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Investigations on the fluorescence quenching of 2,3-diazabicyclo[2.2.2]oct-2-ene by certain flavonoids

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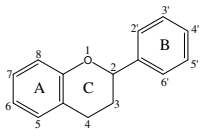
ABSTRACT

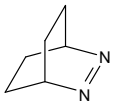
The fluorescence quenching of 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) by seven flavonoids namely flavone, flavanone, quercetin, rutin, genistein, diadzein and chrysin has been investigated in acetonitrile and dichloromethane solvents. The bimolecular quenching rate constants lie in the range of 0.09–5.75 × 10^9 M^{-1} s^{-1} and are explained in terms of structure of the flavonoids studied. The reactivity of flavonoids are in the order: quercetin > rutin > genistein > diadzein > chrysin > flavone > flavanone. The quenching rate constants (k_q) increase with increase in the number of –OH groups. The endergonic thermodynamic values of ΔG_{et} reveal that electron transfer quenching mechanism can be ruled out. Bond dissociation enthalpy calculations reveal that the position of –OH is important. Further *in vitro*-antioxidant activities of flavonoids were evaluated with rat liver catalase by gel electrophoresis. The deuterium isotope effect thus observed in this work provides evidence for hydrogen abstraction involved in the quenching process of singlet excited DBO by flavonoids. The data suggest the involvement of direct hydrogen atom transfer (radical scavenging) in the fluorescence quenching of DBO. Bond dissociation enthalpy calculation performed at B3LYP/6-31G(p′)//B3LYP/3-21G* level are in excellent agreement with the above observations and further reveal that the number OH groups and position of them decide the quenching ability of the flavonoids.

1. Introduction

The azoalkane 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) used as a fluorescent probe for antioxidants, has an extremely long fluorescence lifetime (up to 1 μs) and its strongly fluorescent n,π^* excited state shows radical like behavior [1–6]. Due to the high reactivity of free radicals cellular damage in biological system [7] is induced leading to aging and several degenerative diseases such as heart disease, cataracts, cognitive dysfunction and cancer [8,9]. Antioxidants play a vital role in medicine, biology, polymer chemistry, cosmetics and in food industry. Flavonoids are phenol derivatives with potential antioxidant activity towards free radical species and widely distributed in plants and foods. The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C_6–C_3–C_6), which are labeled A, B and C.



Flavonoids possess pronounced biological activities protecting against coronary heart disease, cancer, inflammation [10–12], etc. Several studies have been performed on the reaction of flavonoids with active free radicals [N_3, HO, $O_2^{+\cdot-}$, *t*-BuO· and LOO·] by pulse radiolysis [13,14] and high performance liquid chromatography (HPLC) [15,16]. Flavonoids exert their protective effects to different extents with different efficiencies depending upon the structure of the molecules [12]. Recently O–H bond dissociation enthalpies (BDEs) have been successfully used to express the free radical scavenging ability of polyphenolic antioxidants [17,18]. DBO shows a pronounced tendency to undergo direct hydrogen atom abstraction reactions with alcohols, ethers, alkanes, phenols, estrogens and catechols [19–23].



2,3-Diazabicyclo[2.2.2]oct-2-ens (DBO)

Fluorescence as a detection method provides high sensitivity down to the single molecule, ease of application, and a subnanosecond temporal and submicrometer spatial resolution. A high

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reactivity combined with selectivity towards antioxidants, the possibility to generate radical mimicking probes *in situ*, i.e., by an instantaneous generation and a sensitive detection method are other essential prerequisites [1]. Fluorescence quenching can be resolved with high sensitivity towards the mechanism and action of antioxidants [24,25] than other conventional methods like absorption measurements involving DPPH [26] or ABTS radicals [27]. In this present study, the fluorescence quenching of n,π^* excited state DBO by seven flavonoids (flavone, flavanone, quercetin, rutin, genistein, diadzein and chrysin) having different number (n = 0–6) of –OH substituents on a flavone skeleton has been measured in two different solvents of varying polarity. The quenching rate constant of flavanone (without 2,3-double bond) was measured to investigate the effect of π conjugation between the A and B rings on the rate of the reaction. We also measured the oxidation potential of flavonoids using cyclic voltametric techniques and Rehm–Weller equation was applied to measure free energies for electron transfer process. The –OH BDEs have been successfully used to express the free radical scavenging ability of flavonoids. For supporting this radical scavenging mechanism, *in vitro*-antioxidant activity was also investigated by gel electrophoresis.

2. Experimental

2.1. Materials

DBO was obtained as a gift sample from Prof. W.M. Nau, Jacobs University, Bremen, Germany. All the flavonoids were obtained from Sigma–Aldrich, USA. Potassium ferricyanide, ferric chloride, H_2O_2, KH_2PO_4, K_2HPO_4, glycerol, Tris-buffer and tetrabutyl ammonium perchlorate were obtained from Merck, India and recrystalised before use. The spectroscopic grade solvent, CH_3CN and CH_2Cl_2 were used for preparing the solutions. Fresh rat liver homogenate was used as enzyme source for gel electrophoresis. All measurements were performed at ambient temperature.

