Evaluation of the Total Peroxyl Radical-Scavenging Capacity of Flavonoids: Structure−Activity Relationships

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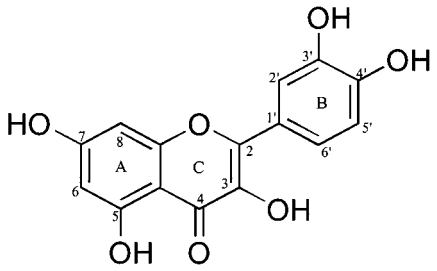
The antioxidant activity of a series of flavonoids against peroxyl radicals generated from thermal homolysis of 2,2′-azobis-amidinopropane was determined by the Total Oxyradical Scavenging Capacity (TOSC) assay. Seven flavonoids with hydroxy and/or methoxy substitution were analyzed and compared to the water-soluble vitamin E analogue Trolox. The most active compound was the flavonol quercetin, followed by its 3-glycoside derivative rutin; these were 7 and 5 times, respectively, better scavengers of peroxyl radical than Trolox. Among the flavones with both hydroxy and methoxy substitution, the most active against peroxyl radicals was the 5,6,4′-trihydroxy-7,8,3′-trimethoxyflavone (thymonin), with a TOSC value 1.5 times greater than that of Trolox. The activity of the remaining flavones was in the following relative order: 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone > 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone (artemetin) > 5,4′-dihydroxy-3,6,7-trimethoxyflavone > 5,6,7,8,2′,3′,4′,5′-octamethoxyflavone (agehoustin A). The data suggest a potential role for dietary intake of flavonoid-containing foods in lowering the risk of certain pathophysiologies that have been associated with free-radical-mediated events.

Flavonoids are polyphenolic compounds that occur commonly in plants;[1] thus, they are frequently components of the diet of numerous herbivores and omnivores, including humans. To date, more than 4000 different flavonoids have been isolated and identified.[2] This list is constantly growing due to the enormous structural diversity associated with these compounds. This diversity arises from the various hydroxylation, methoxylation, sulfation, and glycosylation patterns of ring substitutution. Flavonoids have been shown to elicit antitumoral, antiplatelet, antiischemic, antiallergic, and antiinflammatory activities.[3−11] Along with these activities, flavonoids have also been shown to inhibit the activities of several enzymes, including lipoxygenase and cyclooxygenase,[12,13] monooxygenases,[14] xanthine oxidase,[15] mitochondrial succinoxidase and NADH-oxidase,[16] phospholipase A_2,[17] and protein kinases.[18] The biological activities of the flavonoids are thought to be the result of their antioxidant properties, where the inhibition of the enzymes by flavonoids could be attributed to their ability to react with reactive oxygen species (ROS) formed at or near the reaction center.[19]

The antioxidant activity of the flavonoids varies considerably among the different backbone structures and functional groups. Some of the flavonoids have even proven to be more potent than α-tocopherol in scavenging ROS.[20] The difference in ROS scavenging between the flavonoids can be accounted for by the variation in the number and kind of functional groups present. There are three functional groups that have been attributed to an increase in the ROS-scavenging potential among the flavonoids (Table 1): the *o*-dihydroxy structure of the B ring; the C_2-C_3 double bond in concert with a 4-oxo functionality of the C ring; and the additional presence of both a 3- and a 5-hydroxyl moiety of the C and A rings, respectively.[2] A quantum chemical explanation has been proposed to explain the increase in ROS-scavenging potential of these functional groups, and special attention has been given to those containing the 3-OH functionality.[21]

Table 1. Structure of the Flavonoids Used in This Study



	substituents								
compound	3	5	6	7	8	2′	3′	4′	5′
I	OMe	OH	OMe	OMe	H	H	OMe	OMe	H
II	H	OH	OH	OMe	OMe	H	OMe	OH	H
III	OMe	OH	OMe	OMe	H	H	H	OH	H
IV	H	OH	OMe	OMe	OMe	H	OMe	OH	H
V	H	OMe	OMe	OMe	OMe	OMe	OMe	OMe	OMe
VI	OH	OH	H	OH	H	H	OH	OH	H
VII	ORut[a]	OH	H	OH	H	H	OH	OH	H

[a] Rut: rutinose (=Glu−Rha).

