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Free Radicals Scavenging Efficiency of a Few Naturally Occurring Flavonoids: A Comparative Study

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The interaction of antiperoxidative flavonoids artocarpin (AR), cycloartocarpin (CAR), dalspinin (DP), dalspinosin (DPO), and dalspinin-7-O-galactoside (DPG) with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) and $O_2^{-\bullet}$ was studied in phosphate buffer (pH 7.4). The ability of these compounds to inhibit lipid peroxidation and DNA scission was also investigated. The radical scavenging efficiency of flavonoids is demonstrated by the reduction of nitrogen-centered radical cation ($ABTS^{\bullet+}$). The reduction of $ABTS^{\bullet+}$ follows the order quercetin > morin > Trolox > AR > DPO > CAR > DP. Inhibition of lipid peroxidation was studied by following Mb^{IV} reduction, induced by lipid, arachidonic acid. These results are compared with those obtained for well-known antioxidants such as quercetin, morin, and Trolox. The structure−activity relationships between chemical structures of the flavonoids and their radical scavenging activities are anlayzed. The scavenging of $O_2^{-\bullet}$, inhibition of lipid peroxidation, and DNA damage depend on the oxidation potential of the flavonoids. The possible mechanism for radical scavenging activities of flavonoids in relation to their structure is also outlined.

KEYWORDS: Flavonoids; antioxidants; ABTS; DNA; arachidonic acid; lipid peroxidation

INTRODUCTION

Aerobic respiration stimulated polymorphonuclear leukocytes and macrophages and peroxisomes is thought to be the main endogeneous source of most of the oxidants produced by cells (*1*, *2*). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are implicated in numerous pathological events including inflammation, metabolic disorders, cellular aging, reperfusion damage, artherosclerosis, and carcinogenesis (*3*). The destructive effects on protein in cataract formation, oxidative damage to DNA in the development of certain cancers, and lipid oxidative damage in the occurrence and progression of vascular diseases are attributed to ROS (*4*). However, the human body has several natural defense systems against ROS; but the natural oxidant defense system is not sufficient to deal with the amount of ROS/RNS produced under certain conditions, and it is now thought that a high intake of dietary antioxidants is essential for the low risk of disorders (*5*).

Flavonoids, which are low molecular weight benzo-γ-pyrone derivatives, are present in both lower and higher plants and form a group of naturally occurring antioxidants (*6*). Flavonoids are increasingly appreciated as being an important component of the human diet. Flavonols, particularly the catechin and catechin-gallate ester family and the flavonols quercetin, kaempferol, as well as their glucosides, are constituents of the beverages green and black teas. Quercetin and kaempferol are predominant in vegetables and fruits (*7*). Protective effects of diets rich in fruits and vegetables are attributed to antioxidant nutrients such as vitamin c, β-carotene, and plant phenolics such as flavonoids and phenyl propanoids. Consequently, an adequate intake of food polyphenols may be important to prevent oxidative stress. The antioxidant activities of flavonoids are known to scavenge superoxide anion and hydroxyl, peroxyl, and alkoxyl radicals (*8*, *9*). Flavonoids effectively suppress lipid peroxidation (LPO) in biological tissues and subcellular fractions, such as mitochondria, microsomes, liposomes, low-density lipoprotein, and erythrocyte membranes (*10*−*12*).

Hence, there is a growing interest in the antioxidant activity of flavonoids. Flavonoids function as scavengers of free radicals by rapid donation of hydrogen atoms to radicals. To estimate the antioxidant activity of flavonoids, various methods are used. Each method gives different information about the radical scavenging activity of flavonoids. In this paper, five naturally occurring flavonoids, extracted from medicinal plants, are investigated for their antioxidant potential. The inhibition of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), scavenging of superoxide anion radical, inhibition of LPO, and inhibition of oxidative DNA damage

