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Inhibition of LDL oxidation by flavonoids in relation to their structure and calculated enthalpy

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Abstract

Twenty flavonoid compounds of five different subclasses were selected, and the relationship of their structure to the inhibition of low-density lipoprotein (LDL) oxidation in vitro was investigated. The most effective inhibitors, by either copper ion or 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) induction, were flavonols and/or flavonoids with two adjacent hydroxyl groups at ring B. In the presence of the later catechol group, the contribution of the double bond and the carbonyl group at ring C was negligible. Isoflavonoids were more effective inhibitors than other flavonoid subclasses with similar structure. Substituting ring B with hydroxyl group(s) at 2′ position resulted in a significantly higher inhibitory effect than by substituting ring A or ring B at other positions. The type of LDL inducer had no effect in flavonoids with catechol structure. Calculated heat of formation data ($\Delta\Delta H_f$) revealed that the donation of a hydrogen atom from position 3 was the most likely result, followed by that of a hydroxyl from ring B. Position 3 was favored only in the presence of conjugated double bonds between ring A to ring B. This study makes it possible to assign the contribution of different functional groups among the flavonoid subclasses to in vitro inhibition of LDL oxidation.

Keywords: Flavonoids; Oxidation; LDL; Heat of formation; Free radical

1. Introduction

Flavonoids are compounds which are an integral part of the human diet, with an estimated daily intake of 0.023–1 g/day. They exhibit a wide range of biological activities, of which antioxidation is the most thoroughly explored (see reviews Croft, 1998; Di Carlo et al., 1999). The anti-atherosclerosis effects of flavonoids from red wine (Renaud and de Lorgeril, 1992), licorice (Fuhrman et al., 1997; Vaya et al., 1997), soybean (Ruiz-Larrea et al., 1997), tea (van het Hof et al., 1997), pomegranate (Aviram et al., 2000), olive oil (Visioli and Galli, 1995), ginger (Fuhrman et al., 2000) and garlic (Phelps and Harris, 1993) have been confirmed, as well as their inhibitory effects on low-density lipoprotein (LDL) oxidation in vitro and their ability to decrease ex-vivo LDL susceptibility to oxidation. This protecting property may arise from their ability to scavenge free radicals and thus eliminate reactive oxygen species (ROS), and/or chelate transition metal ions and remove potential initiators (Morel et al., 1993). Flavonoids can also prevent the destruction of LDL endogenous antioxidants, such as tocopherols, β-carotene, lycopene and ubiquinol (Belinky et al., 1998a; Esterbauer, 1995; Esterbauer et al., 1992), inhibit cell-mediated oxidation of LDL or enzymes involved in initiation reactions (xanthine oxidase, glutathion reductase, lipoxygenase and NADPH-oxidase) (Heinecke, 1997), and can preserve the activity of the high-density lipoprotein (HDL)-associated enzyme, paraoxonase (Aviram et al., 1999).

In many publications, the relationship between the structure of flavonoids and their antioxidant activity has been studied, with reference to their ability to reduce ROS generated artificially in non-biological systems

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(Burda and Oleszek, 2001; Heijnen et al., 2001; Lien et al., 1999; Ng et al., 2000; Rice-Evans et al., 1996). The relation between the structure of flavonoids and their ability to retard or to inhibit LDL oxidation has been investigated less fully (Belinky et al., 1998b; Brown et al., 1998). In the present study, we aimed to extend our previous structure-activity relationship (SAR) study among the isoflavans (Belinky et al., 1998b) to other flavonoid subclasses, namely flavones, flavonols, flavanones, flavanols, and isoflavones. The effects of changes in flavonoid structure (Fig. 1) was investigated in relation to their ability to prevent LDL from oxidation and for their capacity to donate electrons to 1,1-diphenyl-2-picrylhydrazyl (DPPH). Experimental data were correlated with the theoretically calculated differences in the heat of formation between each parent flavonoid and