In the present study we have tested several flavonoids with hydroxy and/or methoxy substitution for their antioxidant potential against peroxyl radicals using the TOSC assay.[22] Our interest in this study is two-fold; first, to test the applicability of the TOSC assay to determine the antioxidant activity of the flavonoids and, second, to better understand the structure−activity requirements that govern the antioxidant activity of flavonoids.

Results

The structures of the different flavonoids tested are presented in Table 1. Figure 1 shows the time courses for the antioxidant activity as evaluated in the TOSC assay[22] for different concentrations of 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone (artemetin) [**I**, panel **a**]; 5,6,4′-trihy-

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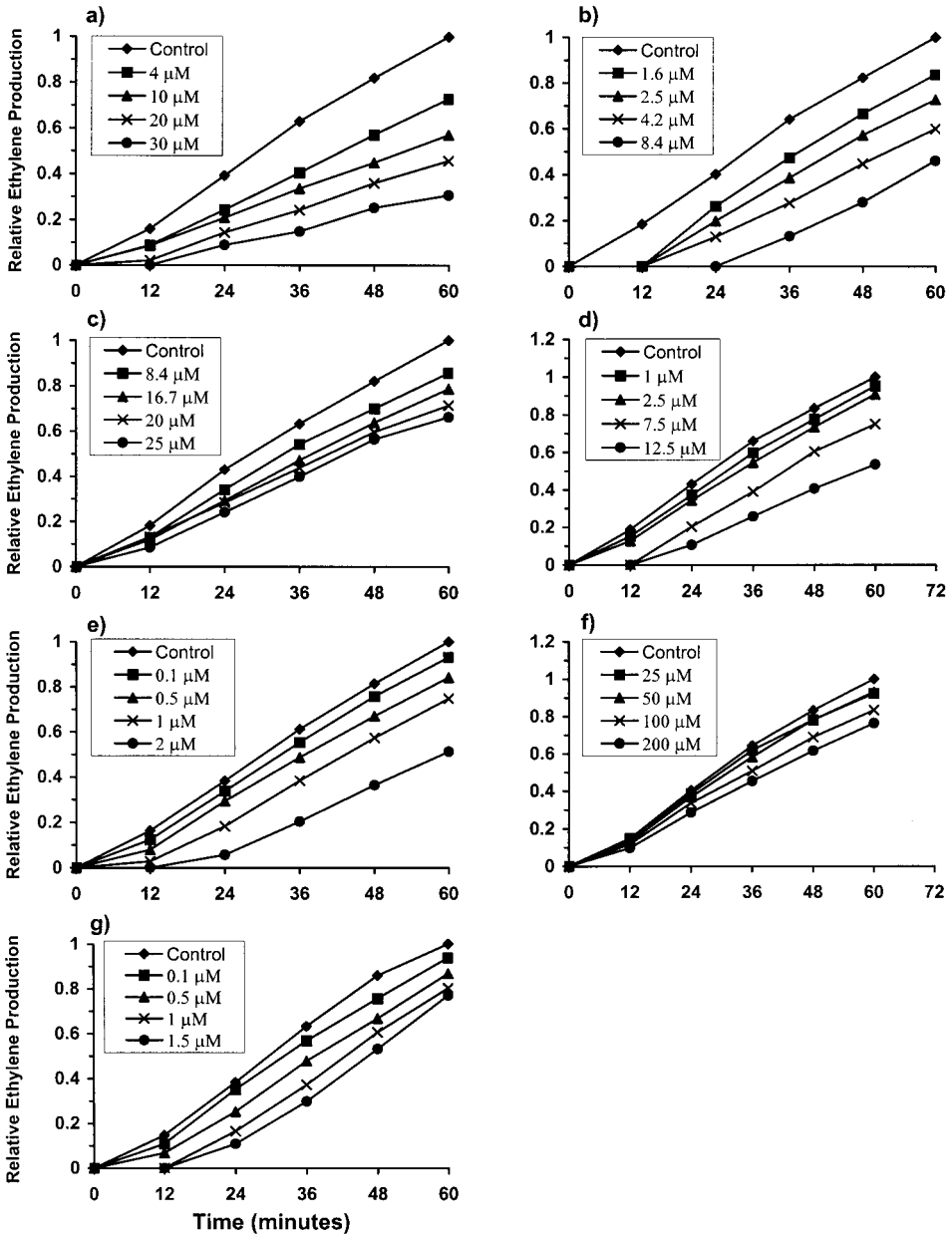

