Antioxidant and Antiradical Activities of Flavonoids

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The relationship between the structure of 42 flavonoids and their antioxidant and antiradical activities was elucidated by heat-induced oxidation in a β-carotene and linoleic acid system and by the 1,1-diphenyl-2-picrylhydrazyl decoloration test. From seven structurally divergent groups of flavonoids, only flavonols with a free hydroxyl group at the C-3 position of the flavonoid skeleton showed high inhibitory activity to β-carotene oxidation. Antiradical activity depended on the presence of a flavonol structure or free hydroxyl group at the C-4′ position. The effect of the 4′-hydroxyl was strongly modified by other structural features, such as the presence of free hydroxyls at C-3 and/or C-3′ and a C2−C3 double bond.

Keywords: *Flavonoids; antioxidant activity; antiradical activity*

INTRODUCTION

Flavonoids, derivatives of benzo-γ-pyrone, are widespread in plants. Several paper (*1*−*8*) have indicated that these compounds have the property of inhibiting autoxidation reactions and scavenging of free radicals, but the relationship between their structure and activity remains unclear. Flavonoids may possess multiple properties for scavenging reactive oxygen and nitrogen species (*1*−*3*). The presence of an ortho-hydroxylation on the B-ring of the flavonoid molecule, the number of free hydroxyl groups, a C2−C3 double bond in the C-ring, or the presence of a 3-hydroxyl group is usually listed as a condition of antioxidant and antiradical activities (*4*−*8*).

The aim of this study was to elucidate the relationship between the chemical structure of the flavonoids and their ability to inhibit oxidation in a β-carotene−linoleic acid model system and their effectiveness as the scavengers of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. The study involved seven structurally different flavonoid groups: flavones, flavonols, flavanones, dihydroflavonols, isoflavones, biflavanones, and one coumestan, a coumestrol.

MATERIALS AND METHODS

Materials. The majority of the flavonoids were isolated in our laboratory (*9*−*15*). Kaempferol and naringenin were obtained by acid hydrolysis of their glycosides (kaempferol 3,7-dirhamnoside and naringin, respectively). The permethyl derivatives (3,5,7,3′,4′-pentamethoxyflavone and 3,5,7,3′,4′,5′-hexamethoxyflavone) were prepared from quercetin and laricytrin by methylation with dimethyl sulfate (*9*). The remaining compounds were purchased from Fluka AG (flavone, naringin, galangin, and kaempferide), Sigma Chemical Co. (morin, hesperetin, 5,7-dihydroxyflavone, BHT, and D,L-α-tocopherol and its acetate), and ICN Pharmaceuticals, Inc. (flavanone, 3-hydroxyflavone, 7-hydroxyflavone, 8-methoxyflavone, 5-hydroxyflavone, apigenin and robinetin).

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Antioxidant Activity Determination. Heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid was used as the antioxidant activity test model (*16*). One milliliter of β-carotene (0.2 mg/mL) dissolved in chloroform was added to an Erlenmeyer flask containing linoleic acid (0.02 mL) and Tween 20 (0.2 mL). The mixture was than dosed with 0.2 mL of the corresponding flavonoid or standard solution in methanol at a concentration of 10^{-3} M. Fifty milliliters of distilled water, saturated for 15 min with oxygen, was added to the flask. The resulting mixture was shaken and kept for 2 h at 50 °C. The absorbance of the samples was measured on a Hewlett-Packard 8453 spectrometer at 470 nm, immediately after their preparation ($t = 0$ min) and at the end of the experiment ($t = 120$ min). Antioxidant activity was calculated as percent inhibition of oxidation versus control sample without flavonoid added, using the equation

$$\%\ \text{antioxidant activity} = 100 \times [1 - (A_s^0 - A_s^{120})/(A_c^0 - A_c^{120})]$$

where A_s^0 is the absorbance of sample at 0 min, A_s^{120} is the absorbance of sample at 120 min, A_c^0 is the absorbance of control sample at 0 min, and A_c^{120} is the absorbance of control sample at 120 min.