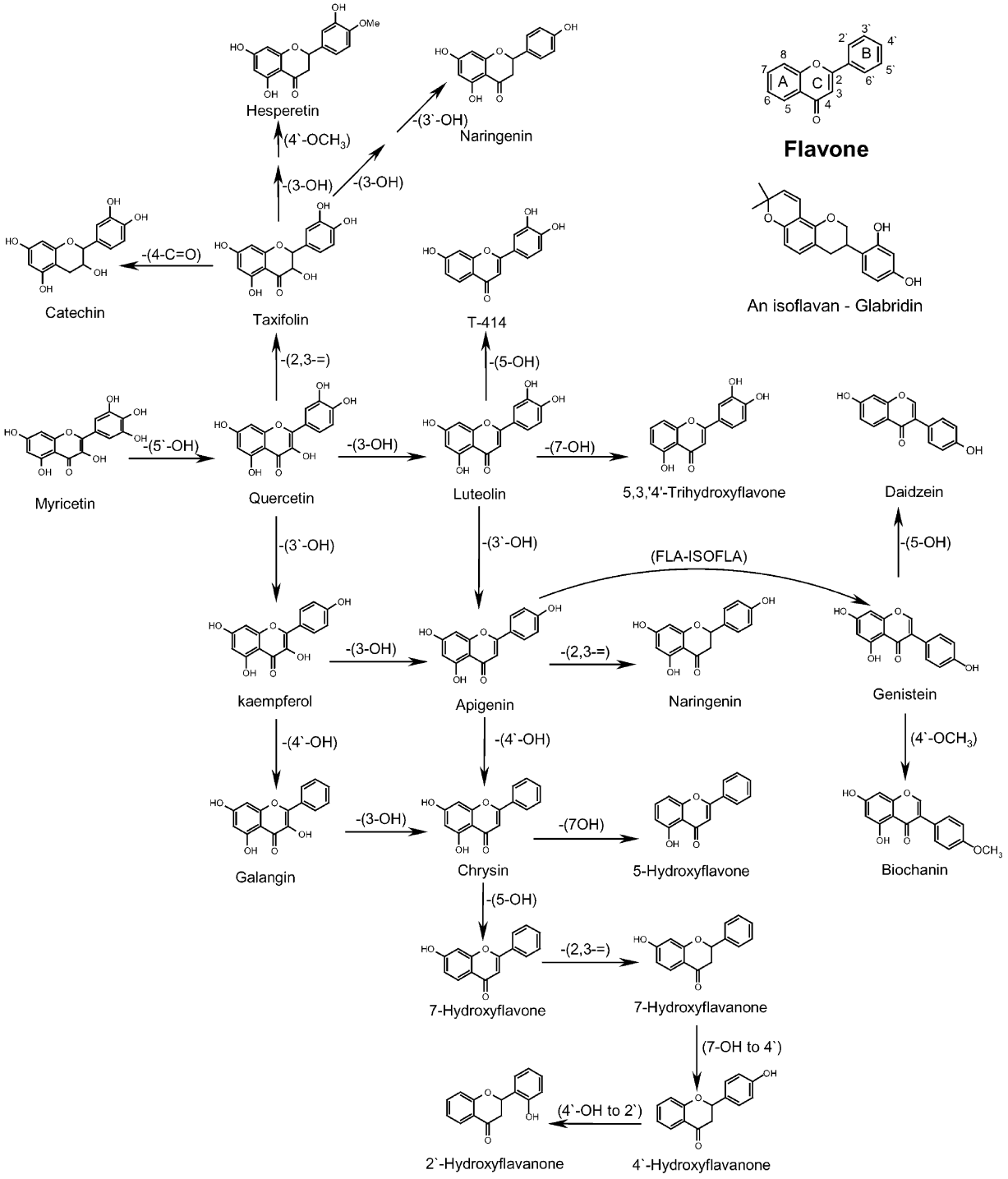


Fig. 1. The molecular structure of selected flavonoids. Arrows show the progressive changes in the structure of the selected flavonoids.

the corresponding radicals ($\Delta\Delta H_f$), produced by abstraction of a hydrogen atom from a hydroxyl moiety in each of the several hydroxyl groups present in the parent molecule.

2. Materials and methods

2.1. Chemicals and apparatus

The following compounds subclasses were selected: from flavones, 7-hydroxyflavone, luteolin (5,7,3′,4′-OH), 5-hydroxyflavone, chrysin (5,7-OH), T-414 (7,3′,4′-trihydroxyflavone) and apigenin (5,7,4′-OH); from flavonol compounds, kaempferol (3,5,7,4′-OH), myricetin (3,5,7,3′,4′,5′-OH), galangin (3,5,7-OH) and quercetin (3,5,7,3′,4′-OH); from flavanones, 2′-hydroxyflavanone, 4′-hydroxyflavanone, taxifolin (3,5,7,3′,4′-OH), naringenin (5,7,4′-OH), hesperetin (5,7,3′-OH, 4′-OMe) and 7-hydroxyflavanone; from isoflavones, daidzein (7,4′-OH), biochanin A (5,7-OH, 4′-OMe) and genistein (5,7,4′-OH); one flavanol, catechin (3,5,7,3′,4′-OH). These compounds were purchased from ICC (Indofine Chemical Company, Inc. Somerville, NJ, USA). 1,1-diphenyl-2-picryl-hydrazyl (DPPH), copper sulfate, thiobarbituric acid (TBA) and Na_2EDTA, were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents were of analytical purity grade. Absorption was determined using a Hewlett Packard model 8552A diode array spectrophotometer (Sunnyvale, CA, USA).

2.2. Human LDL isolation

LDL was prepared from human plasma taken from fasting normolipidemic volunteers. It was separated from the plasma by discontinuous density gradient ultracentrifugation (Aviram, 1983), and dialyzed against saline with Na_2EDTA (1 mM, pH 7.4). The LDL was diluted in PBS to 1000 mg protein/l, and dialyzed overnight against PBS at 4 °C to remove the EDTA.

2.3. Copper ion- and AAPH-induced LDL oxidation

Oxidation of LDL (100 mg protein/l) was carried out in a shaken water bath at 37 °C under air. LDL (1 ml) was incubated with copper ions (5 μM) or 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) (5 mM) for 2 h, in the absence (control) or presence of 8.5 μM of the specific flavonoids tested. All compounds tested were dissolved in ethanol and added to the LDL at the indicated concentration. The final concentration of ethanol in all the LDL samples, including the control, was 0.1%. LDL oxidation was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) (Buege and Aust, 1978) and the amount of total lipid peroxide, which was determined by means of a commercially available kit (cholesterol color reagent, CHOD iodide method, Diagnostica MERCK, Darmstadt, Germany. Conjugated dienes formation (CD) was also measured; LDL (100 μg of protein/ml) was incubated with freshly prepared $CuSO_4$ (5 μM) and LDL oxidation was determined by continuous monitoring the absorbance at 234 nm in the absence (control), or presence of 5 μM of the specific flavonoids tested (Esterbauer et al., 1989). LDL protein concentration was determined by means of Folin phenol reagent (Lowry et al., 1951).

2.4. Free radical scavenging capacity of flavonoids

The free radical scavenging capacity of the compound tested was determined with 1,1-diphenyl-2-picryl-hydrazyl (DPPH). An 0.03 ml aliquot of the compound tested (Fig. 1) in ethanol at various concentrations (0.5, 1, 1.5, 2 or 3 mM) was mixed with 3 ml of 0.1 mM DPPH solution (in ethanol) at 25 °C in a cuvette, and the time course of the optical density change at 517 nm was determined on a diode array spectrophotometer every 5 min, until 60 min and then after 6 h (Stewart, 1989). Where no reaction took place, an additional 6 mole ratio of flavonoid:DPPH was tested. The number of electrons equivalent for each flavonoid was calculated from the test of 0.1 mole equivalent flavonoid/DPPH after 30 min reaction.

2.5. Computer calculations

Molecules were constructed with Cerius^2 1998, (MSI Inc., San Diego, CA) and subsequently subjected to a search for the minimum energy conformation of each. This was achieved by hydroxyl rotations guided by hydrogen bonding ability and by a combination of optimization methods, starting with Steepest Descents and followed by Adopted Basis Newton Raphson, and by the more accurate Truncated Newton method. The semi-empirical quantum mechanical Hamiltonian PM3 (Stewart, 1989) was then employed to determine the final conformations of all the compounds. This gave the heat of formation for all species. All parent and radical species were calculated with the Restricted Hartree-Fock formalism. 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.