Figure 1. Peroxyl-radical-scavenging time course for the flavonoids: (**a**) 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone **I** (armetin); (**b**) 5,6,4′-trihydroxy-7,8,3′-trimethoxyflavone **II** (thymomin); (**c**) 5,4′-dihydroxy-3,6,7′-trimethoxyflavone **III**; (**d**) 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone **IV**; (**e**) quercetin **V**; (**f**) agehoustin A **VI**; (**g**) rutin **VII**.

droxy-7,8,3′-trimethoxyflavone (thymomin) [**II**, panel **b**]; 5,4′-dihydroxy-3,6,7-trimethoxyflavone [**III**, panel **c**]; 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone [**IV**, panel **d**]; 3,3′,4′,5,7-pentahydroxyflavone (quercetin) [**V**, panel **e**]; 5,6,7,8,2′,3′,4′,5′-octamethoxyflavone (agehoustin A) [**VI**, panel **f**]; and 3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside (rutin) [**VII**, panel **g**]. To the best of our knowledge **III** and **IV** have not been assigned common names. The TOSC assay exploits the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas by peroxyl radicals generated by thermal homolysis of 2,2′-azobis-amidinopropane (ABAP) at 39 °C (see Experimental Section). In the presence of the flavonoids, ethylene production was reduced to varying degrees, indicating that KMBA oxidation was differentially inhibited due to the presence of the flavonoids.[22] Ethylene production was found to decrease proportionally as the concentration of the compounds was increased. At the concentrations studied, the ability of the flavonoids to completely prevent KMBA oxidation can be visualized from the time courses of ethylene production. The flavonoids artemetin **I**, thymomin **II**, **IV**, quercetin **V**, and rutin **VII** afforded a finite period of complete protection against KMBA oxidation, as indicated by the absence of ethylene at the 12−24 min time intervals. Beyond these time points, exhaustion of the antioxidant typically occurs, and the reaction proceeds essentially uninterrupted. However, the flavonoids **III** and agehoustin A **VI** did not completely prevent the oxidation of KMBA, as demonstrated by the presence of ethylene at the first injection interval.

TOSC values were calculated for the flavonoid tested, as described in the Experimental Section. From the 60-min time courses of Figure 1, areas under each of the reaction curves were calculated. From these areas, TOSC values were obtained as shown by eq 1 (see below). The TOSC values of the different concentrations of the flavonoids **I**−**VII** are reported in Figure 2. TOSC values can be viewed as inhibition values for antioxidants against peroxyl radicals generated from thermal homolysis of ABAP. The larger