2.2. Methods

2.2.1. Fluorescence quenching experiments

Samples were prepared by dissolving DBO (1×10^{-4} M) in suitable solvents and administering the appropriate amounts of flavonoids with a GC syringe. The concentration of flavone, flavanone, chrysin and genistein were varied from 0 to 5 mM. The concentration of diadzein, rutin and quercetin were varied from 0 to 0.5 mM. The samples were carefully degassed using pure nitrogen gas for 15 min. Quartz cells ($4 \times 1 \times 1$ cm) with high vacuum Teflon stopcocks were used for degassing. For the quenching experiments, various concentrations of quenchers were chosen. Fluorescence intensities were obtained for different quencher concentration and plotted according to the equation

$$I_0/I = 1 + k_q\tau_0[Q]. \quad (1)$$

The slopes afforded the K_{SV} values. The lifetime (τ_0) of DBO in degassed acetonitrile and dichloromethane without any added quencher was 620 and 185 ns, respectively. Similarly, the excited state lifetimes were obtained for different concentration of flavonoids. To extract k_q values, the experimental lifetimes were plotted against quencher concentration according to the Stern–Volmer equation

$$\tau_0/\tau = 1 + k_q\tau_0[Q], \quad (2)$$

where τ_0 and τ are lifetime of DBO in the absence and presence of quenchers. Deuterium isotope effects for flavonoids were measured in wet acetonitrile (either with 10% H_2O or D_2O), assuming a complete and fast OH/OD exchange of protic hydrogens [22].

2.2.2. Steady-state measurements

The steady-state fluorescence quenching measurements were carried out in a Perkin–Elmer LS55 Luminescence spectrometer. The excitation wavelength was 365 nm and the emission was monitored at 420 nm. The excitation and emission slit widths (15 nm) and scan rate (500 nm/s) were constantly maintained for all the experiments. The experimental values are reproducible within ±10% of the experimental error.

2.2.3. Fluorescence lifetime measurement

Fluorescence lifetime measurements were carried out in a FluoroMax-4-time correlated single photon counting (TCSPC) spectrometer. The interchangeable nanoLED (370 nm, Horiba Jobin yvon, USA) was used as excitation source. The fluorescence decay of DBO was measured at 420 nm with a monochromator–photomultiplier setup. The data points were fitted by monoexponential decay functions. The data analysis was carried out by the software provided by the IBH. The error in lifetime measurement is ±5 ns.

2.2.4. Cyclic voltametric measurements

The reduction potential of DBO has been reported as −2.8 V vs SCE in acetonitrile by using hanging mercury drop electrode (HMDE) [28]. The oxidation potential of flavonoids was recorded by using a computer controlled EG&G PAR 283A cyclic voltametric setup in acetonitrile with tetrabutylammonium perchlorate (0.1 M) as supporting electrolyte. The experimental setup consisted of a glassy carbon working electrode (area: 0.07 cm^2), a platinum plate counter electrode and a silver wire as reference electrode. Irreversible peak potentials were measured using cyclic voltammetry setup. The differential pulse voltammogram (DPV) was also measured (5 mV/s). All samples were deaerated by bubbling with nitrogen gas for ca. 20 min at room temperature. The error limit in oxidation potential is ±10 mV.

2.2.5. Computational BDE

All geometry optimizations have been carried out using the density functional method with the popular B3LYP hybrid functional [29] with 3-21G* basis set using the Gaussian 98W program package [30]. Each optimized geometry has been confirmed to be a minimum on the PES by vibrational frequency calculations. Restricted B3LYP functional has been used for the parent molecules and unrestricted B3LYP for the radicals. Energies of various flavonoids used here have been computed at B3LYP/6-31G(p′) level on B3LYP/3-21G* geometries and have been used for BDE calculations. BDE calculations for phenol have been performed at different extended basis set for calibration and it has been found that B3LYP/6-31G(p′) energy closely agree with experiment. Therefore, B3LYP/6-31G(p′) energies have been used for computing various O–H bond dissociation enthalpy of flavonoids employed here.

2.2.6. In vitro-antioxidant activity

Liver sample (100 mg/ml buffer) was homogenized in 50 mM phosphate buffer (pH 7.0); and then centrifuged at 10,000 rpm for 15 min; the supernatant thus obtained was used for this experiment as a source of catalase enzyme. This experiment was designed as follows.

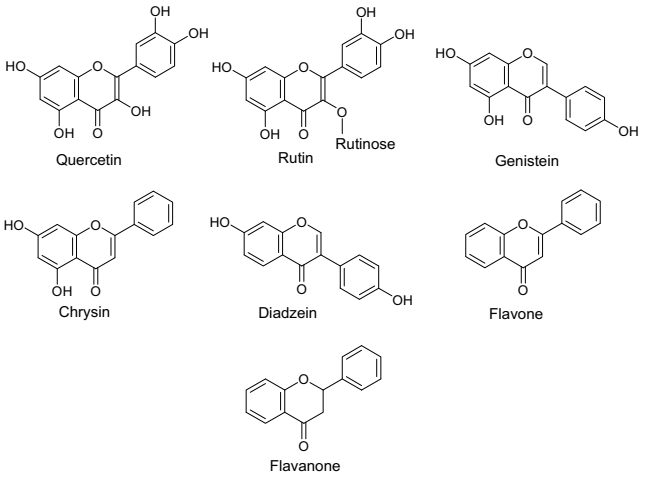
The flavonoid compounds were dissolved in DMSO solvent (about 10 mg/ml). From this solution, 10 μl of each flavonoid was used for gel electrophoresis study. Sample 1 contains liver homogenate (30 μl), Sample 2 contains liver homogenate (30 μl) and 10 μl of H_2O_2 (30%). Samples 3, 4, 5, 6, 7, 8 and 9 contain 30 μl of liver homogenate, 10 μl of H_2O_2 (30%) and 10 μl of antioxidant compounds such as flavone, flavanone, rutin, diadzein, quercetin, gen-

