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DPPH Radical Scavenging Activity of Tricin and Its Conjugates Isolated from "Njavara" Rice Bran: A Density Functional Theory Study

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Supporting Information

ABSTRACT: Structural, electronic, and energetic characteristics of tricin, tricin-4′-*O*-(*erythro*-*β*-guaiacylglyceryl)ether (TEGE), and tricin-4′-*O*-(*threo*-*β*-guaiacylglyceryl)ether (TTGE), isolated from "Njavara" rice bran have been studied using DFT to explain their experimentally determined radical scavenging activity (EC_{50} values) in comparison with known standards such as quercetin, myricetin, and catechin. Among the three mechanisms proposed for explaining the antioxidant activity, proton coupled-electron transfer (PC-ET), sequential proton loss electron transfer (SPLET), and electron transfer-proton transfer (ET-PT), our results support the second one. The O−H bond dissociation enthalpy (BDE) and the spin density on the oxygen with the radical character are excellent descriptors of radical scavenging activity. BDE (in kcal/mol) increased in the order myricetin (74.6) < quercetin (78.1) < catechin (78.3) < tricin (81.5) < TTGE (90.6) < TEGE (91.1), while the EC_{50} increased exponentially with increase in BDE, 20.51, 42.98, 45.07, 90.39, 208.01, and 352.04 μg/mL for myricetin, quercetin, catechin, tricin, TTGE, and TEGE, respectively.

KEYWORDS: *antioxidants, DPPH radical scavenging activity, density functional theory, bond dissociation enthalpy, spin density, ionization potential, proton affinity*

INTRODUCTION

Free radicals formed in the body are believed to be the major cause of most of the diseases including aging because they have the tendency to react with fats, lipids, and proteins.[1,2] Flavonoids, a group of polyphenolic compounds, has received tremendous attention during the past two decades due to their ability to interact quickly with the free radicals formed in the body, thus providing antioxidant activity to such molecules.[3−6] "Njavara", an important medicinal rice variety of Kerala, India, widely used in Ayurveda as a "health-food" and in the treatment of a variety of diseases such as rheumatoid arthritis, paralysis, neurodegenerative diseases, as well as in rejuvenation therapy. Recently, phytochemical investigations of the diethyl ether fraction of methanolic extract of "Njavara" rice bran gave three important flavonoids, tricin and two rare flavonolignans such as tricin 4′-*O*-(*erythro*-*β*-guaiacylglyceryl)ether (TEGE) and tricin 4′-*O*-(*threo*-*β*-guaiacylglyceryl)ether (TTGE).[7] Tricin and its derivatives obtained from "Njavara" rice bran have shown considerable antioxidant and in vivo anti-inflammatory activities. The increasing order of antioxidant activity of compounds is determined as tricin > TTGE > TEGE.[7] The aim of the present work is to explain the antioxidant activity observed for these compounds and some other related flavonoids, from a mechanistic point of view at the molecular level.

Radical scavenging activity of the antioxidants is largely influenced by structural and environmental features of the compounds in the in vivo surroundings.[8−11] Herein, we investigate the radical scavenging activity of a flavonoid (ArOH) based on the following three different mechanisms:[12−16]

$$ROO^{\bullet} + ArOH \rightarrow ROOH + ArO^{\bullet} \quad (1)$$

$$ArOH \rightarrow ArO^{-} + H^{+}$$

$$ROO^{\bullet} + ArO^{-} \rightarrow ROO^{-} + ArO^{\bullet} \quad (2)$$

$$ROO^{\bullet} + ArOH \rightarrow ROO^{-} + ArOH^{\bullet +}$$

$$ROO^{-} + ArOH^{\bullet +} \rightarrow ROOH + ArO^{\bullet} \quad (3)$$

Equation 1 is a proton coupled-electron transfer (PC-ET) mechanism involving a hydroxyl radical and corresponds to the homolytic dissociation of O−H bonds of polyphenolic compounds.[17] Bond dissociation enthalpy (BDE) can be used as an energetic parameter to evaluate the feasibility of PC-ET mechanism.[18] Equation 2 describes a sequential proton loss electron transfer (SPLET) mechanism where a proton is first lost by a heterolytic O−H bond cleavage followed by an electron transfer from ArO^{-} to form $ArO^{\bullet}$.[19,20] This mechanism is highly pH dependent as it involves a proton loss. For SPLET mechanism, proton affinity (PA_1) of the anion formed after the O−H bond cleavage can be used as an efficient parameter to evaluate the proton loss where lower the PA_1 easier will be the heterolytic bond cleavage. The formation of radical from the anion (ArO^{-}) is the second step in SPLET mechanism and can be evaluated by electron transfer enthalpy