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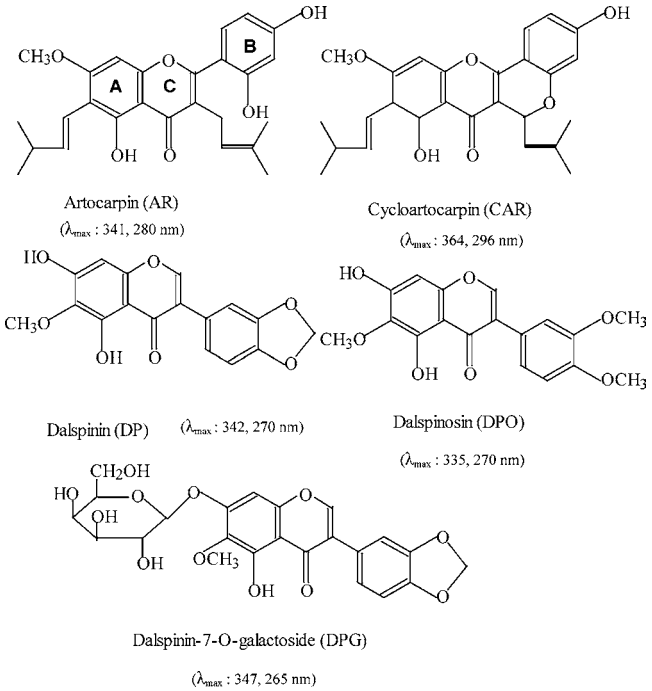

Figure 1. Structures of flavonoids AR, CAR, DP, DPO, and DPG.

are used to estimate the antioxidant activity of five naturally occurring flavonoids activities. The possible relationship between the oxidation potential of the flavonoids and their radical scavenging action is also investigated in vitro.

MATERIALS AND METHODS

Chemicals. The chemical structures of the flavonoids studied are given in **Figure 1**. These natural flavonoids artocarpin (AR), cycloartocarpin (CAR), dalspinin (DP), dalspinosin (DPO), and dalspinin-7-O-galactoside (DPG) were received as gifts from the Department of Natural Products Chemistry, School of Chemistry, Madurai Kamaraj University (Madurai, India). CAR and AR have been extracted from the heartwood of *Artocarpus incisa*. The genus *Artocarpus* belonging to the family of *Moraceae* consists of about 40 species. Several *Artocarpus* species are medicinally used for curing skin diseases and diarrhea and to stop vomiting (*13*, *14*). AR is reported to show a more potent 5-α-reductase inhibitory effect ($IC_{50} = 80\ \mu M$) (*15*). DP, DPO, and DPG were obtained from the roots of *Dalbergia spinosa Roxb*, an Indian medicinal plant (*16*).

ABTS, xanthine (XA), xanthine oxidase (XO), superoxide dismutase (SOD), ferricytochrome *c*, arachidonic acid (AA), horse heart myoglobin (Mb^{III}) were purchased from Sigma Chemical Co. (St. Louis, MO). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Quercetin and morin were purchased from Sarsyntex Lab (France). All absorbance studies were performed in a Specord S100 UV−vis spectrophotometer Analytik Jena AG (Jena, Germany).

Ferricytochrome *c* Reduction Assay. The SOD inhibitable ferricytochrome *c* reduction assay (*17*) was used to evaluate the superoxide scavenging properties of flavonoids. This assay is based on the reduction of ferricytochrome *c* (50 μM) in 10 mM phosphate buffer (pH 7.4) by superoxide anion generated from XA (2 mM)/XO (0.7 U/mL). In this experiment, catalase (100 μg/mL) was included to avoid H_2O_2-mediated oxidation of ferrocytochrome *c*. The reduction was monitored spectrophotometrically at 550 nm using $\Delta OD_{550} = 20000\ M^{-1}\ cm^{-1}$ for reduced−oxidized cytochrome *c*. To determine the inhibition efficiencies on the reduction of ferricytochrome *c*, various flavonoids of the same concentration were added before the addition of XO.

LPO Assay. LPO was performed by incubating Mb^{III} (50 μM), AA (0.4 mM), and H_2O_2 (100 μM) in 10 mM phosphate buffer (pH 7.4). After the addition of H_2O_2, the reaction mixture was incubated at 37 °C under an air atmosphere for 1 h. Mb^{III} was oxidized to Mb^{IV} by H_2O_2. The Mb^{IV} generated was reduced by AA to initiate the formation of lipid alkyl radicals. Aliquots of the incubation mixture were removed and assayed at 532 nm for the thiobarbituric acid reactive substances (TBARS) (*18*−*21*). Flavonoids (0.1 mM) were added to the TBARS, and the decrease in absorbance was compared with a blank. A blank experiment was also carried out during the heating step in the absence of flavonoids.