istein and chrysin, respectively. All samples were shaken well and incubated at 37 °C for 30 min. After the incubation period, equal volume (50 μl) of sample buffer (7.25 ml distilled water, 1.25 ml of 0.5 M TRIS (pH 6.8) and 1 ml of glycerol) were added to all the samples and were subjected to non-denaturing polyacrylamide gel electrophoresis (Native–PAGE). Due to the neutral pH of Tris in the phosphate buffer, it may not interfere with the staining. Non-denaturing polyacrylamide gel electrophoresis was performed essentially as described by Laemmli [31], except that SDS was omitted from all the buffers and the samples were not boiled before electrophoresis. Electrophoretic separation was performed on 8% gel at 4 °C with a constant power supply of 50 V for stacking gel and 100 V for separating gel.

Catalase activity was detected by the method of Woodbury [32]. The gel was soaked in 5 mM H_2O_2 solution for 10 min and was washed with water and stained with a reaction mixture containing 1% potassium ferricyanide and 1% ferric chloride (w/v). The enzyme appeared as a yellow band superimposed on a dark green background. The reaction was terminated by adding water and the gel was photographed at once. Quantification of the enzyme bands was performed by a densitometer (GS-300 transmittance/reflectance scanning densitometer, Hoefer Scientific Instruments, USA).

3. Results and discussion

The fluorescent probe, DBO absorbs at 365 nm and emits at 420 nm. The structures of the quenchers used in the present study are given below.



The ground state complex formation if any between DBO and flavonoids was checked by recording the absorption spectra of a mixture of DBO and flavonoids using concentration similar to those used in quenching studies. The absence of any new peak and the fact that absorption spectrum of DBO was unaltered in the presence of all quenchers eliminate the possibility of ground state charge transfer complex formation. For example, as a typical case, the absorption spectrum of DBO in the presence of chrysin is shown in Fig. 1. It may be noted that other flavonoids also exhibited a similar behavior. The Stern–Volmer quenching plots obtained by the plot of I_0/I or τ_0/τ vs [Q] were linear and gave consistent results, indicating that the quenching process is purely dynamic in nature. Lifetime measurements of DBO with different amount of flavonoids were carried out for evidence of dynamic quenching. In dynamic quenching, $F_0/F = \tau_0/\tau$, where τ_0 and τ are

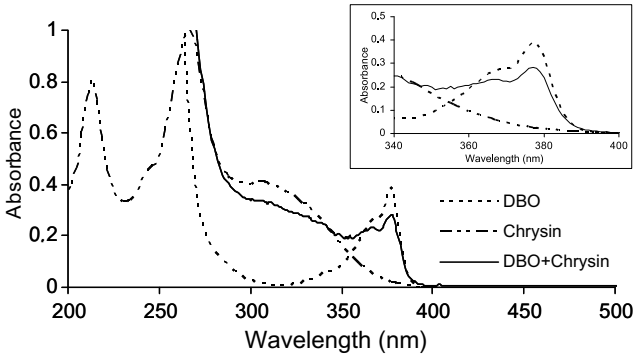


Fig. 1. Absorption spectra of 0.2 mM DBO (dotted line), 0.1 mM chrysin (semisolid line) and mixture of 0.2 mM DBO and 0.1 mM chrysin (solid line) in acetonitrile. The inset shows expansion of the peaks from 340 to 400 nm.

the fluorophore lifetimes in the absence and presence of quencher, respectively, and the fluorescence lifetime decreases on addition of quencher (Fig. 2 and 3).

3.1. Fluorescence quenching of DBO by flavonoids

The time resolved and steady-state quenching plots for the quenching of DBO by flavonoids are given in Figs. 4–7. The bimolecular quenching rate constants (both steady-state and time resolved) and the corresponding electrochemical data are compiled in Table 1. The fluorescence quenching rate constant decreases in the order: quercetin > rutin > genistein > diadzein > chrysin > flavone > flavanone. Quercetin has five –OH groups including 3′ and

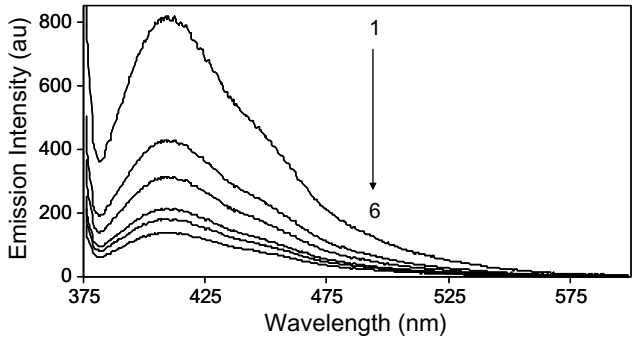


Fig. 2. Steady-state fluorescence spectra of DBO (1×10^{-4} M) in the presence of various concentrations ($0–5 \times 10^{-3}$ M) of chrysin.