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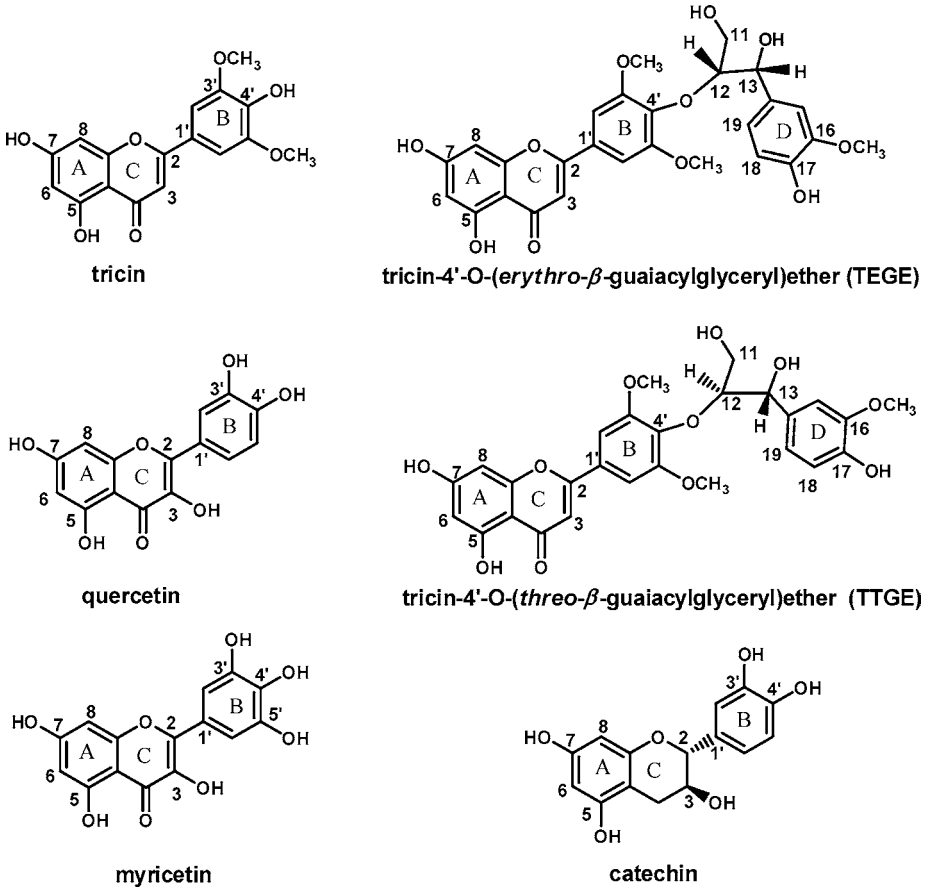

Figure 1. Structural formulas of selected flavonoids.

(ETE) as it involves a single electron transfer to form $ArO^{\bullet}$. Equation 3 describes an electron transfer−proton transfer (ET-PT) mechanism. In ET-PT mechanism, the first step is the formation of a radical, which is governed by an electron transfer, and hence ionization potential (IP) of the molecule can be used as an efficient parameter. The formation of neutral radical ($ArO^{\bullet}$) from the cation radical ($ArOH^{\bullet+}$) is the second step in ET-PT mechanism, which can be evaluated by the proton affinity of the neutral radical (PA_2), and the lower is the PA_2 value, the easier will be the formation of neutral radical from $ArOH^{\bullet+}$. Both ET-PT and SPLET mechanisms are solvent dependent due to the formation of charged species ($ArOH^{\bullet+}$ and ArO^{-}, respectively), whereas the PC-ET mechanism is solvent independent as it passes through charge less radicals and neutral species.[21−24] All of these mechanisms are believed to play important roles in determining the radical scavenging activity of the antioxidants in various environmental conditions.[25−30] Very recently, Foti et al.[31] reported that the kinetics of the reaction of 2,2-diphenyl-1-picrylhydrazil radical ($DPPH^{\bullet}$) with quercetin exhibits a biphasic behavior with both phases following first-order kinetics and suggested that SPLET mechanism takes place rather than PC-ET mechanism.