Inhibition of Oxidative DNA Damage. The inhibition of DNA strand breaks induced by $O_2^{-\bullet}$ (generated by the XA/XO system) by flavonoids was studied in phosphate buffer (pH 7.4). The migration rates of supercoiled (unnicked), relaxed circular (nicked), linear, and degradation plasmid DNA were studied by agarose gel electrophoresis. Circularly closed superhelical plasmid (pUC 19) DNA was prepared and purified by the method of Manniatis et al. (*22*). Supercoiled plasmid DNA, 10 kbp, was purified from alkaline sodium dodecyl sulfate bacterial lysate by cesium chloride gradient centrifugation.

A mixture of plasmid DNA (7 μg) and XA (2 mM)/XO (0.7 U/mL) in 10 mM phosphate buffer (pH 7.4) was incubated for 20 min with various concentrations of DP and DPO in a total volume of 75 μL in a 1.5 mL microfuge tube at 37 °C. After the incubation (20 min at 37 °C) was over, a 20 μL aliquot of the mixture was loaded into strained (ethidium bromide 0.05 μg/mL) 0.7% agarose gel in Tris−acetate−ethylenediaminetetraacetic acid (EDTA) buffer. The electrophoresis was carried out for 2 h at 50 V. After the electrophoresis, the gels were illuminated with UV light and photographed. DNA subjected to electrophoresis in the absence of flavonoids under identical conditions served as the control. The gel electrophoretic mobility of the various forms (I, II, III) of DNA was compared with the control.

ABTS Decolorization Assay. The total antioxidant activity (TAA) or the Trolox equivalent antioxidant activity (TEAC) was measured by the ability of hydrogen-donating antioxidants to scavenge the $ABTS^{\bullet+}$ radical cation using the method developed by Roberta Re et al. (*23*). An aqueous mixture of ABTS (7 mM) and potassium persulfate (2.45 mM) was incubated in the dark at room temperature for 12−16 h. The radical produced ($ABTS^{\bullet+}$) in this form was stable for more than 2 days when stored in the dark at room temperature. The product $ABTS^{\bullet+}$ ($\epsilon_{734} = 15000\ M^{-1}\ cm^{-1}$) was diluted to 50 μM in 10 mM phosphate buffer (pH 7.4). The reduction of $ABTS^{\bullet+}$ (50 μM) by flavonoids (10 μM) was monitored spectrophotometrically by determining the decrease in absorbance at 734 nm. This decolorizing assay was applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants.

Cyclic Voltammetry. Redox potentials of flavonoids were measured using the electrochemical analyzer, EG & G PARC. VERSATAT II. The electrochemical cell consisted of a three-electrode assembly of glassy carbon electrode (working), platinum electrode (auxiliary), and Ag/AgCl (reference) electrode. The working electrode was polished thoroughly with a polishing cloth (P2000 Eagle brand waterproof abrasive paper, Kovax). Flavonoid solutions (0.4 mM in 10 mM phosphate buffer) were deoxygenated for 10 min with nitrogen gas prior to measurement. All potentials are reported against Ag/AgCl, unless otherwise mentioned.

RESULTS AND DISCUSSION

The structure absorption maximum of the flavonoids studied is given in **Figure 1**. The absorption bands occur around 375 (representing the B ring absorption) and around 275 nm (indicating the absorption of the A ring) corresponding to a typical flavonoid structure.

Scavenging of Superoxide Anion Radical. The rate of inhibition of ferricytochrome *c* reduction, measured at 550 nm for various flavonoids, is presented in **Figure 2**. The observed inhibition follows the order morin > ascorbic acid > Trolox > DP > DPO > DPG > CAR > AR.