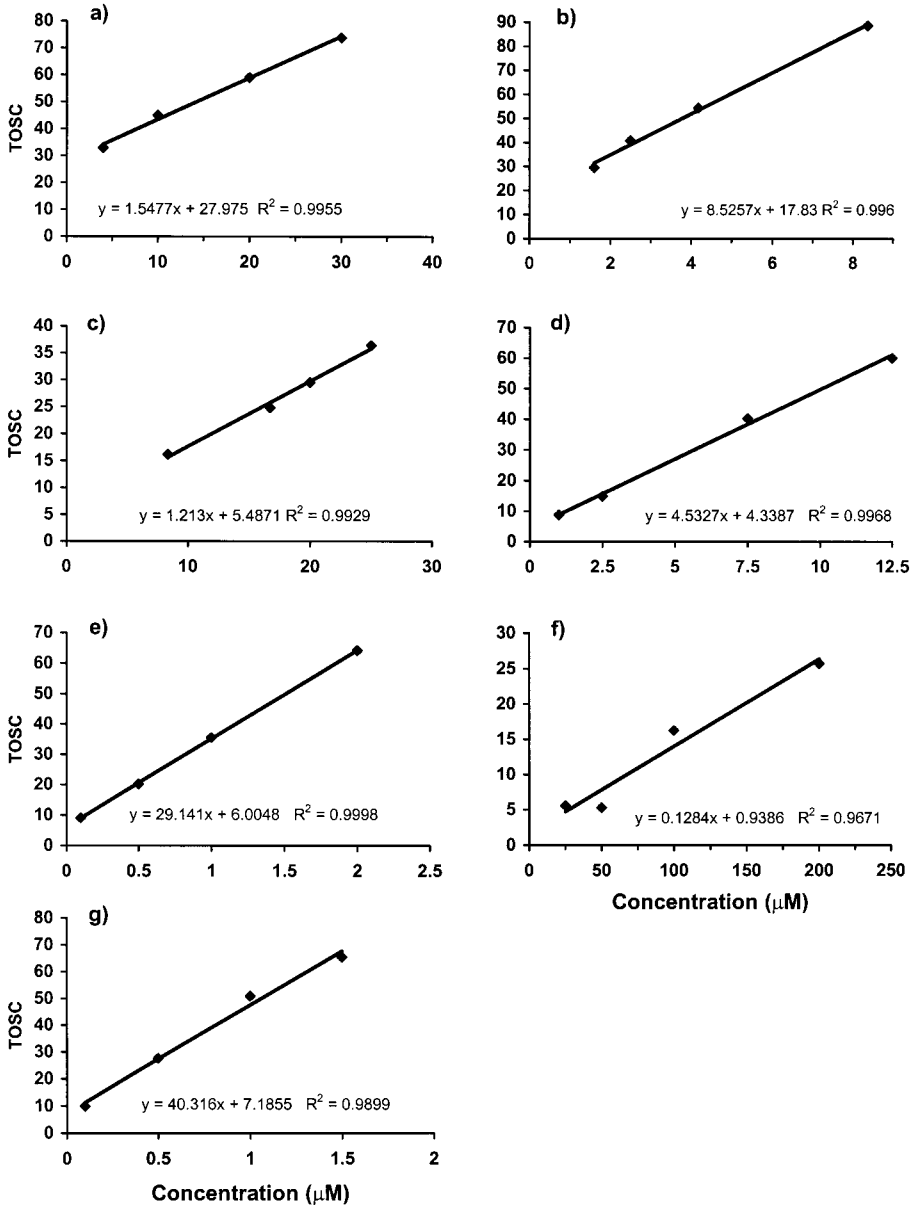

Figure 2. TOSC vs. concentration for the different flavonoids: (**a**) 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone **I**; (**b**) 5,6,4′-trihydroxy-7,8,3′-trimethoxyflavone **II**; (**c**) 5,4′-dihydroxy-3,6,7′-trimethoxyflavone **III**; (**d**) 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone **IV**; (**e**) quercetin **V**; (**f**) agehoustin A **VI**; (**g**) rutin **VII**.

the TOSC value, the higher the degree of inhibition. Experimental TOSC values are then used to determine relative TOSC values (rTOSC). rTOSC values are defined by the assay conditions used; that is, temperature and KMBA concentration.[22] From the TOSC graphs, rTOSC values can be calculated by obtaining the slope of the regression line within the compound's linear range of TOSC values. Because TOSC is unitless, the slope of the regression line must be equal to TOSC per-unit concentration. Specific μM TOSC values are easily obtained by interpolation of the TOSC vs concentration plots in Figure 2.

Comparative TOSC values (cTOSC) were then calculated and can be used as a means of comparison between the different antioxidants relative to the benchmark antioxidant Trolox. The cTOSC and rTOSC values for the flavonoids are listed in Table 2. The higher TOSC values were obtained for the flavonol quercetin **V**, followed by its glycoside derivative, rutin **VII**. From the other compounds tested, the trihydroxyflavone **II** was the more active against peroxyl radicals, with a higher TOSC value than the known antioxidant Trolox. The octamethoxyflavone **VI** was the least active of all the flavonoids tested at the indicated concentrations, which were 10- to 100-fold higher than the rest of flavonoids tested.

Table 2. Relative and Comparative TOSC Values for the Different Flavonoids Tested

compound (common name)	rTOSC	cTOSC
Trolox	5.62	1
I 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone (artemetin)	1.55	0.28
II 5,6,4′-trihydroxy-7,8,3′-trimethoxyflavone (thymonin)	8.53	1.52
III 5,4′-dihydroxy-3,6,7-trimethoxyflavone	1.21	0.22
IV 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone	4.53	0.81
V 3,3′,4′,5,7-pentahydroxyflavone (quercetin)	40.32	7.17
VI 5,6,7,8,2′,3′,4′,5′-octamethoxyflavone (agehoustin A)	0.13	0.02
VII 3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside (rutin)	28.96	5.15

Discussion

The antioxidant potential of several flavonoid compounds against peroxyl radicals was investigated using the TOSC

assay.[22] The TOSC assay measures antioxidant potential based on a compound's ability to protect the oxidative probe KMBA from oxidation by oxidants such as peroxyl and hydroxyl radicals.