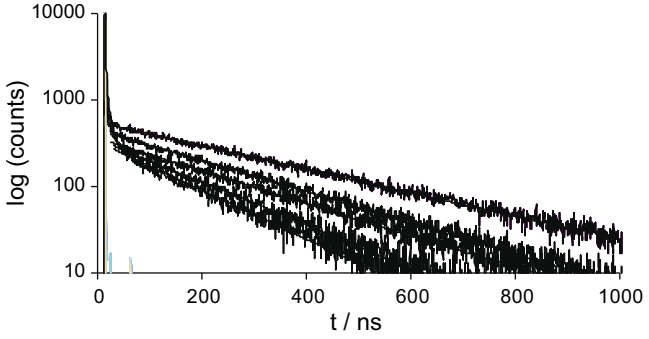


Fig. 3. Time-resolved decays curves of DBO (1×10^{-4} M) in the presence of various concentrations ($0–5 \times 10^{-3}$ M) of chrysin measured by single photon counting technique.

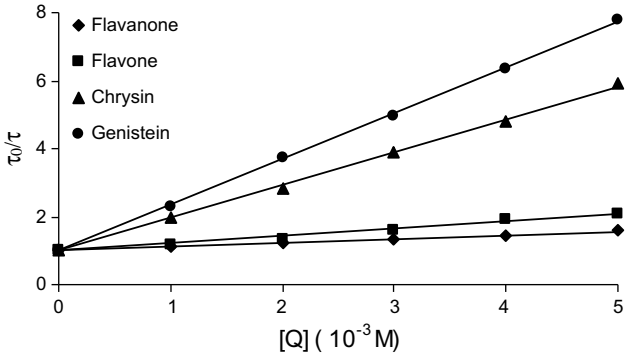


Fig. 4. Stern–Volmer plots for the time-resolved fluorescence quenching of DBO (1×10^{-4} M) by flavone, flavanone, chrysin and genistein (1×10^{-3} M) in acetonitrile.

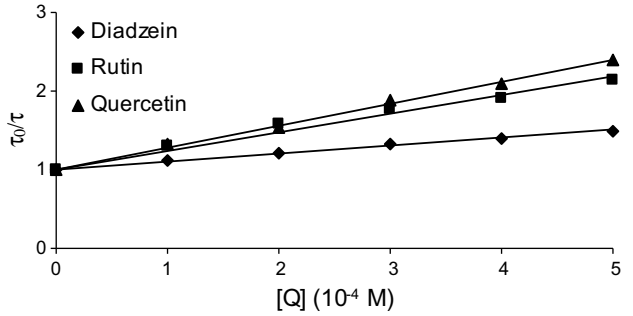


Fig. 5. Stern–Volmer plots for the time-resolved fluorescence quenching of DBO (1×10^{-4} M) by diadzein, rutin and quercetin (1×10^{-4} M) in acetonitrile.

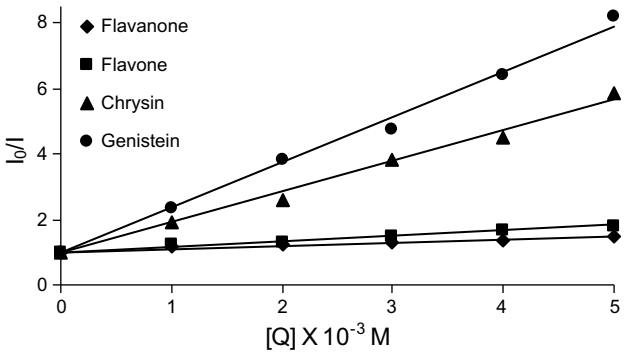


Fig. 6. Stern–Volmer plots for the steady-state fluorescence quenching of DBO (1×10^{-4} M) by flavone, flavanone, chrysin and genistein (1×10^{-3} M) in acetonitrile.

4′ –OH groups (catechol moiety) at the B ring and one –OH group in the C ring. Quercetin having the highest number of –OH groups in the series shows highest k_q. Particularly, the presence of –OH group in the C ring activating the 2,3 double bond is the main factor determining the efficiency of the k_q of quercetin [33,34]. Rutin lacks the –OH group in the C ring unlike quercetin. The lower k_q of rutin is also due to the diminished π conjugation between B and C rings because of the steric repulsion between the 6′ or 2′ ring protons at the B ring and rutinose group (sugar substituents) in the C ring. Flavone and flavanone exhibit the least k_q in the series due to absence of –OH groups at any position. Flavone shows higher k_q than flavanone due to the 2,3 double bond in conjugation with a 4-oxo group, which is responsible for electron delocalization between B and C rings. In the case of flavone, the observed k_q (anti-

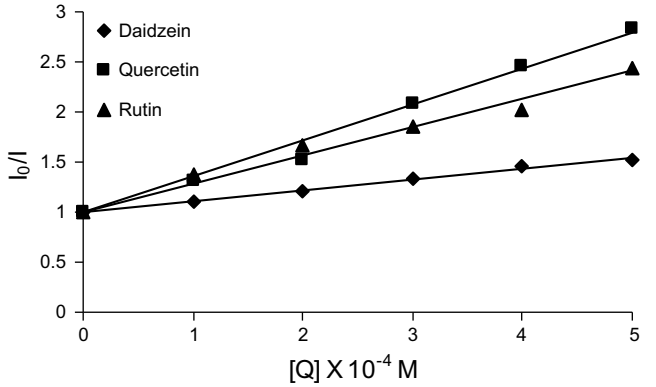


Fig. 7. Stern–Volmer plots for the steady-state fluorescence quenching of DBO (1×10^{-4} M) by diadzein, rutin and quercetin (1×10^{-4} M) in acetonitrile.