The compounds studied in this work broadly fall under two categories, namely, flavonoids and isoflavonoids. The oxidation potentials of three flavonoids, morin, CAR, and AR, are 0.235, 0.614, and 0.620 V respectively (**Table 1**). The order of inhibition of reduction of ferricytochrome *c* by superoxide anion

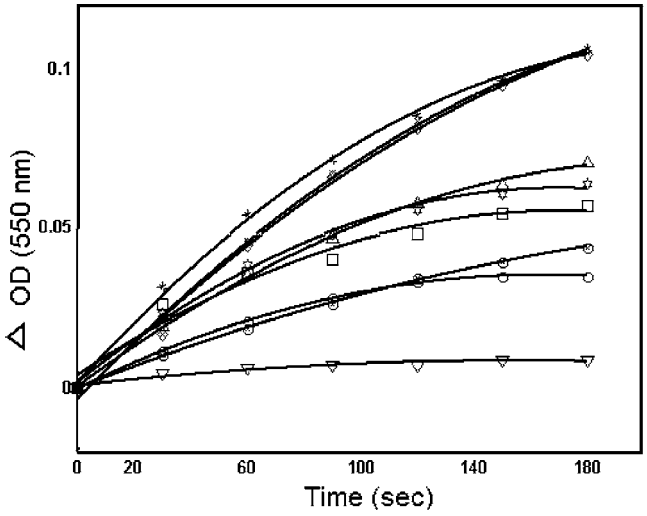

Figure 2. Inhibition of reduction of ferricytochrome *c* by superoxide anion (generated by XA/XO) by (0.1 mM) flavonoids. The change in absorbance in the ferricytochrome *c* assay at 550 nm was followed spectrophotometrically as a function of time. Inhibition of absorbance with control (*), AR (◊), CAR (×), DP (□), DPO (△), DPG (☆), ascorbic acid (⊗), Trolox (○), and morin (▽).

Table 1. Cyclic Voltammetric Data[a] of Antioxidant Substrates

substances	oxidation potential (V)	reduction potential (V)
quercetin	+0.125	+0.025
morin	+0.235	+0.065
AR	+0.620	
CAR	+0.614	
DP	+0.506	
DPO	+0.564	
DPG	+0.190	
ascorbic acid	+0.120	+0.016
Trolox	+0.095	–0.195

[a] Potentials in V against Ag/AgCl; scan rate, 100 mV/s.

is inversely related with the oxidation potential of the flavonoids (*24*, *25*). Morin, which has the lowest oxidation potential, has the highest inhibition rate of these three flavonoids. Similarly, the inhibition rates of other two isoflavonoids, DP and DPO, also show similar trends (**Table 1**). It may be concluded that the flavonoids with the lowest oxidation can be easily reduced by the scavenged $O_2^{-\bullet}$ (*23*). However, DPG shows a reverse trend. Both the oxidation potentials and the inhibition rates are low. The DPG has an additional glucose unit in the A ring; this difference in structure may be a cause for such a deviation.

Inhibition of LPO. The inhibition of LPO may be considered as the first test to determine the antioxidant activity of a compound (*26*). In the present study, natural flavonoids were tested for their AA LPO-inhibiting activity. **Figure 3** shows the inhibition of LPO observed for the various flavonoids of the same concentration. The order of inhibition is found to be 61, 36, 35, 34, 27, 24, and zero percentage for DP, DPO, DPG, CAR, ascorbic acid, AR, and blank, respectively. The inhibition of LPO by flavonoid was also studied for higher concentrations of flavonoids (0.2 and 0.4 mM). The dose-dependent inhibitory effects are observed (data not shown in figure). This order of inhibition is similar to that of $O_2^{-\bullet}$ scavenging efficiency. Hence, it may be observed that the oxidation potential of the compounds may play a major role in the inhibition of LPO. However, it is to be noted that ascorbic acid forms an exception to these