In the present work, theoretical studies have been carried out to find a probable explanation for the observations on the scavenging activity of tricin and its flavonolignan conjugates tricin-4′-*O*-(*erythro*-*β*-guaiacylglyceryl)ether (TEGE) and tricin-4′-*O*-(*threo*-*β*-guaiacylglyceryl)ether (TTGE), isolated from "Njavara" rice bran to understand the structural effects in a molecular way. The ease of radical formation and its stability will give ample insights into the efficiency of the flavonoids.

MATERIALS AND METHODS

Experimental Section. We have used different flavonoids, tricin, tricin 4′-*O*-(*erythro*-*β*-guaiacylglyceryl)ether (TEGE), tricin 4′-*O*-(*threo*-*β*-guaiacylglyceryl)ether (TTGE), quercetin, myricetin, and catechin (flavan-3-ol), for the determination of DPPH radical scavenging activity (Figure 1). Among them, tricin, TEGE, and TTGE were extracted from "Njavara" rice bran, and quercetin, myricetin, and catechin were procured from Sigma-Aldrich Co., U.S. To determine the radical scavenging activity, different concentrations of these compounds in the range 5−1000 μg/mL were prepared in methanol. To 0.5 mL of each of the sample solutions, 5 mL of 0.1 mM DPPH radical solution in methanol was added and shaken well, and the absorbance was measured from 0 to 30 min spectrophotometrically at 517 nm, using a Shimadzu UV-1601. EC_{50} values were determined as detailed in the paper by Mohanlal et al.[7]

Computational. The structures of tricin, TEGE, TTGE, quercetin, myricetin, and catechin were optimized using the B3LYP/6-311++G(d,p) level density functional theory.[32,33] The solvent effects were also computed using the polarizable continuum model (radii = UAHF) for the compounds in gas-phase equilibrium geometries.[34,35] The solvent used is methanol (dielectric constant = 32.63). The geometries were verified as true minima on the potential energy surface (PES) by vibrational frequency calculations. For all of the radical systems, the unrestricted UB3LYP/6-311++G(d,p) method was used. All of the calculations were performed using the Gaussian 09 suite of programs.[36] Total enthalpy at 298 K is taken to obtain the bond dissociation enthalpy (BDE) for the homolytic cleavage of O−H bonds to generate the radicals.[37] For SPLET mechanism, PA_1 is calculated from the zero point energy (ZPE)-corrected energy difference between the parent molecule and the corresponding anion formed from its heterolytic cleavage, and ETE is calculated from the enthalpy difference between the anion and the corresponding radical formed from it. The pK_a values in solution are also computed

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with respect to phenol and reported as further information for polyphenol acidities.[38] For ET-PT mechanism, the IP is obtained from the zero point energy (ZPE)-corrected electronic energy difference between the parent molecule and its radical cation and PA_2 from the zero point energy (ZPE)-corrected electronic energy difference between the radical cation and the most stable neutral radical.

RESULTS AND DISCUSSION

In the UV−vis experiment, formation of DPPH-H is indicated by a decrease in the absorbance value as well as a color change from purple to yellow.[39] In Table 1, the EC_{50} values of all of the compounds are reported, which shows that the DPPH• scavenging activity follows the order myricetin > quercetin > catechin > tricin > tricin-4′-*O*-(*threo*-*β*-guaiacylglyceryl) ether > tricin-4′-*O*-(*erythro*-*β*-guaiacylglyceryl) ether.