oxidant activity) in this work is due to resonance effect of the aromatic nucleus that lends stability to the flavonoid radical [35]. In flavanone, due to the presence of α-hydrogens, keto-enol tautomerism exists which makes one –OH group available in C ring from which H can be abstracted by singlet excited DBO leading to quenching. This observed minimum k_q value may be due to at least weak quenching because of H abstraction from C–H fragments [36]. Chrysin exhibits relatively higher k_q (1.49×10^9 M^{-1} s^{-1}) than flavone and flavanone because of the resorcinol A ring contributing more to the fluorescence quenching of DBO. Owing to an additional –OH group present in B ring, genistein shows higher k_q (2.22×10^9 M^{-1} s^{-1}) than chrysin. Even though chrysin and diadzein possess two –OH groups, diadzein has higher k_q than chrysin due to the possibility of A and B rings in the diadzein to exhibit phenolic character.

3.2. Thermodynamic consideration

The oxidation potential values of flavonoids are in the range of 0.84–1.15 V and given in the Table 1. The variations in the oxidation potential values of flavonoids arise due to their structural changes. In order to recognize the scavenging mechanism, we have used the oxidation potential to distinguish between the sequential electron and proton transfer and H-atom transfer as follows:

$$\text{Flavo-O—H} \rightarrow \text{Flavo-O}^{\cdot} + e^- + H^+ \quad (3)$$

$$\text{Flavo-O—H} \rightarrow \text{Flavo-O}^{\cdot} + H^{\cdot} \quad (4)$$

In a scavenging reaction, a hydrogen atom is donated to the acceptor radical (for example, DBO which resembles [1] alkoxy radicals). As both reactions involve breaking of the same O–H bond and H$^{\cdot}$ consists of e^- and H^+, it is very likely that the oxidisability of a compound is a measure of its ability to scavenge such radicals as mentioned above. The driving force (ΔG_{et}) for photoinduced electron transfer can be estimated according to Rehm–Weller equation [37]

$$\Delta G_{et} = E^{OX}(D) - E^{red}(A) - E^* + C, \quad (5)$$

where $E^{OX}(D)$ is the oxidation potential of donor (flavonoids), $E^{red}(A)$ is the reduction potential of DBO which has been reported as −2.8 V vs SCE [28], E^* is the excitation energy of the acceptor and C is the coulomb term which describes the electrostatic attraction within the contact ion pair and has a value of ca. −0.06 eV in acetonitrile [38] and frequently neglected in polar solvents. The measured electrochemical data imply that the high oxidation potential leads to positive ΔG_{et} values for excited ^{1}DBO with flavonoids (ΔG_{et} values in Table 1). The flavonoids possess lower oxidation potential than phenol [22], but the former was found to

Table 1
Fluorescence quenching rate constants, bond dissociation enthalpy and thermodynamic data of DBO by flavonoids

No.	Quencher (flavonoids)	k_q ($\times 10^9$ M^{-1} s^{-1})[a]			BDE[b] (kcal/mol)	$E_{1/2}$ vs SCE (V)[c]	ΔG_{et} (eV)[d]
		A	B	C			
1	Flavone	0.26	0.35	0.21	–	0.81	0.18
2	Flavanone	0.16	0.18	0.09	–	0.98	0.35
3	Chrysin	1.49	1.58	1.32	89.48	1.02	0.39
4	Diadzein	1.74	1.64	1.54	83.85	1.01	0.38
5	Genistein	2.22	2.15	2.01	84.36	1.01	0.38
6	Rutin	4.56	4.02	3.86	80.35	1.15	0.52
7	Quercetin	5.75	4.54	5.08	73.12	0.84	0.21

[a] Determined by (A) steady-state fluorescence quenching in CH_3CN (τ_0 = 620 ns), (B) time-resolved fluorescence quenching in CH_3CN (τ_0 = 620 ns) (error ± 5 ns) and (C) steady-state fluorescence quenching in CH_2Cl_2 (τ_0 = 185 ns). Error ± 10%.
[b] Lowest O–H bond dissociation enthalpy.
[c] Oxidation potential of flavonoids in V vs SCE in CH_3CN.
[d] Calculated by Rehm–Weller equation $\Delta G_{et} = E^{OX}(D) - E^{red}(A) - E^* + C$, the reduction potential of DBO is −2.8 V vs SCE, E^* = 3.3 eV and C = −0.06 (for acetonitrile), Error ± 10%.

be more reactive (5.75×10^9 M^{-1} s^{-1} for quercetin) than phenol (0.95×10^9 M^{-1} s^{-1}) [22]. This was attributed to a more charge localization for the flavonoids and thus significantly higher reactivity. The driving force for electron transfer is significantly endergonic revealing that quenching through hydrogen abstraction (H·) may be a possible mechanism.

