Investigations on the binding and antioxidant properties of the plant flavonoid fisetin in model biomembranes

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Abstract **Plant flavonoids are emerging as potent therapeutic drugs for free radical mediated diseases, for which cell membranes generally serve as targets for lipid peroxidation and related deleterious effects. Screening and characterization of these ubiquitous, therapeutically potent polyphenolic compounds, require a clear understanding regarding their incorporation and possible location in membranes, as well as quantitative estimates of their antioxidative and radical scavenging capacities. Here, we demonstrate the novel use of the intrinsic fluorescence characteristics of the plant flavonoid fisetin (3,3′,4′,7-OH flavone) to explore its binding and site(s) of solubilisation in egg lecithin liposomal membranes. Spectrophotometric assays have been used to obtain quantitative estimates of its antioxidative capacity. Furthermore, our quantum mechanical semi-empirical calculations provide a quantitative measure for the free radical scavenging activity of fisetin from the OH (at 3, 3′, 4′, 7 positions of the molecule)-bond dissociation enthalpies. Implications of these findings are discussed.**

Keywords: Flavonoid; Fisetin; Intrinsic fluorescence; Lipid peroxidation; Antioxidant; O–H bond dissociation enthalpy

1. Introduction

In 1936, Szent-Györgii [1] first drew attention to the therapeutically beneficial role of dietary flavonoids [2], which are polyphenolic compounds ubiquitously present in common plant based food items and beverages, e.g., onions, apples, tea and red wine. Recent years have witnessed revitalized attention in this area with an explosive growth of research on various bioactive flavonoids having important therapeutic activities, of high potency and low systemic toxicity. In addition, flavonols (which comprise a major class of flavonoid compounds) have emerged as one of the best known molecular systems exhibiting intramolecular excited state proton transfer (ESPT) and dual fluorescence behavior [3–5]. Consequently, there is growing interest on such compounds as useful models for mechanistic studies of ESPT and as exquisitely sensitive fluorescence probes for exploring their binding sites in relevant biological targets, e.g., proteins and biomembranes [6,7].

Flavonoids are known to possess powerful antioxidant properties, which are attributed to the presence of phenolic hydroxyl groups in the flavonoid structure. Free radical scavenging plays a considerable role in the antioxidant activity of such compounds [8–17]. Therefore much recent attention has focused on the potential uses of flavonoid-based drugs for the prevention and therapy of free radical mediated human diseases (e.g., atherosclerosis, ischemia, inflammation, neuronal degeneration, cardiovascular diseases etc.) [13–15]. Fisetin (3,3′,4′,7-tetrahydroxyflavone) (structure shown in Scheme 1), a naturally occurring polyhydroxyflavone, has attracted significant spectroscopic attention for its dual emission behaviour and excellent properties as a laser dye [18]. It has also been of considerable recent interest from biological perspectives for its inhibitory action against protein kinase C, a signal transducing enzyme [19] and HIV-1 proteinase, a virally encoded protein which is indispensable for the maturation and processing of AIDS virus and thus a viable target for HIV therapy [20].

Biological organs contain many polyunsaturated fatty acids (PUFA), such as linoleic and arachidonic acids. These PUFA can undergo lipid peroxidation (LP), which can be interrupted by antioxidants. Cell membranes are important targets of free radicals which induce LP and thereby cause malfunctioning of membranes by altering membrane fluidity and membrane-bound enzyme and receptor functions [21]. Vitamin E (α-tocopherol), a potent endogeneous antioxidant, protects membranes from this oxidative damage by scavenging lipid peroxyl radicals ($LOO^{\cdot}$) [13,22]. Flavonoids, being structurally similar to vitamin E, are known to mimic its antioxidant activity in membranes [13].

Previous workers [12–15,23] have reported a few rather limited attempts to explore structure–activity relationships of plant flavonoids in relation to their antioxidant and radical scavenging potencies in membranes. Moreover, binding affinities and locations of flavonoids in membranes are not clearly known, although such factors appear to be especially crucial in determining their antioxidative efficiencies in membranes. In the present study, we have examined fisetin incorporated in model membranes (liposomes) composed of natural, polyunsaturated phospholipids (egg lecithin). Here, we demonstrate for the first time, novel uses of the intrinsic fluorescence behaviour of fisetin in liposomes, to characterize the binding and location of such compounds in membrane environments. Our next aim was to examine and quantify the antioxidative capacity of the membrane-bound fisetin in terms of the oxidation index. Quantum mechanical semi-empirical calculations based on AM1 and PM3 treatments were performed to characterize the OH bond dissociation enthalpies (BDE) [24,25] occurring at different (namely 3, 3′, 4′, 7) positions of the fisetin molecule,

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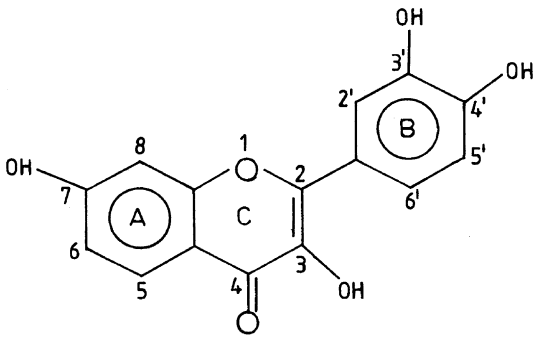

Scheme 1. Structure of fisetin.

and to determine most probable redox sites for scavenging peroxyl radical (OH).