Table 1. Antioxidant Activity of the Compounds Given in Figure 1, Measured with the DPPH Radical

compound	EC_{50} (μg/mL)
tricin	90.39
TEGE	352.04
TTGE	208.01
quercetin	42.98
myricetin	20.51
catechin	45.07

Figure 2 shows the optimized geometries of all of the compounds with some important structural parameters. Tricin, TEGE, TTGE, and catechin show a twist angle ($\angle C_3C_2C_1'C_6$) of 16.0, 19.4, 19.0, and 94.4, respectively, with respect to the central $C_2-C_{1'}$ bond, whereas both myricetin and quercetin show planar geometry (twist angle = 0.0). Catechin lacks a double bond between C_2 and C_3, but is included in the present work, to analyze more deeply the influence of the different structural characteristics on the mechanism of antioxidant activity. Recently, the charge transfer analysis of various flavonoids has demonstrated the relative independence of each structural motif when hydrogen abstraction processes are considered.[29] In all of the systems, the hydroxyl groups interact with nearby oxygen atoms for intramolecular hydrogen bonds (shown by dotted lines in Figure 2), and this will enhance the *π*-delocalization in the aromatic ring.[13,14,40,41]

The OH groups present in these molecules are prone to undergo homolytic cleavage when they interact with a radical such as DPPH•. For tricin molecule, three different phenoxide type radicals are possible by the cleavage of O−H bonds at C_5, C_7, and $C_{4'}$ positions. Myricetin has six different possibilities for the O−H bond cleavage, while the rest of the molecules have five different options. The BDE values of all of these possibilities are presented in Table 2. For a given system, the most stable radical formed by the O−H bond cleavage is the one that shows the lowest BDE. In the case of tricin, the most stable radical is formed by the cleavage of the $C_{4'}O-H$ bond in the B ring as it shows the lowest BDE of 81.5 kcal/mol as compared to the BDE for the C_5O-H (91.0 kcal/mol) and the C_7O-H (90.8 kcal/mol) bonds. Similarly, for TEGE and TTGE, the O−H bond cleavage is the easiest at the C_{17} position in the D ring, and their values are 78.2 and 79.2 kcal/mol, respectively. The O−H bond cleavage of quercetin, myricetin, and catechin is favored at $C_{4'}$ position in the B ring, and the corresponding BDE values are 78.1, 74.6, and 78.3 kcal/mol, respectively.

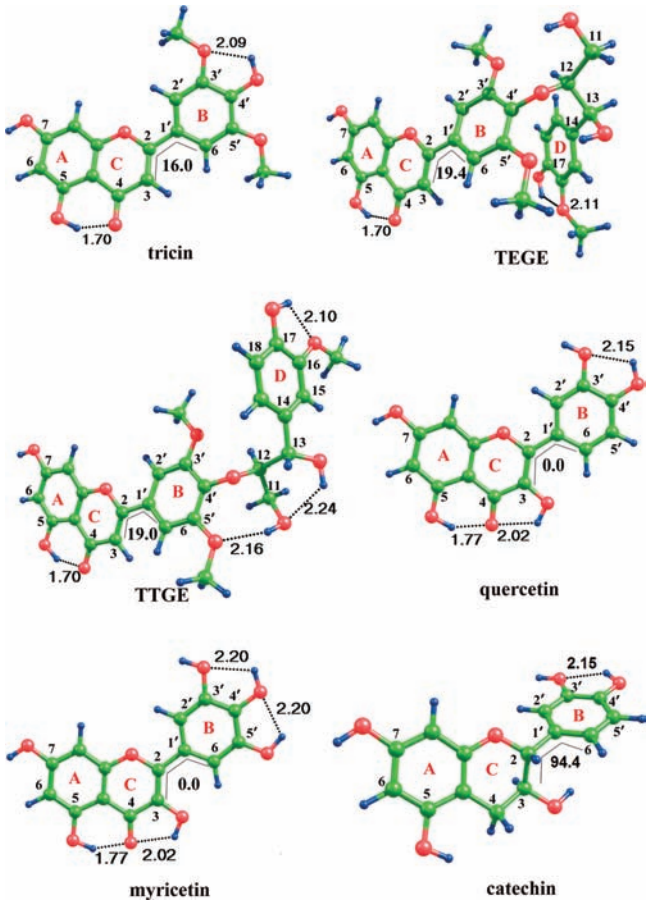

Figure 2. The optimized geometries of the flavonoids with their structural features. Bond distances are given in angstroms, and torsion angles are in degrees.