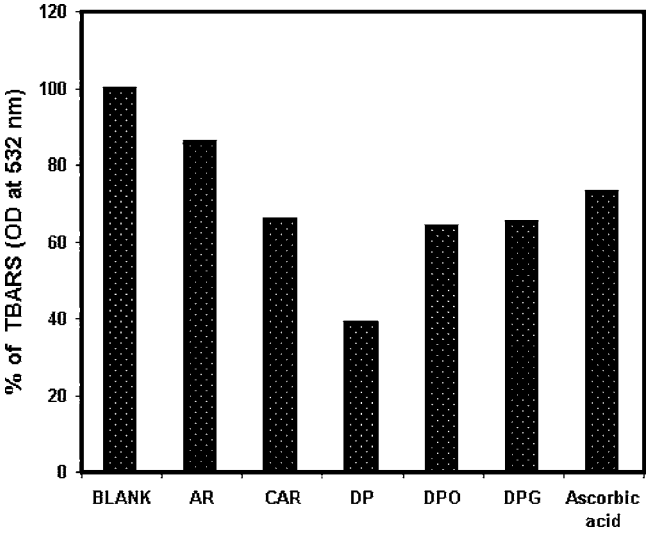

Figure 3. Inhibition of LPO by flavonoids (0.1 mM). Reaction mixtures contained AA (0.4 mM), Mb^{III} (50 μM), EDTA (100 mM), and H_2O_2 (100 μM) in 10 mM phosphate buffer (pH 7.4). The peroxidation was initiated after the addition of H_2O_2, and the mixture was incubated for 1 h at 37 °C. After the incubation, aliquots from the reaction mixture were assayed for MDA content as described under the Materials and Methods.

observations. Although ascorbic acid is a strong antioxidant, the inhibition order differs from other flavonoids. It may be due to some pro-oxidant effect (*27*).

Inhibition of Oxidative DNA Damage. ROS have been known to damage many biological macromolecules, with DNA being a significant target. One of the abundant oxidized DNA bases is 8-hydroxy-2′-deoxyguananosine (8-OHdG), a well-established biomarker relevant to carcinogenesis and aging (*28*). Increased levels of 8-OHdG have been found in cancerous tissues (*29*), and they are thought to contribute to the development of DNA base mutations (*30*) and activation of certain oncogens, such as H-ras and K-ras (*31*). The formation of 8-OHdG can be induced by various environmental factors, such as chemical modification, ionizing irradiation, and UV radiation. In view of the potential cancinogenecity of 8-OHdG, it can be argued that dietary habits that reduce this type of DNA damage may reduce the risk of certain cancers and other degenerative disorders.

The ability of DP and DPO to inhibit the DNA damage caused by $O_2^{-\bullet}$ (generated by XA/XO) was investigated by the agarose gel electrophoresis method. **Figure 4** shows the protection effect of DP and DPO on DNA damage, by $O_2^{-\bullet}$. Lane 1 has plasmid DNA of forms I, II, and III, and it served as a control. However, in the presence of XA/XO (lane 2), the plasmid DNA was damaged. Additions of SOD (100 μg/mL) to the XA/XO system inhibited the DNA damage (lane 8) completely, confirming the role of $O_2^{-\bullet}$ in the DNA damage. The form I was completely degraded to III in lane 2. The form III percentage was increased to a higher level than the control. Flavonoids DP and DPO (5 mM) inhibited the DNA scission (lanes 4 and 7) significantly. However, lanes 3 and 6 show an inhibition effect on DNA scission for lower concentrations (2.5 mM). A comparison of results of DP and DPO in lanes 4 and 7 shows that DP inhibits the DNA damage more effectively than DPO. This behavior may be correlated to the oxidation potential of the flavonoids (**Table 1**). Compounds with a lower oxidation potential show a higher inhibition effect.

TAA. The $ABTS^{\bullet+}$ radical anion assay is useful to study the TAA of a range of carotenoids, phenolics, and plasma antioxi-

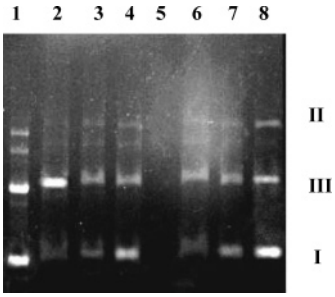

Figure 4. Inhibition of DNA strand breaks induced by $O_2^{-\bullet}$ (generated by XA/XO) in the presence of flavonoids studied by gel electrophoresis. Plasmid DNA (7 μg) in phosphate buffer (pH 7.4) solution was incubated for 20 min with XA/XO acting as the control. Lane 1, DNA (without XA/XO); lane 2, control; lane 3, control + DP (2.5 mM); lane 4, control + DP (5 mM); lane 5, none; lane 6, control + DPO (2.5 mM); lane 7, control + DPO (5 mM); and lane 8, control + SOD (100 μg/mL).