Flavonoids are hydrophobic aromatic compounds. The flavonoids become more water-soluble by an increased level of hydroxylation. Similarly, hydrophobicity increases as the number of methoxyl groups is increased. The solubility of the flavonoids has an impact on their ROS-scavenging ability due to phase partitioning.[23] The position of hydroxyl groups also plays a significant role in ROS scavenging. Efficient ROS scavenging has been attributed to the ability of antioxidants to delocalize electron distribution, giving the antioxidant radical species more stability.[21] Flavonoid radical stability is thought to be increased by the creation of a completely conjugated electron system. This can be accomplished through structural planarity of the flavonoid structure. For the flavonoids, structural planarity can be accomplished by the presence of a hydroxyl group at the 3 position on the C-ring, resulting in a flavonol backbone structure.[21] A methoxy substituent at this position perturbs this planarity due to steric hindrance imparted by the methyl group; the result is to render the flavonoids less active as antioxidants. The flavonols such as quercetin and kaempferol are theoretically more potent scavengers of ROS than flavones such as luteolin, which lacks a 3-OH group. However, caution should be exercised in this quantum explanation because recent findings indicate that luteolin (3′,4′5,7-tetrahydroxyflavone) is a better peroxyl radical scavenger than quercetin (3,5,7,3′,4′-pentahydroxyflavone) and kaempferol (3,4′,5,7-tetrahdroxyflavone), which do contain 3-OH groups.[20] This finding is consistent with previous observations from our laboratory that indicate that the oxyradical-scavenging capacity of an antioxidant is very much dependent upon the attacking oxidant.[25]

The flavonoids 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone (artemetin) **I**; 5,6,4′-trihydroxy-7,8,3′-trimethoxyflavone (thymonin) **II**; 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone **IV**; quercetin **V**; and rutin **VII** all behaved as fast-acting antioxidants.[22,24] Fast-acting antioxidants are those that produce reaction profiles with a distinct induction period marked by complete inhibition of ethylene production for a finite period in the time course of inhibition until the antioxidant reaches a threshold value[22,24] (Figure 1). On the other hand 5,4′-dihydroxy-3,6,7-trimethoxyflavone **III** and agehoustin A **VI** behaved as slow-acting antioxidants or retardants[22,24] at the concentrations tested. These antioxidants lack an induction period but are inhibitory throughout the time course of the reaction.[22,24] All hexa-*O* substituted flavones tested are fast-acting antioxidants, while the penta-*O* subtituted flavonol **III** is a slow-acting antioxidant. The flavonol quercetin and its 3-glycoside derivative rutin are fast-acting antioxidants. Agehoustin A, with fully *O*-methylated groups, is a poor antioxidant.

The peroxyl-radical-scavenging capacity of the flavonoids **I**−**VII** were of the relative order: quercetin > rutin >> 5,6,4′-trihydroxy-7,8,3′-trimethoxyflavone > 5,4′-hydroxy-6,7,8,3′-tetramethoxyflavone > 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone ≅ 5,4′-dihydroxy-3,6,7-trimethoxyflavone >> agehoustin A. The substitution of *O*-methoxy groups for hydroxyl groups at the 3 position of the C-ring significantly decreased the peroxyl-radical-scavenging ability of flavonols **I** and **III**. The absence of the *O*-methoxy group increased the ability of **II** and **IV** to effectively scavenge peroxyl radicals relative to the compounds that possess a methoxy group at the 3 position.

All the flavonoids tested in this study have the 2,3-double bond with the 4-oxo functionality in the C ring, which is considered an essential functionality for antioxidant activity.[26] It is generally accepted that an increase in the number of hydroxy groups increases the antioxidant activity of the flavonoids.[20] In the present study we used compounds ranging from zero to five hydroxyl groups. In all instances where hydroxyl group substituents are discussed herein, reference is made to phenolic (SP^2-OH) hydroxyl groups. There are hydroxyl groups present in rutin that are not phenolic; however, these are not implicated in the antioxidant capacity of the flavonoids. It is interesting to note that armetin **I**, which posseses only one hydroxyl group, is more active than compound **III**, which has two hydroxyl groups. Both compounds have a hydroxyl group at the C5 position; the other hydroxy group in compound **III** is situated at the 4′ position in ring B. These data suggest that the *O*-dimethoxy functionality in ring B is more important than the mere presence of a single hydroxy group at the 4′ position, as in compound **III**. If we compare both dihydroxyflavonoids **III** and **IV**, it can be seen that the position of the hydroxyl groups is identical, the only difference being the methoxy substituents. The presence of the methoxy group in the B ring ortho to the hydroxy group seems to play an important role in the activity of **IV**. The agehoustin A **VII** is practically inactive, indicating that the presence of the C2−C3 double bond and the 4-oxo functionality are not sufficient for the antioxidant activity of the flavones.