Antiradical Activity Determination. Scavenging free radical potentials were tested in a methanolic solution of DPPH (*17*). The degree of decoloration of the solution indicates the scavenging efficiency of the added substance. For each compound, 1 mL of a 10^{-4} M solution in methanol was added to 2 mL of DPPH solution (10 mg/L). Five minutes later, the absorbance was measured at 517 nm. A reference sample was prepared with 1 mL of methanol. The antiradical activity was calculated as a percentage of DPPH decoloration using the following equation:

$$\text{antiradical activity} = 100 \times (1 - \text{absorbance of sample/absorbance of reference})$$

Determination of the Rate of Flavonoid Oxidation. The spectra of flavonols exhibit two major absorption peaks, which are commonly referred to as band I (350−385 nm) and band II (240−280 nm). The UV absorbance of band I of the flavonoid spectra was measured at the beginning and after 2 h of keeping of the flavonoid solution in the same conditions as the antioxidant activity determination, but without addition of β-carotene.

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Table 1. Antioxidant Activity of Flavonoids (3.9×10^{-6} M)[a] in an Aqueous Emulsion of Linoleic Acid/β-Carotene at 50 °C and Water/Chloroform Partition Coefficient for Flavonoids at 50 °C

compound	antioxidant activity (%)	partition coefficient ($K_{w/c}$)
BHT	95.3[a]	nd
D,L-α-tocopherol	95.8[a]	nd
D,L-α-tocopherol acetate	88.6[a]	nd
kaempferol (**1**)	65.3[b]	0.52
galangin (**2**)	64.9[b]	0.04
quercetin (**3**)	63.6[b]	5.48
morin (**18**)	63.5[b]	10.39
robinetin (**5**)	61.7[b]	15.44
fisetin (**6**)	61.6[b]	9.02
kaempferide (**7**)	60.0[b]	0.07
3-hydroxyflavone (**8**)	59.4[b]	0.18
coumestrol (**42**)	38.7[c]	0.34
laricytrin (**9**)	28.5[d]	0.84
laricytrin 3′-*O*-glucoside (**10**)	26.2[d,e]	2.36
myricetin (**11**)	18.4[e]	6.69
hesperetin (**31**)	4.7[f]	0.02
3,5,7,3′,4′,5′-hexamethoxyflavone (**12**)	2.6[e,f]	0.05
3,5,7,3′,4′-pentamethoxyflavone (**13**)	1.1[f,g]	nd
laricytrin 3,3′-*O*-diglucoside (**14**)	1.1[f,g]	1.07
7-hydroxyflavone(**21**)	0.0[f,g]	0.08
flavone (**19**)	−1.5[f,g,h]	0.01
5-hydroxyflavone (**20**)	−4.0[f,g,h]	0.14
quercetin 3-*O*-glucoside-7-*O*-rhamnoside (**15**)	−6.2[g,h]	92.44
laricytrin 3,7,3′-*O*-triglucoside (**16**)	−6.2[g,h]	16.32
rutin (**17**)	−10.2[h,i]	113.47
taxifolin (dihydroquercetin) (**33**)	−16.8[i,j]	21.31
naringenin (**29**)	−16.8[i,j]	0.55
GB-1a (biflavanone) (**35**)	−16.9[i,j]	nd
kaempferol 3,7-*O*-dirhamnoside (**4**)	−17.5[i,j]	48.58
formononetin (**39**)	−20.4[j,k]	0.04
biochanin A (**41**)	−20.4[j,k]	0.06
chrysin (**22**)	−20.8[j,k,l]	0.08
flavanone (**28**)	−23.0[j,k,l]	0.16
fustin (dihydrofisetin) (**32**)	−23.4[j,k,l]	13.21
genistein (**40**)	−24.6[j,k,l,m]	0.75
luteolin 7-*O*-glucoside (**27**)	−25.3[j,k,l,m]	63.86
8-methoxyflavone (**23**)	−29.2[k,l,m]	0.01
apigenin 8-*C*-glucoside (vitexin) (**25**)	−29.6[k,l,m]	nd
GB-1 (biflavanone) (**34**)	−30.1[l,m]	nd
daidzein (**38**)	32.9[m]	7.16
naringin (**30**)	47.4[n]	55.90
apigenin 7-*O*-glucoside (**26**)	−63.9[o]	10.13
apigenin (**24**)	−78.8[p]	1.21

[a] The concentration is calculated on the basis of the total volume of the emulsion. Means followed by the same letter are not significantly different by LSD multiple-range test at 5% level. For flavonoid structure see Figures 1 and 2. nd, not determined.