3.3. Bond dissociation enthalpy (radical scavenging potential)

Free radical scavenging activity of polyphenols (ArO–H) is characterized by its hydrogen atom donating ability to scavenge the radicals [39].

$$\mathrm{ArO{-}H} + \mathrm{ROO^{\cdot}} \rightarrow \mathrm{ROOH} + \mathrm{ArO^{\cdot}} \quad (6)$$

The ability to donate a hydrogen atom is mainly governed by the O–H bond dissociation enthalpy [40,41]. The O–H bond dissociation energy can be calculated by the following equation:

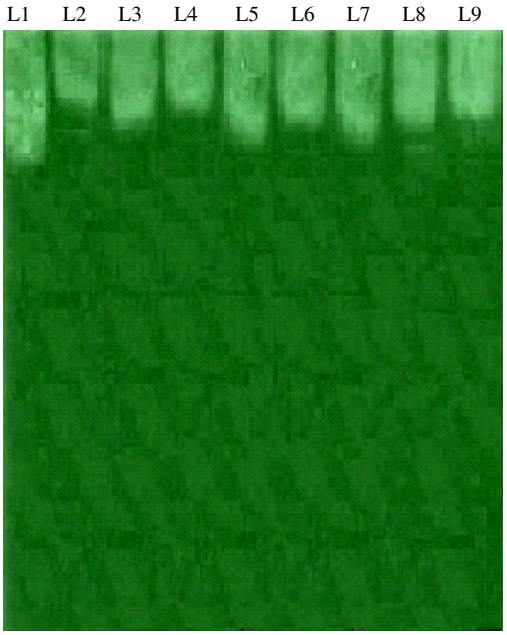
$$\mathrm{BDE(O{-}H)} = H(\mathrm{ArO^{\cdot}}) + H(\mathrm{H}) - H(\mathrm{ArO{-}H}), \quad (7)$$

where H(ArO·) is the enthalpy for a radical generated after H· abstraction, H(H) is the enthalpy for a H atom (−0.5 a.u.) and H(ArO–H) is the enthalpy for the parent molecule. The above equation helps to establish which O–H bond is weakest, since the weaker the O–H bond, the smaller the BDE and the greater the free radical scavenging ability of antioxidant. We obtained evidence for the weakest nature of ArO–H bond by computing the BDE for all O–H sites at the B3LYP/6-31G(p′)//B3LYP/3-21G* level. After identifying the weakest OH site (Fig. 10) in all of the compounds, the computed BDE is compared with the corresponding k_q values. The compound having smallest BDE shows highest k_q values and there is a good correlation between BDE and k_q values. Among the flavonoids, one of the OH group in the C-ring of quercetin shows lowest BDE (73.12 kcal/mol). The radical formed from O–H group of the C-ring is stabilized by the adjacent electron rich olefine bond by resonance as olefine is more electron rich than aromatic ring and this OH group is responsible for the highest antioxidant activity of quercetin. In the case of rutin, the hydroxyl group is replaced by rutinose group (lack of OH group in the C-ring), the catechol moiety in the B-ring possessing the lowest BDE among the four hydroxyl groups in this compound. Therefore, this OH group could be responsible for the free radical scavenging ability. Among the diadzein and genistein, diadzein shows slightly lower BDE (83.85 kcal/mol) than genistein (84.36 kcal/mol). Even though two hydroxyl groups are present in the A-ring of genistein, there is no significant difference in the BDE. Hence, these hydroxyl groups do not affect the antioxidant activity of the flavonoids. Hence, genistein and diadzein donate hydrogen atom from the hydroxyl group present in the B-ring. In the case of chrysin, the OH groups are present in A-ring only. Hence the BDE (89.48 kcal/mol) is higher than that of other flavonoids. It may donate hydrogen atom from its A-ring only. The above study reveals that mainly the position of the OH group determines the BDE. This finding on the order of radical scavenging ability by calculating BDE is in good agreement with experimentally obtained k_q values.

3.4. Gel electrophoresis

Gel electrophoresis is also a method for screening the antioxidant activity [31]. The primary aim of this study is to determine the radical scavenging ability of flavonoid compounds in enzyme models. Antioxidant activity of various flavonoids has been determined by the electrophoretic method [42]. An analysis of the electrophoretic pattern of catalase enzyme in the liver homogenate revealed one band with variations in the staining intensity of the band among the nine samples of flavonoids (Fig. 8 or 9), the lane 1 (only liver homogenate) exhibited a single band of high intensity (band area 230.54), while that of lane 3, 4, 5, 6, 7, 8 and 9 exhibited a band of almost similar intensity in flavonoid compounds (Fig. 8 or 9), i.e., band area 219.03, 215.62, 225.78, 221.07, 227.26, 222.94 and 220.13, respectively [43]. However, lane 2 (liver homogenate damaged by H_2O_2) revealed a single band with less intensity (band area 211.68). The intensity of band values was compared with the rest of the studies.

