPH solution (in MeOH). After a 30 min incubation period at room temp. in the dark, the absorbance was read against a blank at 517 nm using a Jenway 6505 UV/Vis spectrophotometer. For flavonoids that were not completely soluble in MeOH, similar procedure was followed with only the MeOH substituted with MeOH–DMSO (1:1) mixture (MD) for preparing solutions of the flavonoids and 0.004% DPPH solution. For each flavonoid, not less than four different concentrations were tested. In order to make the comparison of activity based on the half-maximal inhibitory concentration (IC_{50}) uniform and meaningful, for each flavonoid, the slope of its linear regression curve of concentration vs. absorbance was taken to construct a new linear curve with a *Y*-intercept (absorbance at concentration = 0) equals to 1. Activities were then expressed as the micromolar concentrations giving 50% reduction in the absorbance of a DPPH solution with an initial absorbance of 1.000. For flavonoids that were not soluble in MeOH, their theoretical IC_{50} values in MeOH were approximated by:

$$\frac{IC_{50} \text{ in MD}}{IC_{50} \text{ in MeOH}} = 2.17 \quad (1)$$

3.3. Calculating heat of formation (H_f) and spin density

Molecules were constructed with Chem3D® version 8.0. [Chemoffice® package, 1986-2003 CambridgeSoft Corporation (Cambridge Scientific Computing, Inc.)]. The models were pre-optimised by minimizing the energy with the semi-empirical modelling application, MOPAC/Chem3D and also by rotating around C2–C1′ and bonds connecting methoxyl groups or hydroxyl groups which are not involved in hydrogen bonding. Pre-optimised models were then minimized using the ab initio program, Gaussian 98W/Chem3D® interface. Some models were constructed by modifying others that have passed through the above steps. In most cases, radicals were constructed by deleting hydrogen from a specific hydroxyl. In all species, the maximum number of hydrogen bonds was constructed initially, and the rotations of the hydroxyls were adjusted according to the changes in the positions of radicals (Vaya et al., 2003). Minimizing using Gaussian 98W, was done at the default procedures; restricted Hartree Fock Hamiltonian with the 6-31G minimal basis set, spin 1, for parent structures and unrestricted Hartree–Fock Hamiltonian with the aforementioned basis set and spin 2, for radicals (Jones et al., 2002). The heat of formation (H_f) of all the parent structures and radicals were estimated on the final optimised geometries using MOPAC/Chem3D® (AM1, restricted shell) (Dangles et al., 2000; Jones et al., 2002; Krishnamachari et al., 2004; Wang and Zhang, 2004). Maximum effort was exerted to obtain the global energy minima of a model. The relative change in energy (ΔH_f) in kcal/mol associated with the formation of a radical from its parent structure was calculated by subtracting the heat of formation of the radical from that of the parent structure. The spin density at each atom of a radical was calculated using Gaussian 98W/Chem3D® interface (unrestricted Hartree Fock/6-31G, spin 2). The 3D map of the spin density of the Gaussian-optimised radical species at the isocharge value of 0.00500 a.u was obtained by using Chem3D®.

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References

Amić, D., Davidović-Amić, D., Bešlo, D., Trinajstić, N., 2003. Structure–radical scavenging activity relationships of flavonoids. Croat. Chem. Acta 76, 55–61.

Blokhina, O., Virolainen, E., Fagerstedt, K.V., 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann. Bot. 91, 179–194.

Burits, M., Asres, K., Bucar, F., 2001. The antioxidant activity of the essential oils of *Artemisia afra*, *Artemisia abyssinica* and *Juniperus procera*. Phytother. Res. 15, 103–108.

Cai, Y.Z., Mei Sun, Jie Xing, Luo Q., Corke, H., 2005. Structure–radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. Life Sci. Available online November 30 [Epub ahead of print].

Chen, J.W., Zhu, Z.Q., Hu, T.X., Zhu, D.Y., 2002. Structure–activity relationship of natural flavonoids in hydroxyl radical-scavenging effects. Acta Pharmacol. Sin. 23, 667–672.

Cotelle, N., 2001. Role of flavonoids in oxidative stress. Curr. Top. Med. Chem. 1, 569–590.

Dangles, O., Fargeix, G., Dufour, C., 1999. One-electron oxidation of quercetin and quercetin derivatives in protic and non protic media. J. Chem. Soc., Perkin Trans. 2, 1387–1395.