3. Results

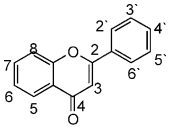
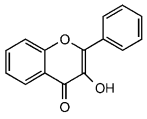
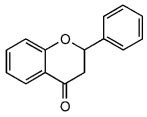
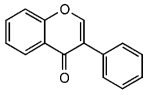
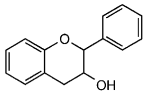
3.1. Inhibition of copper ion- and AAPH-induced LDL oxidation by flavonoids

A series of 20 flavonoids, comprising five different subclasses, was selected on the basis of their structural

differences (Fig. 1), to allow the attribution of specific activity to specific structural variations and functional groups. Out of the 20 flavonoids, six possessed a flavone structure, there were four flavonols, six flavanones, three isoflavones and one flavanol structure (catechin). These flavonoid subclasses were tested for their ability to retard LDL oxidation induced by copper ions or AAPH. The inhibitory effect of each compound is expressed quantitatively. Thus, LDL (100 μg protein/ml) was incubated with copper ions (5 μM) or with AAPH (5 mM) for 2 h at 37 °C, in the absence (control) or presence of 8.5 μM of the specific flavonoid and the level of LDL oxidation was measured, using the malondialdehyde (TBARS, Table 1 and Fig. 2) and lipid peroxides (PD, Fig. 3) methods. In a separate set of experiments LDL (100 μg protein/ml) was incubated with copper ions (5 μM) and the increase in the absorbance at 234 nm (conjugated dienes, CD) was measured continuously, in the absence (control, lag time 37 min) or presence of 5 μM of the specific flavonoid (Table 1). The results obtained demonstrated that:

(a) The most efficient functional group in flavonoids for retarding LDL oxidation by either copper ion or AAPH induction had two hydroxyl groups on ring B in a mutual ortho position. The flavones luteolin and T-414, both without a hydroxyl group at position 3, inhibited LDL from oxidation to a higher degree than the flavonols (CD), due to the two *ortho* hydroxyl groups on ring B. The dominant contribution of the catechol structure to the inhibition of LDL oxidation is exist also among the flavonols (quercetin and myricetin vs. galanging and kaempferol) and flavanone (taxifolin vs. the other). This high inhibitory effect was largely retained even when one of the two ortho hydroxyls (at position 4′) was protected by a methyl group (catechin vs. hesperetin, lag time of 110 vs. 115 min, measuring

Table 1
The antioxidative effect of selected flavonoids on copper ions and AAPH- induced LDL oxidation (% of inhibition, and lag time)

Name of flavonoids		CuSO4		AAPH
		TBARS	CD(lag time)	TBARS
Flavone				
	5-hydroxy (5-OH)	9.6±1.3	63±3	11.8±7.4
	7-hydroxy (7-OH)	(−6.1±4.0)	64±2	9.4±9.0
	Chrysin (5,7-OH)	17.4±1.4	58±1	7.9±4.2
	Apigenin (5,7,4′-OH)	37.8±3.1	60±2	(−9.1±9.4)
	T-414 (7,3′,4′-OH)	94.2±1.1	115±3	93.1±1.1
	Luteolin (5,7,3′,4′-OH)	95.1±0.4	>250	95.3±0.6
Flavon-3-ol				
	Galangin (3,5,7-OH)	93.0±2.0	82±3	74.0±1.6
	Kaempferol (3,5,7,4′-OH)	89.8±3.2	75±5	74.1±2.1
	Quercetin (3,5,7,3′,4′-OH)	94.2±1.7	90±5	92.4±1.0
	Myricetin(3,5,7,3′,4′,5′-OH)	91.4±1.3	92±7	91.4±1.0
Flavanone				
	7-hydroxy(7-OH)	(−10.5±7.1)	62±2	27.3±1.5
	2′-hydroxy(2′-OH)	66.0±4.1	88±4	13.2±4.5
	4′-hydroxy(4′-OH)	18.0±4.8	62±2	21.4±8.7
	Naringenin(5,7,4′-OH)	17.5±1.7	62±3	39.7±1.6
	Hesperetin(5,7,3′-OH,4′-OMe)	77.3±4.3	115±6	74.2±2.4
	Taxifolin(3,5,7,3′,4′-OH)	96.9±3.2	>250	95.3±0.8
Isoflavone				
	Daidzein(7,4′-OH)	78.3±1.2	71±7	21.4±3.2
	Biochanin(5,7-OH,4′-OMe)	0.6±2.0	65±3	34.1±6.9
	Genistein(5,7,4′-OH)	71.0±2.3	85±5	31.6±1.7
Flavanol				
	Catechin (3,5,7,3′,4′-OH)	91.5±1.4	110±6	93.6±0.4

LDL (1 ml of 100 mg protein/l) was incubated for 2 h at 37 °C with 5 μM $CuSO_4$ or with 5 mM AAPH in the absence (control) or presence of each specific flavonoid (8.5 μM), measuring TBARS. % LDL inhibition of oxidation was determined by measurement of TBARS. In separate experiments, LDL (100 μg of protein/ml) was incubated with 5 μM $CuSO_4$ and LDL oxidation was determined by continuous monitoring the absorbance at 234 nm in the absence (control, lag time 37 min), or presence of 5 μM of the specific flavonoids. Results are given as the mean±S.D. ($n=3$).