Catalase is an antioxidant enzyme, which is present in most cells and catalyses the decomposition of H_2O_2 to water and O_2. The addition of H_2O_2 to catalase enzyme leads to formation of free radicals. These radicals are scavenged by the antioxidant compounds. In the present study, the enzyme catalase was inactivated by H_2O_2 (lane 2) and then activated by the flavonoid compounds (Fig. 8 or 9). This might be due to the antioxidant potential of the flavonoids and the H_2O_2 could be scavenged by these compounds. This might be the reason for increasing intensity of the bands in lanes 3, 4, 5, 6, 7, 8 and 9. In a previous study by Geraldine and coworkers [42], antioxidant properties of mushroom *Pleurotus ostretaus* in an CCl_4-induced hepatotoxicity of rats, extract of mushroom, when administered to CCl_4-intoxicatedrats, were found to increase the band intensity of catalase enzyme. Since the mushroom contains potential antioxidant components like flavonoids, carotenoids and vitamins, this study is in agreement with the present investigation. The results from this gel electrophoresis method agreed with those of the steady-state and time resolved fluorescence quenching experiments in this work. Among the flavonoid compounds tested quercetin and rutin possess highest antioxidant activity, whereas



Where L1 - Liver homogenate (catalase)
L2 - Liver homogenate + H_2O_2
L3 - Liver homogenate + H_2O_2 + Flavone
L4 - Liver homogenate + H_2O_2 + Flavanone
L5 - Liver homogenate + H_2O_2 + Rutin
L6 - Liver homogenate + H_2O_2 + Diadzein
L7 - Liver homogenate + H_2O_2 + Quercetin
L8 - Liver homogenate + H_2O_2 + Genistein
L9 - Liver homogenate + H_2O_2 + Chrysin

Fig. 8. In vitro-antioxidant activity of flavonoids by gel electrophoresis.

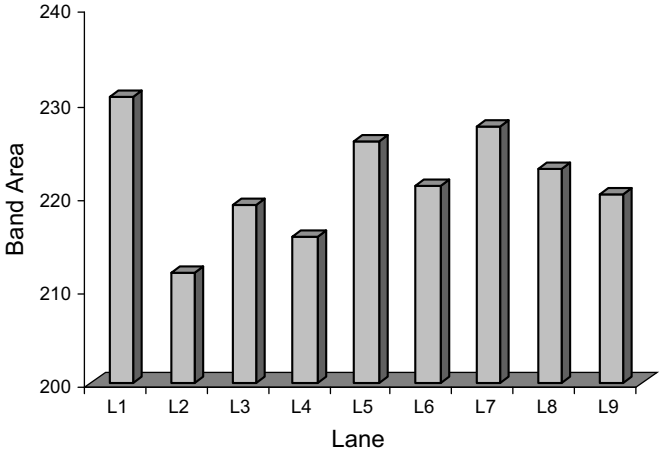


Fig. 9. Densitometric pattern of catalase enzyme from liver homogenate treated with flavonoids.

compounds flavone and flavanone show lowest activity. However, chrysin, diadzein and genistein show moderate activity than the other compounds. The ability to scavenge free radicals by the flavonoid compounds are correlated with the number of hydroxyl group bounded with the aromatic ring and their structure, position of aromatic –OH ring [33,34]. This result indicates that the flavonoids possess free radical scavenging ability.

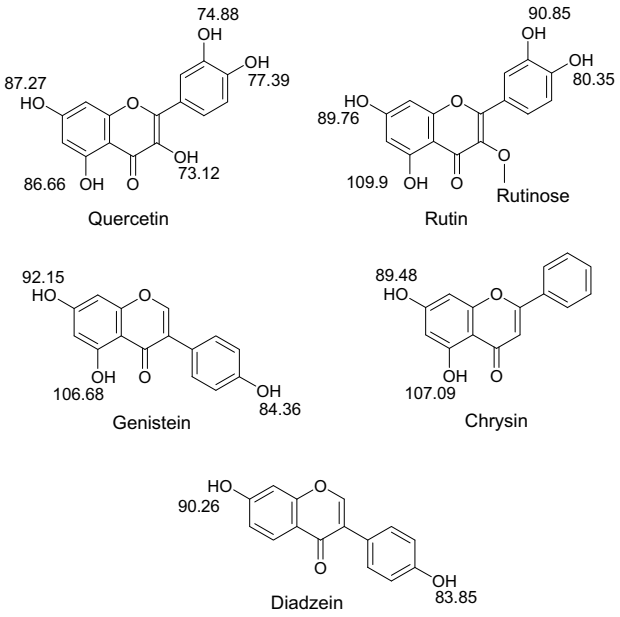


Fig. 10. Bond dissociation enthalpy of different O–H groups in flavonoids.