Dangles, O., Fargeix, G., Dufour, C., 2000. Antioxidant properties of anthocyanins and tannins: a mechanistic investigation with catechin and the 3′,4′,7-trihydroxyflavylium ion. J. Chem. Soc., Perkin Trans. 2, 1653–1663.

Havsteen, B.H., 2002. The biochemistry and medical significance of the flavonoids. Pharmacol. Ther. 96, 67–202.

Heijnen, C.G., Haenen, G.R., van Acker, F.A., van der Vijgh, W.J., Bast, A., 2001. Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups. Toxicol. In Vitro 15, 3–6.

Heim, K.E., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. J. Nutr. Biochem. 13, 572–584.

Hollman, P.C., Katan, M.B., 1999. Dietary flavonoids: intake, health effects and bioavailability. Food Chem. Toxicol. 37, 937–942.

Jones, J.P., Mysinger, M., Korzekwa, K.R., 2002. Computational models for cytochrome P450: a predictive electronic model for aromatic oxidation and hydrogen atom abstraction. Drug Metab. Dispos. 30, 7–12.

Kaur, C., Kapoor, H., 2001. Antioxidants in fruits and vegetables – the millennium's health. Int. J. Food Sci. Technol. 36, 703–725.

Knekt, P., Kumpulainen, J., Jarvinen, R., Rissanen, H., Heliovaara, M., Reunanen, A., Hakulinen, T., Aromaa, A., 2002. Flavonoid intake and risk of chronic diseases. Am. J. Clin. Nutr. 76, 560–568.

Krishnamachari, V., Levine, L.H., Zhou, C., Pare, P.W., 2004. In vitro flavon-3-ol oxidation mediated by a B ring hydroxylation pattern. Chem. Res. Toxicol. 17, 795–804.

Lugasi, A., Hóvári, J., Sági, K.V., Bíró, L., 2003. The role of antioxidant phytonutrients in the prevention of diseases. Acta Biol. Szeged. 47, 119–125.

Middleton Jr., E., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52, 673–751.

Miller, A.L., 1996. Antioxidant flavonoids: structure, function and clinical usage. Alt. Med. Rev. 1, 103–111.

Mojžišová, G., Kuchta, M., 2001. Dietary flavonoids and risk of coronary heart disease. Physiol. Res. 50, 529–535.

Narayana, K.R., Reddy, M.S., Chaluvadi, M.R., Krishna, D.R., 2001. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. Indian J. Pharmacol. 33, 2–16.

Pannala, A.S., Chan, T.S., O'Brien, P.J., Rice-Evans, C.A., 2001. Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. Biochem. Biophys. Res. Commun. 282, 1161–1168.

Peterson, J., Dwyer, J., 1998. Flavonoids: dietary occurrence and biochemical activity. Nutr. Res. 18, 1995–2018.

Pietta, P., 2000. Flavonoids as antioxidants (Review). J. Nat. Prod. 63, 1035–1042.

Sánchez-Moreno, C., 2002. Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Sci. Tech. Int. 8, 121–137.

Soobrattee, M.A., Neergheen, V.S., Luximon-Ramma, A., Aruoma, O.I., Bahorun, T., 2005. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. Mutat. Res. 579, 200–213.

van Acker, S.A., de Groot, M.J., van den Berg, D.J., Tromp, M.N., Donné-Op den Kelder, G., van der Vijgh, W.J., Bast, A., 1996. A quantum chemical explanation of the antioxidant activity of flavonoids. Chem. Res. Toxicol. 9, 1305–1312.

Vaya, J., Mahmood, S., Goldblum, A., Aviram, M., Volkova, N., Shaalan, A., Musa, R., Tamir, S., 2003. Inhibition of LDL oxidation by flavonoids in relation to their structure and calculated enthalpy. Phytochemistry 62, 89–99.

Wall, J., 2000. Antioxidants in prevention of reperfusion damage df vascular endothelium. TSMJ 1, 67–71.

Wang, L.F., Zhang, H.Y., 2004. Unexpected role of 5-OH in DPPH radical-scavenging activity of 4-thiaflavans. Revealed by theoretical calculations. Bioorg. Med. Chem. Lett. 14, 2609–2611.

Yang, B., Kotani, A., Arai, K., Kusu, F., 2001. Estimation of the antioxidant activities of flavonoids from their oxidation potentials. Anal. Sci. 17, 599–604.

Yokozawa, T., Chen, C.P., Dong, E., Tanaka, T., Nonaka, G.I., Nishioka, I., 1998. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2 picrylhydrazyl radical. Biochem. Pharmacol. 56, 213–222.