Water/Chloroform Partition Coefficient Determination. The coefficient $K_{w/c}$ was calculated as the ratio of the absorbance of a water solution of an individual compound, measured at the maximum of absorption, before and after extraction with chloroform at 50 °C.

RESULTS AND DISCUSSION

The highest antioxidant activities were established for the synthetic antioxidant 2,6-bis(1,1-dimethylethyl-4-methylphenol) (BHT), D,L-α-tocopherol, and its acetate (Table 1; Figures 1 and 2). Slightly lower, but still high, antioxidant activity was shown by a homogeneous group that included fisetin, kaempferol, galangin, quercetin, robinetin, morin, and kaempferide. All of these compounds are flavonols with a free hydroxyl group at the C-3 position. This suggested that the flavonol C-3 hydroxyl group is responsible for the high inhibition of β-carotene oxidation in the heterogeneous system. Comparison of the antioxidant activity of flavonol aglycons with the activity of their glycosides or methyl derivatives showed that the blockage of the C-3 hydroxyl group resulted in a total loss of antioxidant activity (Table 2). Glycosylation or methylation of other flavonol hydroxyls did not produce such an effect.

These results are in agreement with those presented by Chung et al. (*18*) for flavonoids of *Chorizante diffusa* in which the flavonol with a free 3-OH group had a higher antioxidant potential than its substituted derivatives. Joyeux et al. (*8*) also showed the highest antilipoperoxidant activity for free flavonols; taxifolin (dihydroflavonol), rutin, and flavones had distinctly lower activity.

Some apparent discrepancies with the conclusion that the ability of flavonoids to inhibit oxidation processes is controlled by the presence of the double bond between C-2 and C-3 and a free hydroxyl in C-3 position were found in the present study. The antioxidant activities obtained for 3,5,7,3′,4′-pentahydroxy-5′-methoxyflavone (laricytrin), laricytrin 3′-*O*-glucoside, and 3,5,7,3′,4′,5′-pentahydroxyflavone (myricetin) were low. The lower than expected antioxidant activity values obtained for these compounds can be explained by their high sensitivity to oxidation, which causes their rapid oxidation and partial decomposition during measurement. Oxidation was also observed for the other flavonols but to a lesser extent (Table 3).

The flavonoids examined showed different solubility patterns in chloroform. As suggested previously, these differences in solubility may influence results of tests (*19*), but in the present experiments the antioxidant activity values obtained did not correlate with water−chloroform partition coefficients. This may suggest that partition of the compounds between two phases did not significantly influence oxidation results.

As shown by Russo and co-workers (*20*), on the basis of semiempirical calculations, radicals formed by H• removal from hydroxyls at C-3 and C-4′ may be involved in the antioxidant properties of quercetin. The results

Table 2. Effect of Glycosylation or Methylation of Flavonols on Their Antioxidant Activity[a]

flavonol (aglycon)	antioxidant activity (%)	glucoside or methoxyl derivative	antioxidant activity (%)
kaempferol (**1**)	65.3	kaempferide (**7**)	60.0
kaempferol (**1**)	65.3	kaempferol 3,7-*O*-dirhamnoside (**4**)	−17.5
quercetin (**3**)	63.6	quercetin 3-*O*-glucoside-7-*O*-rhamnoside (**15**)	−6.2
quercetin (**3**)	63.6	quercetin 3-*O*-rhamnoglucoside (rutin) (**17**)	−10.2
quercetin (**3**)	63.6	3,5,7,3′,4′-pentamethoxyflavone (**13**)	1.1
laricytrin (**9**)	28.5	laricytrin 3′-*O*-glucoside (**10**)	26.2
laricytrin (**9**)	28.5	laricytrin 3,3′-*O*-diglucoside (**14**)	1.1
laricytrin (**9**)	28.5	laricytrin 3,7,3′-*O*-triglucoside (**16**)	−6.2
laricytrin (**9**)	28.5	3,5,7,3′,4′,5′-hexamethoxyflavone (**12**)	2.6
myricetin (**11**)	18.4	3,5,7,3′,4′,5′-hexamethoxyflavone (**12**)	2.6

[a] For flavonoid structures see Figure 1.