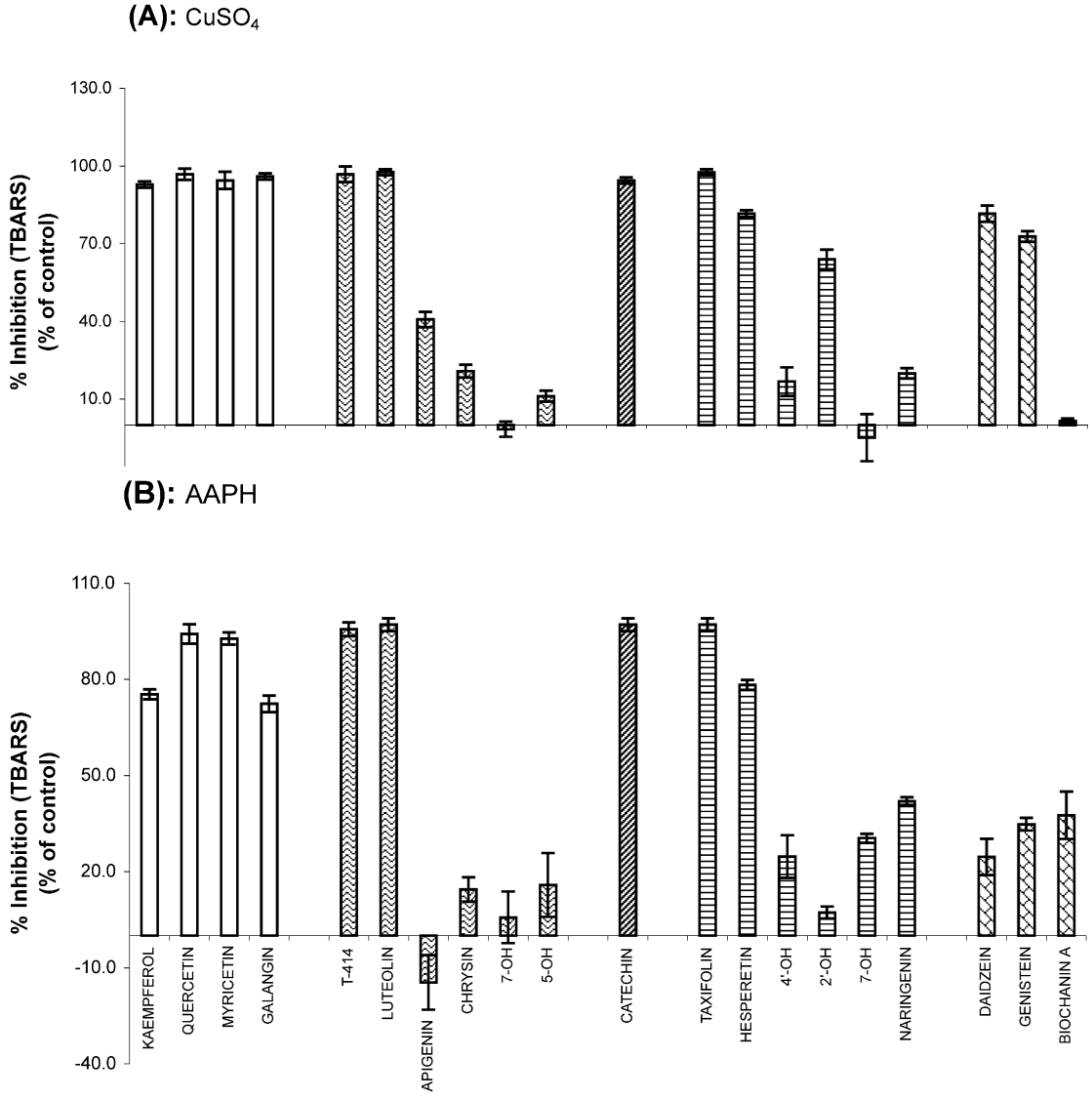


Fig. 2. The inhibitory effects of selected flavonoid compounds on inhibition of LDL oxidation by copper ions (A) and AAPH (B). LDL (1 ml of 100 mg protein/l) was incubated for 2 h at 37 °C with 5 μM $CuSO_4$ (A) or with 5 mM AAPH (B) in the presence of each specific flavonoid (8.5 μM). The% of LDL inhibition was calculated relative to control (0% inhibition) and was determined by measurement of TBARS associated with the LDL. Results are given as the mean ± S.D. ($n = 3$) for both experiments.

conjugated dienes formation, or 91.5 and 77.3% inhibition measuring TBARS, respectively). The inhibitory effect was also maintained when the copper ions were replaced by AAPH as inducer (Table 1 and Fig. 2).

(b) The second most efficient functional group is the presence of a hydroxyl group at position 3 in the flavonoid molecule. Thus, the flavonols; kaempferol, myricetin, galangin and quercetin delayed LDL oxidation by 75–92 min (CD) or 90–94% inhibition, measuring TBARS. The degree of inhibitory activity obtained was independent of the number of hydroxyls at the B ring, unless this increases forms two ortho hydroxyls, measuring TBARS (none in galangin, 1 in kaempferol, 2 in quercetin and 3 in myricetin) and of the same order of magnitude (90–97% inhibition) in different flavonoid subclasses (flavonols—quercetin, flavanone—taxifolin, flavanol—catechin) (Fig. 2). This last conclusion needs further evaluation, since quercetin, taxifolin and catechin although have same hydroxyl groups are differ in their structure, and other factors may influence the observed activity.

(c) A hydroxyl at position 4′, without the adjacent hydroxyl at 3′, gave a lower inhibitory effect, with weak to moderate activity by measuring TBARS (naringenin—17% inhibition vs. hesperetin—77% and apigenin—38% vs. taxifolin—97%, or luteolin—95% inhibition), or by measuring PD (data no shown), or by measuring lag time (naringenin—62 min vs. hesperetin—115 and apigenin—60 vs. taxifolin or luteolin—>250 min).