Flavonoids have been shown to scavenge various ROS and have been implicated as inhibitors of lipid peroxidation.[27] Herein we demonstrate the ability of a few select flavonoids to scavenge peroxyl radicals. The scavenging of peroxyl radicals is a key step in the prevention of lipid peroxidation by breaking the chain of propagation of free-radical reactions. Indeed, the evidence presented herein suggests that a dietary intake of flavonoid-containing foods may be of benefit in lowering the risk of certain pathophysiologies that have been associated with free-radical-mediated events, including coronary heart disease and ischemia−reperfusion injury.[28−30] Therefore, the further study of the mechanistic properties of flavonoids is of potential importance in understanding and preventing ROS-linked diseases.

Experimental Section

Chemicals. KMBA and quercetin were purchased from Sigma Chemical Co. (St Louis, MO). ABAP was obtained from Wako Chemicals (Richmond, VA). Agehoustin A (**VI**), obtained from *Ageratum houstonianum*,[31] was kindly provided by Dr. Leovigildo Quijano (Instituto de Química, Universidad Nacional Autónoma de Mexico). Artemetin (**I**) was isolated from *Melampodium argophyllum*,[32] and thymonin (**II**) and 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone (**IV**) were obtained from *Calamintha ashei*.[33,34] All other flavonoids used in this study were from the natural products repository in the Department of Chemistry, Louisiana State University (N. H. F.). The purity of all seven flavonoids was determined by high-field ^{1}H NMR analysis.[35−37]

TOSC Assay. TOSC assay of Winston et al.[22] was used to evaluate antioxidant behavior of the flavonoids. Essentially, peroxyl radicals were generated by thermal homolysis of ABAP at 39 °C. The assay conditions used were 0.1 mM KMBA and 10 mM ABAP in 100 mM potassium phosphate buffer, pH 7.4. Under these conditions, the rate of radical input in the reaction is 1.5×10^{-8} M s^{-1}.[22] The TOSC assay was performed on compounds **I**−**VII**. Reactions were carried out in 10-mL rubber septum-sealed vials in a final reaction volume of 1 mL. The

reactions were initiated by the injection of 100 μL of 100 mM ABAP in water directly through the rubber septum. Ethylene production was measured by GC analysis of 1-mL aliquots taken directly from the headspace of the reaction vials. Samples were monitored in sequence at 12-min intervals. Analyses were performed with a Hach-Carle (Series 100 AGC) gas chromatograph equipped with a 6-ft Poropack N column (Supelco) and a flame ionization detector (FID). The oven, injection, and FID temperatures were respectively, 60, 280, and 190 °C. Helium was used as the carrier gas at a flow rate of 30 mL/min.

Quantification of TOSC. The area under the kinetic curve was calculated from the integral of the equation that best defines the experimental points for both the control and sample reactions. TOSC is then quantified according to eq 1, where

$$\mathrm{TOSC} = 100 - \left(\frac{\int_{\mathrm{SA}}}{\int_{\mathrm{CA}}} \times 100 \right) \quad (1)$$

$\int_{SA}$ and $\int_{CA}$ are the integrated areas from the curve defining the sample and control reactions, respectively. Thus, a sample that displays no oxyradical-scavenging capacity would give an area equal to the control, making the ($\int_{SA}/\int_{CA}$) equal to 1, and hence a corresponding TOSC value is zero. On the other hand, as the $\int_{SA}$ approaches 0, the hypothetical TOSC approaches 100. Relative TOSC (rTOSC) values were calculated from the slope of the linear regression lines for the TOSC curves. Comparative TOSC (cTOSC) values were calculated as shown in eq 2 by dividing the rTOSC of the antioxidants tested by

$$\mathrm{cTOSC} = \frac{\mathrm{rTOSC\ (antioxidant)}}{\mathrm{rTOSC\ (Trolox)}} \quad (2)$$

the rTOSC obtained for Trolox, a water-soluble analogue of α-tocopherol (vitamin E), thus establishing a scale based on Trolox equivalents. Statistical analysis was performed using the spreadsheet program Excel by Microsoft and Mathematica 3.0 for students by Wolfram.

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