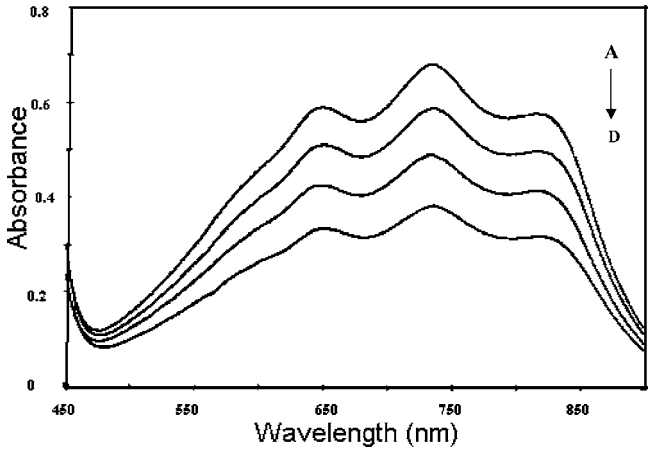

Figure 5. Concentration-dependent decrease in the near-IR spectrum of $ABTS^{\bullet+}$ (50 μM) radical cation as a function of increasing concentration of AR (A, 2.5; B, 5.0; C, 7.5; and D, 10 μM) measured after 1 min of initial mixing in 10 mM phosphate buffer (pH 7.4).

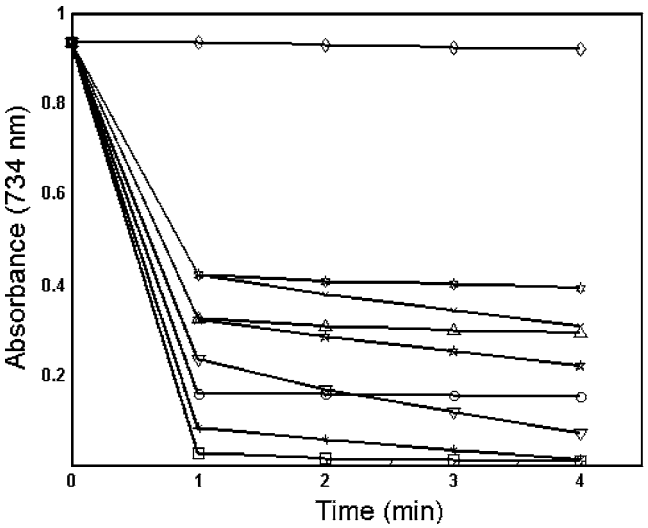

Figure 6. Reduction of $ABTS^{\bullet+}$ [measured after 1 min of initial mixing of antioxidants (10 μM)] by DP (×), CAR (✱), DPG (△), DPO (☆), AR (▽), Trolox (○), morin (*), quercetin (□), and control (◇).

Table 2. Trolox Equivalent Antioxidant Capacity of Flavonoids and Related Phenolic Compounds[a]

compounds	persulfate decolorization assay TEAC (mM)
quercetin	1.23
morin	1.13
AR	0.91
DPO	0.79
DPG	0.79
CAR	0.69
DP	0.67

[a] Measured by $ABTS^{\bullet+}$ decolorization assay (using potassium persulfate as the oxidant).

dants (*21*). The antioxidant activity of all of the five flavonoids was determined by following the decolorization (reduction) of $ABTS^{\bullet+}$ radical cation. In **Figure 5**, the suppression of the absorbance of $ABTS^{\bullet+}$ in a concentration-dependent manner is typically shown for flavonoid AR. **Figure 6** shows the decrease in absorbance of the $ABTS^{\bullet+}$ radical cation at 734 nm for flavonoids AR, CAR, DP, DPO, and DPG and for the reference compounds quercetin, morin, and Trolox. The experimental results demonstrate that the reaction with $ABTS^{\bullet+}$ is completed within 1 min, except for CAR, DPO, AR, and morin that show small inhibitory effects even up to 4 min of reaction. The decrease in the absorbance of $ABTS^{\bullet+}$ was completed within a very short time (<1 min). Hence, the rate of bleaching could not be calculated. Nevertheless, the inhibition follows the order 1:0.92:0.81:0.74:0.64:0.64:0.56:0.54 for quercetin, morin, Trolox, AR, DPO, DPG, CAR, and DP, respectively. This order of inhibition can be explained by the structure−activity relationship of flavonoids as follows.