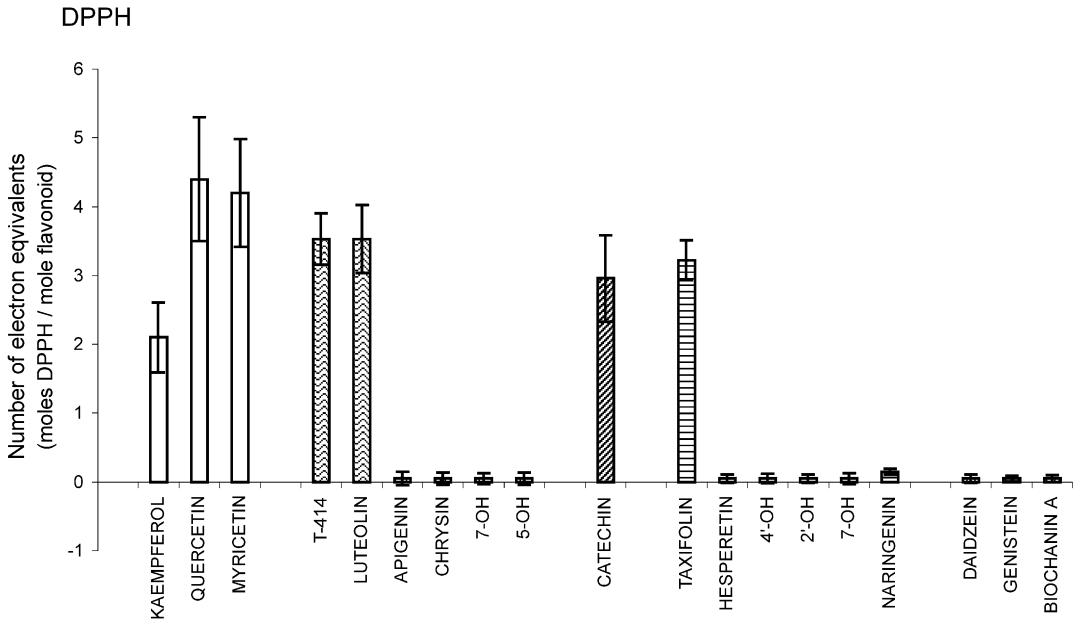


Fig. 3. Effect of selected flavonoid compounds on their ability to donate an electron (B) to DPPH. The number of electrons each molecule of these compounds can donate to a molecule of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was calculated. A 0.03 ml aliquot of the compound (1 mM) in ethanol was mixed with 3 ml of 0.1 mM DPPH solution (in ethanol) and the time course of the optical density change at 517 nm was determined after 30 min. Number of electron equivalent was calculated from the molar ratio of reacted flavonoid/DPPH. Results are given as the mean ± S.D. ($n = 3$) for both experiments.

(d) A hydroxyls at position 7 (flavone 7-OH, flavanone 7-OH) or position 5 (flavone 5-OH), or at both 5 and 7 positions (chrysin) were not sufficient to preserve inhibitory activity (not active, pro-oxidant, 10 and 17% inhibition, respectively, comparing TBARS, or lag time of 58–62 min). A hydroxyl group at position 4′ (flavanonoe 4′-OH) or at both 4′ and 5 and 7 (apigenin, naringinin) afforded compounds almost not active, measuring TBARS or lag time. On the other hand, a hydroxyl group alone at 2′ position (flavanone 2′-OH) presented moderate activity when measuring TBARS (66%) and high when measuring lag time (88 min). The unexpectedly high antioxidant activity of hydroxyl at position 2′, relative to other positions on ring A or B is further evident by testing glabridin, the major antioxidant constituent of the licorice root (Vaya et al., 1997). Glabridin is an isoflavan containing two hydroxyl groups at positions 2′ and 4′, under identical conditions, prevent LDL oxidation with lag time of 115 min (data not shown) (Belinky et al., 1998b; Vaya et al., 1997).

(e) Comparing the different flavonoid subclasses revealed that the isoflavone structure (ring B attached to carbon 3) presented stronger inhibitory activity than flavone and flavanone structures, containing the same number of hydroxyl groups and at the same positions, e.g. genistein (71%, or 85 min) vs. apigenin (38%, or 60 min) and naringenin (17%, or 62 min), measuring TBARS or lag time, respectively (Table 1 and Fig. 2).

(f) A comparison between the two LDL inducers, copper ions and AAPH measuring TBARS, demonstrated that, when the hydroxyl group was at position 3 and/or at positions 3′, 4′, the effect of the flavonoids were generally the same, irrespective of the inducer type (the flavonols, or T-414, luteolin, catechin, hesperetin). However, when there was neither a hydroxyl group at position 3 present in the molecule nor the couple 3′, 4′ hydroxyl groups, the inducer type became of major important, and hence the inhibitory effect of specific flavonoid became more complicated, with some specificity. Thus, apigenin with OH at positions 5 and 7 of ring A and in position 4′ of ring B, demonstrated pro-oxidation when AAPH was the inducer, but became moderately anti-oxidant with Cu^{+2} (38% inhibition). Activity of the flavanone 7-OH changed from low in AAPH (27% inhibition) to slightly pro-oxidant activity with $Cu+^{2}$. The flavanone 2′-OH was practically inactive in AAPH, but became a strong inhibitor with Cu^{+2} (13 vs. 66% inhibition), while biochanin (isoflavone 5,7-OH, 4′-OMe) changed from a moderate anti-oxidant with AAPH (34% inhibition) to inactive with Cu^{+2}.

3.2. Donation of electron(s) to 1,1-diphenyl-2-picrylhydrazyl (DPPH) by flavonoids

DPPH is a molecule containing a stable free radical. In the presence of an antioxidant which can donate an

electron to DPPH, the purple color typical of the free DPPH radical decays, a change which can be followed either spectrophoto metrically (517 nm) or by detecting changes in concentration of starting materials and/or end reaction products, using HPLC analysis (Belinky et al., 1998a). This simple test can provide information on the ability of a compound to donate an electron, the number of electrons a given molecule can donate and on the mechanism of antioxidant action. In cases where the structure of the electron donor is not known (e.g. a plant extract), this method can afford data on the reduction potential of the sample, and hence can be helpful in comparing the reduction potential of unknown materials. Fig. 4 summarizes the results obtained. The number of electrons donated by a given flavonoid was calculated by reacting the flavonoid with DPPH in a range of 0.05–0.3 flavonoid to 1.0 DPPH molar ratios. In instances in which the flavonoid tested was not active even at 0.3 molar ratio after 30 min of incubation at 25 °C, an additional experiment was performed, using flavonoids/DPPH of 6.0 molar ratio. Thus, compounds designated in Fig. 4 as not active, were not active even at that high ratio. Glabridin donated an average of 0.7 electrons/molecule, while quercetin, with five hydroxyl groups, donated an average of 4.4 electrons/molecule. Results shown in Fig. 4 demonstrate that flavonoids which react with DPPH and donate an electron are those with a hydroxyl group at position 3 and/or those with the two adjacent hydroxyl groups at positions 3′, 4′. Thus, compounds which were able to donate electrons to the DPPH molecule were the same as those showed high activity in inhibiting LDL from oxidation induced by copper ion and AAPH.