Table 3. Rate of Oxidation of Selected Flavonoids Measured by the Decrease of Absorption in Band I[a]

compound	antioxidant activity (%)	absorption decline in band I of flavonoid spectra (%)
3,5,7-trihydroxyflavone[b] (**2**)	64.9	20.6
3,7,3′,4′-tetrahydroxyflavone[c] (**6**)	67.2	23.0
3,5,7,4′-tetrahydroxyflavone[d] (**1**)	65.3	14.9
3,5,7,3′,4′-pentahydroxyflavone[e] (**3**)	63.6	20.7
3,5,7,5′,4′-pentahydroxy-3′-methoxyflavone[f] (**9**)	28.5	46.5
3,5,7,3′,4′,5′-hexahydroxyflavone[g] (**11**)	18.4	42.7

[a] Band I absorption peak at 350−385 nm. For flavonoid structures see Figure 1. [b] Trivial name: galangin. [c] Triviail name: fisetin. [d] Trivial name: kaempferol. [e] Trivial name: quercetin. [f] Trivial name: laricitrin. [g] Trivial name: myricetin.

Table 4. Antiradical Activities of Flavones, Flavanones, and Biflavanones (3.3×10^{-5} M) in a Methanol Solution of DPPH (1.6×10^{-5} M)[a]

compound	antiradical activity (%)	double bond C2−C3	C3-OH	C4′-OH	*o*-di-OH B-ring
morin (**18**)	96.5[a]	+	+	+	+
taxifolin (dihydroquercetin) (**33**)	94.8[a,b]		+	+	+
kaempferol (**1**)	93.5[b,c]	+	+	+	
GB-2 (biflavanone) (**36**)	92.8[b,c]		+	+	+
fustin (dihydrofisetin) (**32**)	91.9[c,d]		+	+	+
galangin (**2**)	91.8[c,d]	+	+		
rutin (**17**)	90.9[c,d]	+		+	+
quercetin (**3**)	89.8[d,e]	+	+	+	+
luteolin 7-*O*-glucoside (**27**)	87.6[e,f]	+		+	+
quercetin 3-*O*-glucoside-7-*O*-rhamnoside (**15**)	86.8[f,g]	+		+	+
laricytrin (**9**)	84.6[g,h]	+	+	+	+
laricytrin 3′-*O*-glucoside (**10**)	83.8[h]	+	+	+	
robinetin (**5**)	82.3[h]	+	+	+	+
fisetin (**6**)	79.0[i]	+	+	+	+
myricetin (**11**)	72.8[j]	+	+	+	+
kaempferol 3,7-dirhamnoside (**4**)	70.6[j]	+		+	
3-hydroxyflavone (**8**)	66.0[k]	+	+		
apigenin 7-*O*-glucoside (**26**)	34.8[l]	+		+	
hesperetin (**31**)	30.0[l]			+	
vitexin (**25**)	21.0[m]	+		+	
3,5,7,3′,4′,5′-hexamethoxyflavone (**12**)	12.6	+			
GB-1a (biflavanone) (**35**)	11.2[n,o]			+	
GB-1 (biflavanone) (**34**)	9.5[o]			+	+
naringenin (**29**)	6.3[p]			+	
GB-2a (biflavanone) (**37**)	5.6[p]			+	
naringin (**30**)	4.7[p,r]			+	
7-hydroxyflavone (**21**)	2.8[r,s]	+			
flavanone (**28**)	2.6[r,s]				
flavone (**19**)	1.5[s]	+			
chrysin (**22**)	1.1[s]	+			
apigenin (**24**)	0.7[s]	+		+	
8-methoxyflavone (**23**)	0.7[s]	+			
5-hydroxyflavone (**20**)	0.6[s]	+			

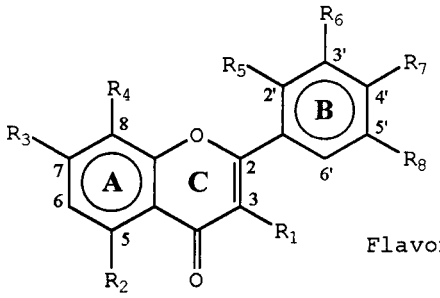
[a] Means followed by the same letter are not significantly different by LSD multiple-range test at the 5% level; see Figures 1 and 2 for flavonoid structure.

of the present experiments reveal that in the heterophasic modeling system used the ability to inhibit β-carotene oxidation by flavonoids depends primarily on the free hydroxyl at C-3 and the double bond between C-2 and C-3.