Structure−Activity Relationship. In general, the antioxidant activity of flavonoids depends on the structure and substitution pattern of the hydroxyl groups. The essential requirement for effective radical scavenging is the 3′,4′-orthodihydroxy configuration in the B ring and the 4-carbonyl group in the C ring (*32*). The presence of the 3-OH group or 3- and 5-OH groups, giving a catechol-like structure in ring C, is also beneficial for the antioxidant activity of flavonoids. The presence of the C2−C3 double bond configurated with a 4-keto is known to be responsible for electron delocalization from the B ring, and it increases the radical scavenging activity (*33*). In the absence of the *o*-dihydroxy structure in the B ring, a catechol structure on the A ring can compensate for the flavonoid antioxidant activity (*34*).

To obtain the relationship between the chemical structure and their radical scavenging activities, the molecular structure is analyzed. Quercetin possesses a catechol structure in the B ring as well as a 2,3-double bond in conjunction with a 4-carbonyl group on the C ring, allowing delocalization of the phenoxyl radical electron to the flavonoid nucleus. The combined presence of a 3-hydroxy group with a 2,3-double bond additionally increases the resonance stabilization for electron delocalization; hence, it has a higher TEAC value (*35*, *36*). Quercetin and morin have an identical number of hydroxyl groups, with 3′,4′- and 3′,5′-dihydroxyl groups, respectively, in the B ring. It is observed that the *o*-dihydroxy structure in the B ring increases the stability of the radical form and participates in electron delocalization. Hence, morin has a lower TEAC value than quercetin (**Table 2**).

The flavonoid AR has two hydroxyl groups in the B ring at 4′ and 6′ with a TEAC value 0.91 lower only to morin. The isoflavonoid DPO has a TEAC value of 0.79, due to the absence of the OH group in the B ring. However, its value is higher than isoflavonoid DP. This may perhaps be due to the presence

of the electron-donating methoxy group in the B ring. The isoflavonoids DP and DPO have two hydroxyl groups in the A ring at the 5- and 7-positions. These hydroxyl groups in the A ring have little influence on the stability of radical, due to steric hindrance by 6-OCH_3 bulky substituents (*37*). The lower value of DP may be attributed to the presence of a saturated cyclic ring in the B ring (*38*).

The isoflavonoid DPG has one hydroxyl 5-OH group in the A ring with glycosylation substitution at the 7-OH group in the saturated heterocyclic ring. In addition, there is a hydroxyl group in the B ring. As compared to DP, DPG has a higher antioxidant activity, maybe due to the participation of the hydroxyl group in the glycosylation unit. Similarly, flavonoid CAR has a fused partially saturated heterocyclic six member ring between rings C and B. The stability of its radical form is decreased (TEAC is 0.69) as compared to flavonoid AR. These results demonstrate the importance of electron delocalization across the molecule for stabilization of the aryloxyl radical.

Even in the absence of the 3-OH group in the C ring and the catechol-like structure in the A and B rings, the flavonoids studied in this work have high antioxidant activities. However, all five compounds, AR, CAR, DP, DPO, and DPG, have 5-OH and 4-keto groups. Hence, an intramolecular rearrangement may take place leading to a catechol-like structure in ring C, resulting in a higher antioxidant activity (*39*).

Two flavonoids and three isoflavonoids are tested for their antioxidant activity by various assays. The evidence presented herein suggests that the electrochemical characteristics of flavonoids may play a crucial role in their antioxidant activity. The inhibition of DNA scission by flavonoids shows that the lower oxidation potential effectively inhibits DNA scission. Structure−activity relationships indicate that highly active flavonoids possess 3′,4′-dihydroxy groups in the B ring. Even in the absence of catechol-like structures in A and B rings, the presence of 5-OH and 4-keto groups leads to higher antioxidant activities.

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