3.5. Deuterium isotope effects

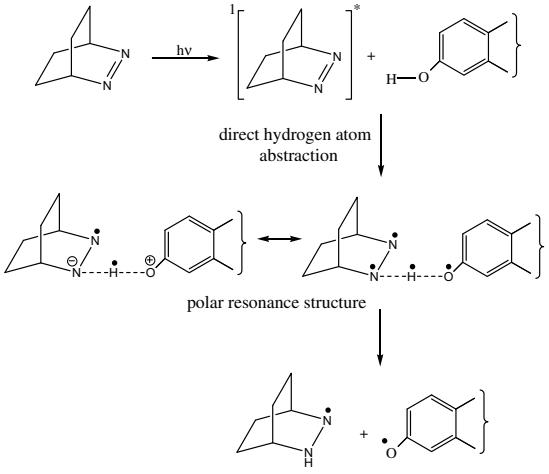
Fluorescence quenching of DBO by flavonoids shows appreciable deuterium isotope effects in (10% H_2O or D_2O) CH_3CN/H_2O and CH_3CN/D_2O mixtures (Table 2). The addition of water (10%) to acetonitrile decreases the quenching rate constants (Table 1) indicating a competitive effect of hydrogen atom donation by added H_2O against the flavonoids (in donation H to DBO) hinting that the quenching is mediated by hydrogen atom transfer in the present study. The observed deuterium isotope effect for flavonoids (Table 2) is in the range of 1.02–2.86, which are characteristic for hydrogen abstraction close to diffusion controlled limit as reported earlier [44,45]. In the case of flavone, the deuterium isotope value has been observed around unity (1.02). This clearly indicates the absence of OH/OD hydrogen exchange in the fluorescence quenching. Hence, the very low k_q value of flavone may be due to hydrogen atom abstraction from C–H fragment [36]. A small but significant isotope effect has been observed for flavanone (1.29). This may be due to the presence of α-hydrogen at C2 position (keto-enol tautomerism) which makes one OH group available in the C-ring. Hence, this OH may undergo fast OH/OD exchange. Large isotope effect (1.75–2.86) has been observed for other flavonoids (quercetin, rutin, genistein, diadzein and chrysin) related to the high quenching rate constants ($1.49–5.75 \times 10^9\ M^{-1}\ s^{-1}$) (Table 1). This possibly suggests a predominant role of hydrogen abstraction [22] in the quenching mechanism. The deuterium isotope effect thus observed in this work provides evidence for hydrogen abstraction involved in the quenching process of singlet excited DBO by flavonoids.

The overall mechanistic pathways of the photoreduction of DBO by flavonoids are illustrated in Scheme 1. The quenching of DBO by flavonoids can be explained by a number of possible mechanisms such as (i) sequential electron and proton transfer (ii) exciplex mediated H-atom abstraction or (iii) by direct H-atom abstraction [22]. The photoinduced electron transfer can be excluded on the basis of endergonic energetics calculated form the Rehm–Weller equation. Hence, the mechanism involving sequential electron and proton transfer can also be ruled out. Excited-state hydrogen abstraction not only may be achieved by direct abstraction, but also may occur from exciplex (mediated abstraction) involving

Table 2
Deuterium isotope effect

No.	Flavonoids	[a]k_q ($\times 10^9$ M^{-1} s^{-1})	[b]k_q ($\times 10^9$ M^{-1} s^{-1})	[c]k_q(O–H)/k_q(O–D)
1	Flavone	0.12	0.12	1.02
2	Flavanone	0.08	0.06	1.29
3	Chrysin	0.90	0.51	1.75
4	Diadzein	1.28	0.70	1.82
5	Genistein	1.93	0.86	2.23
6	Rutin	3.89	1.42	2.74
7	Quercetin	4.23	1.48	2.86

[a] Measured by steady-state method in wet acetonitrile (10% H_2O).
[b] Measured by steady-state method in wet acetonitrile (10% D_2O).
[c] Deuterium isotopic effect measured by steady-state fluorescence quenching in CH_3CN containing 10% H_2O or D_2O, i.e., [k_q(O–H)/k_q(O–D)]. Errors in all data are with in ±10%.



Scheme 1. Fluorescence quenching mechanism of DBO by flavonoids through a direct hydrogen atom transfer.

lone pair coordination. The quenching rate constants increase with solvent polarity. The faster quenching in polar solvent is attributed to stabilization of primary reaction intermediates. In the present study, exciplex mediated hydrogen abstraction may be ruled out based on the absence of inverted solvent effect on k_q, as reported earlier in similar study [46].

From the quenching rate constant, endergonic thermodynamics and bond dissociation energy calculations, it is suggested that the fluorescence quenching of DBO by flavonoids occurs via direct hydrogen atom abstraction mechanism. The flavonoid phenoxyl radical formed after the donation of hydrogen atom to DBO [15,18,19] would be relatively long lived and stabilized by internal delocalization of the electrons around its aromatic structure. The experimental data obtained from various experiments, i.e., quenching studies (steady state and time resolved), cyclic voltametric studies, theoretical calculations and gel electrophoresis studies are fully consistent with this interpretation.

4. Conclusions

The fluorophore DBO is quenched by flavonoids in acetonitrile and the result indicates that fluorescence quenching rate constant (k_q) increases with increasing number of –OH substitutions on the flavone skeleton. Quercetin shows highest fluorescence quenching rate constant than other flavonoids studied in this work and follows the trend: quercetin > rutin > genistein > diadzein > chrysin > flavone > flavanone. Especially, the existence of 2, 3-double bond in the C ring and catechol moiety in the B ring is essential for higher antioxidant activity of flavonoids. Fluorescence quenching of DBO by flavonoids shows substantial deuterium isotope effects provides evidence for hydrogen abstraction involved in the quenching process of singlet excited DBO by flavonoids. The radical scavenging ability of flavonoid compounds has been determined by the electrophoretic method as well as BDE calculations. The bond dissociation enthalpy calculation reveals that the number and position of the OH groups in the flavone skeleton determine the radical scavenging ability. Based on the experimental data obtained from steady state, time resolved, cyclic voltametric, deuterium isotope effects and *in vitro*-antioxidant activity studies a quenching process involving direct hydrogen atom abstraction by singlet excited DBO is suggested.

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