Among all of the systems, myricetin shows the lowest BDE for the $C_{4'}OH$ bond. It has the highest number of three hydroxyl groups in the B ring, and the radical formed (myricetin $C_{4'}O$•) is stabilized by two hydrogen bonds from either $C_{3'}$ and $C_{5'}$ positions as well as the conjugation effect from the coplanar arrangement of the aromatic rings A, B, and C. The second lowest BDE is observed in quercetin where, apart from one hydrogen bond on the $C_{4'}O$•, the planarity of the whole molecule is helpful in delocalizing the unpaired electron. In catechin, the radical formed from the cleavage of $C_{4'}-OH$ in the B ring (catechin $C_{4'}O$•) experiences stabilization from one hydrogen bond, while the conjugation effect is restricted only in the B ring due to the saturated C_2 atom. In the case of tricin, the radical formed at $C_{4'}$ position lacks stabilization by hydrogen-bond interactions, and hence a higher BDE is observed as compared to myricetin, quercetin, and catechin. In general, a radical is more stabilized by the nearby hydrogen bond/s than the planar nature of the entire molecule. Table 2 lists the BDE values of all of the O−H bonds of the corresponding molecules.

Foti et al. supported the SPLET mechanism for the reaction of DPPH radical with the flavonoids in polar solvents such as methanol.[31] According to them, at first, flavonoid will undergo a proton loss from the corresponding anion (ArO^-) in ionizing solvents, and in the second step, the anion will transfer an electron to the DPPH radical to form ArO•.[42] To understand this mechanism, we have considered the heterolytic bond dissociation of all of the ArO−H bonds and calculated the

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Table 2. BDE Values for the Formation of Radicals and PA_1 Values for the Formation of Anions[a]

compound	position of homolytic cleavage	BDE (kcal/mol)	position of heterolytic cleavage	PA_1 of ArO^- (kcal/mol)	relative pK_a for ArO^- (kcal/mol)
tricin	$C_5O^\bullet$	91.0	C_5O^-	293.4	−0.7
	$C_7O^\bullet$	90.8	C_7O^-	289.3	−3.1
	$C_{4'}O^\bullet$	81.5	$C_{4'}O^-$	288.8	−3.1
	$C_5O^\bullet$	104.7	C_5O^-	294.7	0.2
	$C_7O^\bullet$	91.1	C_7O^-	290.5	−2.2
	$C_{11}O^\bullet$	102.9	$C_{11}O^-$	311.3	12.8
TEGE	$C_{13}O^\bullet$	97.8	$C_{13}O^-$	314.0	14.9
	$C_{17}O^\bullet$	78.2	$C_{17}O^-$	299.1	4.6
	$C_5O^\bullet$	90.8	C_5O^-	295.7	1.2
	$C_7O^\bullet$	90.6	C_7O^-	291.5	−1.4
	$C_{11}O^\bullet$	102.5	$C_{11}O^-$	306.9	9.8
TTGE	$C_{13}O^\bullet$	96.9	$C_{13}O^-$	307.9	10.2
	$C_{17}O^\bullet$	79.2	$C_{17}O^-$	294.5	1.9
	$C_3O^\bullet$	78.4	C_3O^-	291.2	−1.7
	$C_5O^\bullet$	88.0	C_5O^-	292.1	−1.2
quercetin	$C_7O^\bullet$	88.2	C_7O^-	289.3	−2.9
	$C_{3'}O^\bullet$	80.8	$C_{3'}O^-$	292.2	−0.7
	$C_{4'}O^\bullet$	78.1	$C_{4'}O^-$	288.8	−3.0
myricetin	$C_3O^\bullet$	78.4	C_3O^-	290.9	−2.0
	$C_5O^\bullet$	88.4	C_5O^-	291.7	−1.5
	$C_7O^\bullet$	88.8	C_7O^-	289.2	−3.1
	$C_{3'}O^\bullet$	81.6	$C_{3'}O^-$	291.8	−1.1
	$C_{4'}O^\bullet$	74.6	$C_{4'}O^-$	287.0	−4.2
	$C_{5'}O^\bullet$	81.6	$C_{5'}O^-$	292.1	−0.8
catechin	$C_3O^\bullet$	102.2	C_3O^-	305.3	8.7
	$C_5O^\bullet$	82.0	C_5O^-	293.7	0.3
	$C_7O^\bullet$	83.3	C_7O^-	296.1	1.8
	$C_{3'}O^\bullet$	78.9	$C_{3'}O^-$	291.8	−1.1
	$C_{4'}O^\bullet$	78.3	$C_{4'}O^-$	291.4	−1.2

[a]Numbering of carbon atoms is as given in Figure 1. The relative pK_a values are also reported with respect to phenol.