3.3. Calculation of radical stability by quantum mechanical parameters

Possible explanations of some of the experimental results obtained could be derived from calculating the heat of formation differences ($\Delta\Delta H_f$) between radicals and their parent flavonoids (bond dissociation energy). All heats of formation were calculated by the PM3 semi-empirical Hamiltonian, for energy optimized species (Stewart, 1989). The $\Delta\Delta H_f$ of a given radical (Table 2) represents the difference between the parent flavonoid and the appropriate radical, which was constructed by an abstraction of a hydrogen atom from the assigned hydroxyl moiety. This value may represent the relative stability of a radical with respect to its parent compound, and it enables a comparison to be made between the stabilization achieved by hydrogen abstraction (toward radical formation) from alternative positions within an individual molecule, as well as between molecules. A summary of calculated $\Delta\Delta H_f$ for the H-abstraction from hydroxyl groups in all the flavonoids tested is shown in Table 2. Myricetin contains six hydroxyl groups, two attached to ring A (positions 5 and 7), three to ring B (positions 3′, 4′ and 5′) and one to ring C (position 3). The $\Delta\Delta H_f$ for each of the five possible radicals (two out of the six hydroxyls are

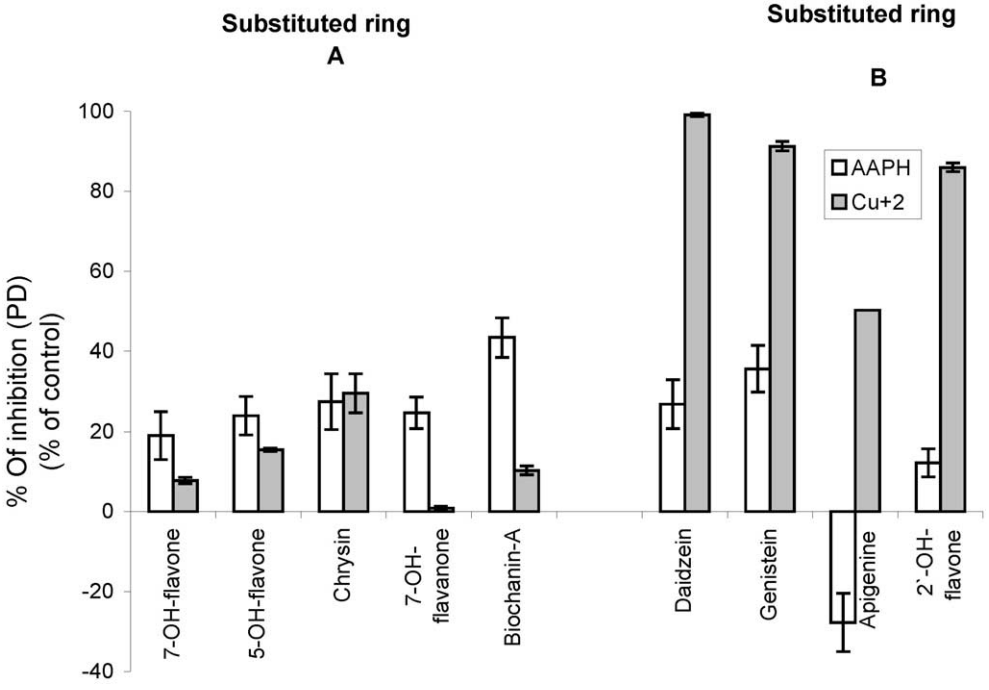


Fig. 4. The inhibitory effect of selected flavonoids on LDL oxidation. % Inhibition of selected flavonoids substituted with OH group(s) at ring A (A) and/or at ring B (B) on LDL oxidation induced by $CuSO_4$ or AAPH, measuring total lipid peroxides (PD). Results are given as the mean ± S.D. ($n = 3$).

Table 2
Calculated differences in heat of formation between the parent flavonoid and each possible corresponding relative radical ($\Delta\Delta H_f$)

Molecule Name	$\Delta\Delta H_f$				
	3r	5r	7r	3′r	4′r
Flavone					
7-OH			38.11		
Luteolin		46.12	41.4	30.19	33.16
5-OH		43.83			
Chrysin		45.71	41.29		
7,3′,4′-OH			38.3	29.94	32.89
Apigenin		45.74	41.27		36.63
Flavonol					
Kaempferol	27.13	45	42.25		36.04
Myricetin	26.95	44.97	42.43	33.24	29.11
Galangin	27.12	44.96	42.31		
Quercetin	27.02	45.29	42.38	29.66	32.70
Flavanone					
2′-OH				36.81	
4′-OH					35.60
Taxifolin	48.86	43.69	41.23	28.64	28.51
Naringenin		44.67	46.91		35.76
Hesperetin		43.64	39.88	30.06	
7-OH			38.30		
Isoflavone					
Daidzein			38.43		34.75
Biochanin		46.86	42.39		
Genistein		45.83	41.46		35.00
Flavanol					
Catechin	47.95	34.39	35.82	28.09	32.01

Structural optimization of each flavonoid and its radical was determined by calculating the minimum energy conformation. The Steepest Descent method was used, followed by the ABNR and Quasi Newton methods and ending with the Truncated Newton method (smart minimizer). MOPAC93 was used to determine the final minimum energy conformation of the compounds and was generated by using the semi-empirical Hamiltonian PM3 in the electronic part of the calculation to obtain molecular orbital, ΔH_f and its derivative.

equivalent) was calculated. The results show that in myricetin, the lowest $\Delta\Delta H_f$ is attached to radical formation at position 3 of ring C (26.9 Kcal/mol), while the next most stable radicals are at positions 3′ (or 5′) and 4′, both of these on ring B (33.2 and 29.1 Kcal/mol, respectively). The two remaining hydroxyls, on ring A, have significantly higher $\Delta\Delta H_f$ values. Thus, the order of preference of positions from which a hydrogen atom is most likely to be donated in the myricetin molecule is 3,4′,3′(or 5′),7,5. As shown in Table 2, hydrogen atom donation from ring B is more likely to occur than from ring A, i.e. abstraction of hydrogen from positions 2′ or 4′ is more likely than from position 5 or 7; and that in ring A, position 7 is favored over position 5.