Of the remaining flavonoids of different structures only coumestrol had moderate antioxidant activity (38.7%). Other flavonoids showed no antioxidant properties under these experimental conditions. It should be noted that there were numerous flavonoids that, instead of inhibiting oxidation, were able to enhance this process, an aspect that requires further elucidation. Flavonol 3-*O*-glycosides have a blocked C-3 hydroxyl group, which in line with the theory presented here, results in their inability to inhibit oxidation. The antioxidant activity for these compounds falls within the range of 1 to −20%, which classifies them as totally inactive. However, in biological systems glycosides may undergo enzymatic hydrolysis, resulting in the formation of active aglycons (*21*). For example, rutin (quercetin 3-*O*-rhamnoglucoside), which is commonly found in plants, upon the hydrolysis of the glycoside bond produces quercetin, a highly antioxidative aglycon.

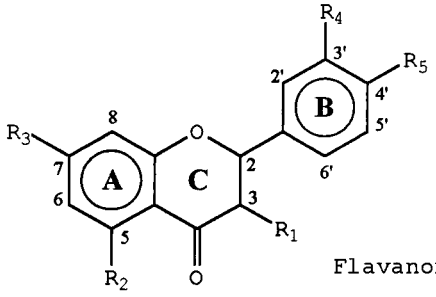
Table 4 show results obtained for the DPPH decoloration test. The flavonoids without any hydroxyl group (flavone, flavanone, and 8-methoxyflavone) or with the free hydroxyls only at C-5 and/or at C-7 (5-hydroxyflavone, 7-hydroxyflavone, and 5,7-dihydroxyflavone) had no effect on scavenging of free radicals. All flavonols with a free hydroxyl in the C-3 position, which were very effective antioxidants, also had high ability to scavenge DPPH radicals. If the compounds that are very sensitive to oxidation (myricetin, laricytrin, and its 3′-*O*-glucoside) are ignored, the correlation between antioxidant activity and antiradical activity is very strong ($r = 0.92$) for this group of flavonoids.

The remaining flavonoids examined showed antiradical activity within a wide range from a few percent to >90%. All of these compounds had a free hydroxyl group at the C-4′ position. It appears that the presence of this hydroxyl group is essential for the antiradical activity of this group of flavonoids. The antiradical effectiveness



Flavonols and flavones

No	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	-OH	-OH	-OH	-H	-H	-H	-OH	-H
2	-OH	-OH	-OH	-H	-H	-H	-H	-H
3	-OH	-OH	-OH	-H	-H	-OH	-OH	-H
4	-O-rha	-OH	-O-rha	-H	-H	-H	-OH	-H
5	-OH	-H	-OH	-H	-H	-OH	-OH	-OH
6	-OH	-H	-OH	-H	-H	-OH	-OH	-H
7	-OH	-OH	-OH	-H	-H	-H	-OMe	-H
8	-OH	-H	-H	-H	-H	-H	-H	-H
9	-OH	-OH	-OH	-H	-H	-OH	-OH	-OMe
10	-OH	-OH	-OH	-H	-H	-O-glu	-OH	-OMe
11	-OH	-OH	-OH	-H	-H	-OH	-OH	-OH
12	-OMe	-OMe	-OMe	-H	-H	-OMe	-OMe	-OMe
13	-OMe	-OMe	-OMe	-H	-H	-OMe	-OMe	-OH
14	-O-glu	-OH	-OH	-H	-H	-O-glu	-OH	-OMe
15	-O-glu	-OH	-O-rha	-H	-H	-OH	-OH	-H
16	-O-glu	-OH	-O-glu	-H	-H	-O-glu	-OH	-OMe
17	-O-rut	-OH	-OH	-H	-H	-OH	-OH	-H
18	-OH	-OH	-OH	-H	-OH	-H	-OH	-H
19	-H	-H	-H	-H	-H	-H	-H	-H
20	-H	-OH	-H	-H	-H	-H	-H	-H
21	-H	-H	-OH	-H	-H	-H	-H	-H
22	-H	-OH	-OH	-H	-H	-H	-H	-H
23	-H	-H	-OMe	-H	-H	-H	-H	-H
24	-H	-OH	-OH	-H	-H	-H	-OH	-H
25	-H	-OH	-OH	-glu	-H	-H	-OH	-H
26	-H	-OH	-O-glu	-H	-H	-H	-OH	-H
27	-H	-OH	-O-glu	-H	-H	-OH	-OH	-H