Table 3. ETE Values for the Radical Formation from ArO^- by an Electron Loss, IP Values for the Radical Cation Formed by an Electron Loss, and PA_2 Values for the Radical Formation from Radical Cation by a Proton Loss

radical formed from ArO^-	ETE of ArO^- (kcal/mol)	radical formed from ArOH	IP of ArOH (kcal/mol)	radical formed from $ArOH^{+\bullet}$	PA_2 of $ArO^\bullet$ (kcal/mol)
tricin-$C_{4'}O^\bullet$	104.8	tricin$^{+\bullet}$	127.6	tricin-$C_{4'}O^\bullet$	267.6
TEGE-$C_7O^\bullet$	114.4	TEGE$^{+\bullet}$	123.1	TEGE-$C_{17}O^\bullet$	268.9
TTGE-$C_7O^\bullet$	113.0	TTGE$^{+\bullet}$	124.6	TTGE-$C_{17}O^\bullet$	268.5
quercetin-$C_{4'}O^\bullet$	103.4	quercetin$^{+\bullet}$	123.4	quercetin-$C_{4'}O^\bullet$	268.8
myricetin-$C_{4'}O^\bullet$	101.2	myricetin$^{+\bullet}$	122.7	myricetin-$C_{4'}O^\bullet$	265.6
catechin-$C_{4'}O^\bullet$	101.1	catechin$^{+\bullet}$	128.3	catechin-$C_{4'}O^\bullet$	264.1

proton affinity (PA_1) and pK_a values of various ArO^- species (Table 2). The values of PA_1 and pK_a show that the proton loss preferably happens at C_7 position for both TEGE and TTGE and at $C_{4'}$ for all other systems. It may be noted that for homolytic O−H bond cleavage, the preferred position was C_{17} for both TEGE and TTGE and $C_{4'}$ for the rest. Also calculated are the ETE values, the enthalpy required for the formation of a phenoxide radical from the most stable ArO^- (Table 3). Tricin, quercetin, myricetin, and catechin show ∼103 ± 2 kcal/mol of ETE, whereas the ETE values of TEGE and TTGE are 114.4 and 113.0 kcal/mol, respectively.

The ET-PT mechanism is evaluated by analyzing the IP of ArOH and PA_2 of $ArO^\bullet$ (Table 3). IP values are ∼123.5 ± 1 kcal/mol for most of the systems and 127.6 kcal/mol for tricin and 128.3 kcal/mol for catechin. These values do not show a correlation with the EC_{50} values. Hence, this mechanism may not operate in the reaction.

In all three mechanisms, PC-ET, SPLET, and ET-PT, the formation of $ArO^\bullet$ must take place. Therefore, irrespective of which mechanism is operating, the radical scavenging activity or the antioxidant activity of the flavonoids can also be related to the stability of the radicals formed. To assess this, the delocalization of the unpaired electron in $ArO^\bullet$ is analyzed on the basis of spin density distribution (Figure 3). The spin contamination of the wave function is negligible in every case studied herein as the expectation value for the total spin (S^2) was very close to 0.75. In the cases of quercetin, myricetin, and tricin, the spin density is delocalized significantly even up to the C ring, whereas for the rest of the systems, the spin density is confined within only one aromatic ring. In general, a decrease in the spin density at the oxygen of $ArO^\bullet$ shows an increase in the radical scavenging activity, in terms of its EC_{50} values.

The BDE values show a correlation with EC_{50} values (Figure 4). As indicated in Figure 4, the $DPPH^\bullet$ radical scavenging activity increases almost exponentially with the increase in BDE