A correlation has been found between the calculated $\Delta\Delta H_f$ values and the experimental results of antioxidant activity (Fig. 5). Taking the values from the first column of Table 1 ($CuSO_4$, TBARS) for the percent of inhibition in the compounds' heats of formation for all species, we find from the regression analysis a correlation with $R^2 = 0.78$ ($R \sim 0.88$) for the following equation:

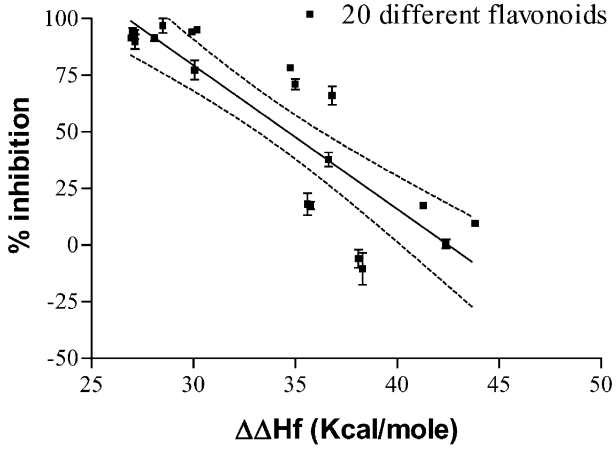


Fig. 5. Regression analysis of the effect of 20 different flavonoids on the% inhibition of LDL oxidation vs. the differences in the enthalpy between each flavonoid's parent compound and its radical ($\Delta\Delta H_f$).

$$\%\text{inhibition} = 270.1 - 6.35\Delta\Delta H_f \ (n = 20) \quad (1)$$

4. Discussion

In continuation of our previous structure–activity relationship study among the flavonoid subclass of isoflavans (Belinky et al., 1998b), the present study illustrates the contribution of different functional groups of the flavonoid subclasses to the inhibition of LDL oxidation induced by copper ions or AAPH and to donate electrons to DPPH. It shows good correlation between the inhibitory effects of the compounds tested and their calculated $\Delta\Delta H_f$.

Twenty commercially available compounds, representing five different flavonoid subclasses, were selected for this study, having such variations in their structure as to enable the attribution of specific LDL oxidation inhibition, to specific functional groups. Under the experimental conditions used, the most effective inhibitors of LDL in vitro oxidation, induced by either copper ions or AAPH, were compounds containing two adjacent hydroxyls at B ring followed by compounds of the flavonols substructure.

Using the oxygen radical absorbance capacity (ORAC), Cao et al. (1997) tested the antioxidant and pro-oxidant behaviors of some flavonoids to determine their structure-activity relationship. They found that the more OH substitutions, the stronger the ORAC activity against peroxyl radical. In the present study on the LDL system, myricetin, with six OH groups, showed the same inhibitory effect on LDL oxidation as galangin and T-414, with three OH groups, or luteolin, with four OH groups measuring TBARS and myricetin was less active than T-414 measuring lag time (or 5,3′,4′-trihydroxyflavone with lag time >250 min, data not shown). This may indicate that, in the LDL system, the presence of

the two adjacent 3′, 4′ OH groups at ring B or 3-OH (when ring C contains a double bond between C2–C3 and the carbonyl group at position 4), are sufficient to obtain strong inhibition of LDL oxidation. Results also show that the contribution of the catechol moiety at the B ring to antioxidant activity was higher than the 25% attributed to this structure in a study, uses the trolox equivalent antioxidant activity assay (Rice-Evans et al., 1996), measuring the ability of an antioxidant to donate a hydrogen atom in an aqueous medium. As the contribution of catechol structure is also significant in AAPH-induced oxidation, although not to the same extent, we can infer that the inhibitory effect of the catechol substructure is not entirely due to a chelation of copper ions, thereby eliminating the oxidative catalyst. The influence of the double bond between C2 and C3 on the inhibition of LDL oxidation was shown to be negligible in the presence of the two adjacent hydroxyl groups. Thus, T-414 and quercetin demonstrated inhibitory activity similar to that of catechin, and luteolin to taxifolin. This finding also indicates that the presence of the means of delocalization of electrons from the aryloxyl radical on the B ring to the ring A is not necessary to obtain high inhibition of LDL from oxidation in these molecules.

As generally accepted, the antioxidative activity of flavonoids was reduced in the absence of the catechol structure at the B ring or 3-OH. The present study showed that in a flavonoid lacking both of these functional groups, minor changes in its structure, changed significantly the inhibition of LDL oxidation. Thus, a hydroxyl at position 2′ reflected significantly higher antioxidative activity in the LDL particle than at position 4′ among the isoflavan subclasses (Belinky et al., 1998b) and among the flavanones (2′-OH, vs. 4′-OH) this is less evident. More apparent is the preference of hydroxyl group(s) on ring B for antioxidant activity, over ring A. Flavone 7- or 5-OH and flavanone 7-OH showed less inhibitory activity (inactive, 10% inhibition and pro-oxidant, respectively) than flavanone 2′- or 4′-OH (64% and 18% inhibition, respectively, measuring TBARS and with copper ion as inducer). Jovanovic et al. (1994) observed that, as ring B is richer in electrons than ring A, it is also the preferred target of any oxidant; and thus ring A reflects an lower antioxidant ability toward a superoxide radical. The present study showed that this preference of OH substitution on ring B over ring A is also valid in the in vitro inhibition of LDL oxidation and when other ROS are involved.

A comparison between isoflavonoids, in which ring B is attached to carbon 3, with flavone or flavanone (ring B attached to carbon 2) revealed the superiority of the isoflavonoids in inhibiting LDL oxidation induced by copper ions. Thus, genistein, with hydroxyl groups at positions 5, 7 and 4′, was a better inhibitor (73 or 91% inhibition, TBARS or PD, respectively) than apigenin, with same three hydroxyl groups (41 or 50% inhibition, TBARS or PD, respectively). Daidzein, with only two hydroxyl groups (7, 4′- OH), showed higher inhibitory activity (78%) than naringenin, with an additional hydroxyl at position 5 (20%). Similar data obtained when lag time was measured; genistein and daidzein 85 and 71 min, respectively relative to apigenin and naringenin of 60 and 62 min, respectively.