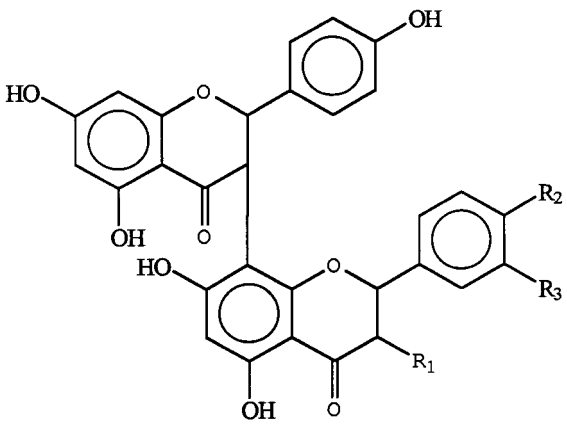


Flavanones and dihydroflavonols

No	R_1	R_2	R_3	R_4	R_5
28	-H	-H	-H	-H	-H
29	-H	-OH	-OH	-H	-OH
30	-H	-OH	-O-neohesp	-H	-OH
31	-H	-OH	-OH	-OH	-OMe
32	-OH	-H	-OH	-OH	-OH
33	-OH	-OH	-OH	-OH	-OH

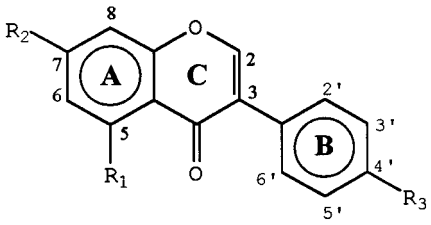
Figure 1. Structures of flavonols, flavones, flavanones, and dihydrovlavonols tested.

of this group can be strengthened by certain other structural features of the flavonoid molecule such as a double bond in the C-ring (C2−C3) and hydroxyl groups at C-3 and/or C-3′ positions. Flavanones with a single bond at C2−C3, which have only one hydroxyl group in the B-ring at C-4′, showed very low antiradical activity (naringin and naringenin). Flavones and flavonols with a substituted hydroxyl group at the C-3 position, which have only a C-4′ hydroxyl in the B-ring, showed distinctly higher activity, ranging from 20 to 70%. Only apigenin showed no antiradical properties. Strong antiradical activity, comparable to that expressed by free flavonols, was shown by compounds with an *o*-dihydroxy system in the B-ring. This group includes dihydroflavonols, flavonol 3-*O*-glycosides, and flavones. It appears that the reason for their high activity is a strong effect of the C-3′ hydroxyl on the reactivity of the hydroxyl at C-4′. This is in agreement with conclusions that the *o*-dihydroxy system in the B-ring of flavonoids is highly effective against free radicals (*8*). We were



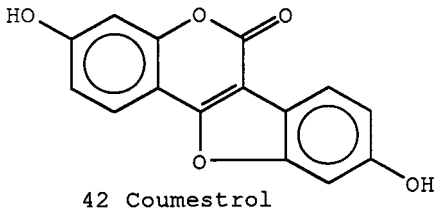
Biflavanones

No	R_1	R_2	R_3
34	-OH	-OH	-H
35	-H	-OH	-H
36	-OH	-OH	-OH
37	-H	-OH	-OH



Isoflavones

No	R_1	R_2	R_3
38	-H	-OH	-OH
39	-H	-OH	-OMe
40	-OH	-OH	-OH
41	-OH	-OH	-OMe



42 Coumestrol

Figure 2. Structures of biflavanones, isoflavones, and coumestrol tested.

unable to explain the influence of the methoxyl groups on antiradical properties. The slight activity found for hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone) suggested that a methoxyl substituted in certain positions can increase the antiradical activity of flavonoids.

The antiradical activity of biflavanones can be explained by the same factors. From the four compounds examined, only GB-2, which consists of naringenin and dihydroquercetin moieties, had the same activity as dihydroquercetin itself. The other biflavanones, which comprised only inactive monomers, showed very low activity. Three isoflavones examined showed very low antiradical activity. We have insufficient results to discuss any structure−activity relationship for this flavonoid group. The moderate antiradical activity of coumestrol correlated with the antioxidant activity of this compound.

ABBREVIATIONS USED

BHT, 2,6-bis(1,1-dimethylethyl-4-methylphenol); DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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