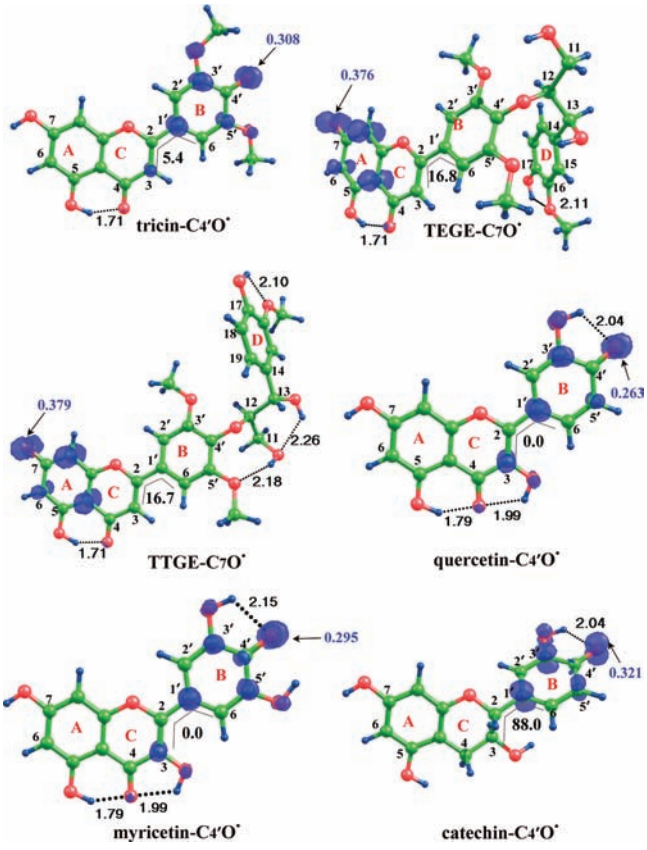

Figure 3. The spin density distribution (blue surface) in the most stable radicals of tricin, TEGE, TTGE, quercetin, myricetin, and catechin. The bond distances are given in angstroms, and twist angles are in degrees.

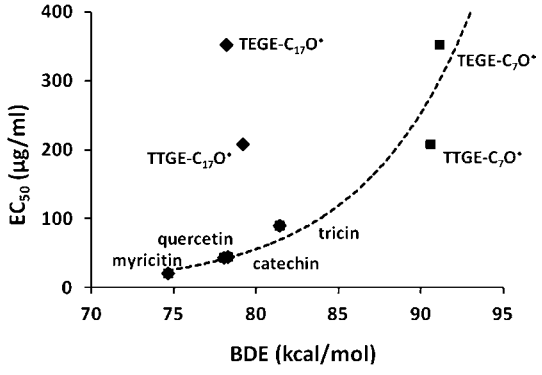

Figure 4. Correlation between BDE values and DPPH radical scavenging activity (EC_{50} values).

values. The BDE values of TEGE and TTGE show better correlation with experimentally determined EC_{50} values when the phenoxy radicals are formed at C_7 position (correlation coefficient = 0.98; Figure 4), and it does not show any particular correlation when the radicals are formed at C_{17} position. Hence, this result favors the SPLET mechanism more when the radical formation preferably happens at the OH bond present at C_7 position of TEGE and TTGE. This is further supported by a correlation between ETE and BDE with a correlation coefficient of 0.95. Kinetic factors may also play an important role in the mechanism of the activity of certain antioxidants,[43,44] particularly when tunnelling effects of hydrogen transfer reactions are significant.[45]

In conclusion, the free radical scavenging activities of the flavonoids tricin, quercetin, myricetin, catechin, and flavonolignan conjugate of tricin isolated from "Njavara" rice bran, TEGE and TTGE, have been investigated on the basis of theoretical calculation using B3LYP/6-311++G(d,p) DFT method. The experimental order of the free radical scavenging activity of these compounds almost exponentially increased with respect to the decrease in BDE values. The lower are the BDE values, easier is the O−H bond cleavage and the higher will be the radical scavenging activity. Analysis of spin density is useful to evaluate the delocalization of the unpaired electron in the radical species. In general, a radical system with more delocalized spin density is more active for free radical scavenging. Hydrogen-bond interaction around the phenoxide radical center increases the radical scavenging activity. Moreover, extended conjugation of the π-electrons to more than one aromatic ring enhances the stability of the radical systems as well as the radical scavenging activity. The BDE values and spin density distribution serve as useful descriptors to assess the radical scavenging activity of the selected antioxidants. Lower BDE value and higher delocalization of spin density increases the ease of radical formation as well as the stability of the radicals formed. Increasing the stability of the flavonoid radical increases its antioxidant activity. Although we do not have conclusive evidence to suggest a specific mechanism for the antioxidant activity of flavonoids, the accumulated results point to the fact that the most important species in the mechanism is the ArO$^{\bullet}$ radical and the most probable mechanism is the SPLET mechanism.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Thermodynamic parameters and Cartesian coordinates of all of the geometries. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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