Copper ions and AAPH, two in vitro inducers of LDL oxidation, showed differences in their modes of action. While AAPH generated free radicals at a constant rate, at a certain temperature, with a random attack on the endogenous constituents of LDL (Esterbauer et al., 1989), copper ion-induced LDL oxidation required the presence of preformed lipid hydroperoxide, and the rate of LDL oxidation was related to this initial peroxide concentration (Frei and Gaziano, 1993). Different mechanisms by which flavonoids inhibit LDL oxidation may be involved. They may donate a hydrogen atom and thus reduce the free radical generated via the formation of reactive oxygen species (ROS) and/or they may chelate metals ions, such as iron or copper. Brown et al. (1998) showed that during interaction between flavonoids (such as quercetin) with copper ion, it is most likely that an oxidation of the flavonoids occurs at the free 3-OH group, with additional oxidation of the 4′-OH group, and the new flavonoid structure does not revert upon elimination of the metal ion (with EDTA). This finding implies that, during the formation of a copper-flavonoid chelate, an oxidation reduction reaction takes place between them, which further complicates the mode of copper ion mechanism of oxidation. In the present study, some of the flavonoids tested were able both to chelate metal ions and to donate a hydrogen atom, e.g. flavonoids containing the catechol structure at ring B and/or 3-OH, together with C-4 carbonyl group at ring C. The present study demonstrated that, when the OH group is at positions 3′, 4′ and/or at position 3, the effect of the flavonoids (flavonols, T-414, luteolin, catechin, hesperetin) was generally the same, irrespective of the inducer type (TBARS). However, when none of these functional groups were present in the flavonoid structure, a clear difference between the effect of the two inducers arose. Flavonoids with only ring A substituted with OH group(s) inhibited LDL oxidation under both inducers, to about the same level or with slight superiority for AAPH measuring TBARS or PD (Fig. 3). Thus, flavone 5,7-OH presented about the same effect with both inducers; flavanone 7-OH was not active with Cu^{+2} as inducer, but showed 25% inhibitory effect with AAPH (PD). Biochanin showed 10% inhibition with Cu^{+2}, but 44% inhibitory effect with AAPH (PD). On the other hand, when ring B was substituted by a hydroxyl group, the substitution of ring A was of no importance, and the inhibitory effect of these flavonoids was significantly

higher with Cu^{+2} as inducer than with AAPH. Thus, daidzein and genistein strongly inhibited LDL oxidation with Cu^{+2} as inducer, but showed only a weak effect with AAPH. Apigenin showed moderate activity with Cu^{+2} and pro-oxidant with AAPH and the flavanone 2′-OH was almost inactive with AAPH and strong inhibitor with copper ions. Among the isoflavan subclass, glabridin, with OH groups at ring B (at 40 μM), caused 92% inhibition of lipid peroxides vs. 65% inhibition with AAPH (Belinky et al., 1998a). This suggests that, in the presence of a hydroxyl group at B ring, a reduction of the copper ion may take place, while a similar reaction does not occur with the hydroxyl at A ring.

Results from the DPPH experiments revealed that only compounds presenting high inhibitory effect of LDL oxidation, were able to donate electrons to DPPH. The number of electron mole equivalents each flavonoid donated did not entirely correlate with the number of its hydroxyls. Thus, kaempferol with four hydroxyl groups, donated two electrons, whereas luteolin (four OH groups) and T-414 (three OH groups) donated an average of 3.5 electrons each.

Quantum chemical calculations of the geometry of the flavonoids and their corresponding radicals gave their heat of formation. The $\Delta\Delta H_f$ calculated between each flavonoid and its corresponding radicals provides an estimation of the ease with which radicals may be formed (Lien et al., 1999), based on their relative stability (Table 2). Van Acker et al. (1996a) correlated the calculated $\Delta\Delta H_f$ for a group of flavonoids with experimental data from ESR and from their electrochemical oxidation (Ep/1/2) and found the results to be in good agreement. The results shown in Table 2 are also in agreement with our collected experimental data on the inhibitory effect of the selected flavonoids on LDL oxidation and their capability to donate electrons to DPPH. The calculated $\Delta\Delta H_f$ shows that the least energy required for abstracting a hydrogen atom is from a hydroxyl at position 3, when ring C contains a double bond and C-4 carbonyl group (flavonols). This is also the case when in addition to 3-OH, ring B has a catechol structure. Thus, abstraction of a hydrogen atom from position 3 is still slightly favorable (by about 2 Kcal/mole), e.g. kaempferol and galangin, lacking a catechol structure, compared to myricetin and quercetin, which have two adjacent OH groups at ring B. van Acker et al. (1996b) attributed the high activity of quercetin in inhibiting enzymatic and non-enzymatic microsomal lipid peroxidation (LPO) to the combination of the catechol structure with the presence of 3-OH, with an advantage to the former. The present study showed that the presence of catechol structure ensure high activity in inhibition of LDL oxidation but no advantage was found to the combination of both, 3-OH and catechol structure (Table 1 measuring lag time, and Table 2 measuring $\Delta\Delta H_f$). In the absence of flavonol structure, the most favored position for donating a hydrogen atom is from the two adjacent hydroxyls at ring B, with position 3′ preferred over position 4′ (Table 2). In myricetin, in which hydroxyl 4′ is adjacent to two hydroxyl groups at positions 3′ and 5′, the donation of a hydrogen atom from hydroxyl 4′ is favored over position 3′ (or 5′). This may be explained by an additional available hydrogen bond next to an aryloxyl radical (from 3′ and 5′-OH), which confers high stability to the radical, and by further delocalization of the radical due to expanded electron scattering (Bors et al., 1990). In addition, we show clearly, for the first time (Table 2), that hydroxyl 3 is the favored position for donating a hydrogen atom only when ring C contains both, a double bond and a C-4 carbonyl group. Thus, taxifolin, containing the carbonyl group, but without the double bond between C2–C3 and catechin, lacking the double bond and the carbonyl group, both have very high $\Delta\Delta H_f$ values (48.9 and 47.9 Kcal/mole, respectively) for abstracting a hydrogen from 3-OH, and thus hydroxyl 3′ becomes the favored position for the donation of a hydrogen. These findings emphasize the contribution of the conjugation of the double bonds between ring A and B to make position 3 dominant, due to the delocalization effect of the free radical formed.

In summary, the present study suggests the possibility of predicting the degree of contribution of different functional groups among the flavonoid subclasses to their in vitro inhibition of LDL oxidation.

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