We report that fisetin strongly binds around the interfacial region of the egg PC liposomes (between the polar head and hydrophobic tail of the phospholipids, where the fisetin molecules are expected to be adequately accessible to the incoming free radicals [15]), and inhibits lipid peroxidation with an estimated efficiency of 50%. The semi-empirical calculations confirm significantly high antioxidant capacity (as indicated by the calculated BDE values) for fisetin, with the potency for extracting OH from the surrounding medium predicted to decrease in the order 3-OH > 3′-OH > 4′-OH > 7-OH.

2. Materials and methods

2.1. Reagents

Fisetin was a product of Aldrich Chemical Company. Dipalmitoyl phosphatidylcholine (DPPC) and egg lecithin (egg PC) were obtained from Sigma. Solvents used were of spectroscopic grade and were preliminarily checked for absence of absorbing and fluorescent impurities. All other chemicals used in this work were obtained from Sisco Research Laboratories, India. Triple distilled water was used for all liposomal experiments.

2.2. Liposome preparation

Small unilamellar liposomes of DPPC and egg PC phospholipids (in absence and presence of fisetin) were prepared by probe sonication technique as described previously [7]. For all spectroscopic measurements fisetin:phospholipid molar ratio was kept at 1:20 (25 μM fisetin, 500 μM lipid).

2.3. Spectrophotometric measurements

Steady state absorption and fluorescence spectra were recorded with Hitachi models U-2000 spectrophotometer and F-4010 spectrofluorometer, respectively. Temperature-dependent fluorescence studies were carried out using a Neslab RTE 110 refrigerated circulating water bath. The fluorescence polarization anisotropy (r) values were obtained from the expression $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I_{VV} and I_{VH} are the vertically and horizontally polarized components of fisetin emission with excitation by vertically polarized light at 370 nm and G is the sensitivity factor of the detection system [26].

2.4. Detection of oxidation in liposomal membranes

It is well known that free radicals can cause LP of polyunsaturated lipids (such as egg lecithin) which can occur in air (autoxidation), or can be induced by metal ions, e.g., Fe^{2+} [8,11,27]. The relevant reaction mechanisms have been described by Farmer and Sutton [27,28]. The essential stages in the peroxidation process include formation of conjugated diene (CD) followed by hydroperoxide formation. Ring formation and subsequent cleavage of the chain to give malondialdehyde (MDA) complete the process. Monitoring such reactions involve the absorption spectrophotometric measurements of CD at 234 nm, and of MDA equivalents formed, with thiobarbituric acid (TBA) and trichloroacetic acid (TCA) [27,29], at the absorption maximum of MDA (534 nm).

The oxidation index (defined as the ratio of the absorption at 232 nm to the absorption at 210 nm, A_{232}/A_{210}) provides a rapid and sensitive method for detecting oxidation in liposome preparations during their preparation or subsequent experiments [27,29].

For measuring MDA, TBA and TCA were added to aliquots of the egg lecithin liposomal solution (both in presence and absence of fisetin), a portion of each solution was subjected to autoxidation by air, while other portions were used for studies of peroxidation induced by adding 40 μM Fe^{2+}/ascorbate solutions [8,29]. All the mixtures were heated in a boiling water bath for 30 min. After cooling, the supernatant was extracted with n-butanol and the absorbance was checked at 534 nm (for MDA–TBA adduct) after specific time intervals of oxidation using thiobarbituric acid reactive substances (TBARS) assay [29].

2.5. Quantum mechanical calculations

The lowest energy conformation of fisetin was evaluated by using a combination of MM+ force field and quantum-mechanical semi-empirical methods implemented in the HYPERCHEM 7.5 package v. 2004. Both AM1 and PM3 calculations were done using the unrestricted Hartree–Fock method to optimize the parent molecule and its radicals. Previous studies have associated BDE, corresponding to the O–H bond breaking and abstraction of H, to the free radical scavenging activity. The lower the BDE value, more efficient is the radical scavenging capacity [24,25]. BDE was evaluated from the difference in the heat of formation (H_f) of the flavonoid (ROH) and its corresponding radical (RO·) formed according to the following reaction:

$$\mathrm{ROH} + {}^{\cdot}\mathrm{OH} \rightarrow \mathrm{RO}^{\cdot} + \mathrm{HOH} \quad (\mathrm{ROH\ is\ the\ antioxidant, fisetin})$$

3. Results and discussions

Fig. 1A presents absorption (inset) and fluorescence emission spectra of fisetin incorporated in egg PC liposomes. The emission spectra are shown at two different temperatures, below and above the phase transition temperature (T_m) of egg PC, as reported in the literature [30,31]. Dual emission behavior is observed at both the temperatures, below and above T_m, the emission yield being higher in the former case (Fig. 1A). The high energy band having emission maximum ≈488 nm can be assigned to the $S_1 \rightarrow S_0$ normal emission, whereas the large Stokes shifted green fluorescence band can be attributed to the emission from the tautomer species (PT fluorescence) generated by an ESPT process [3–5,7,32]. It is noteworthy that the emission profiles of fisetin recorded in the liposomal membranes resemble the situation in aprotic environment where dual emission behavior is prominent [7,32]. Moreover, this is consistent with the spectral characteristics of the excitation profile (monitored for the PT fluorescence, below T_m) which reveals a weak but clearly perceptible vibrational shoulder (Fig. 1B) [32] typical of a predominantly aprotic environment. Fig. 1B (inset) depicts the excitation spectrum monitored in the normal emission region (460 nm) of fisetin which shows the presence of a long wavelength shoulder at around 430 nm, attributable to a ground state complex formation of fisetin in the membrane environment. This suggests presence of strong specific interaction of fisetin with the phospholipid molecules. Such behavior is reminiscent of similar findings reported by us recently, in case of a closely related naturally occurring flavonol, quercetin, bound to a protein (human serum albumin) [33].

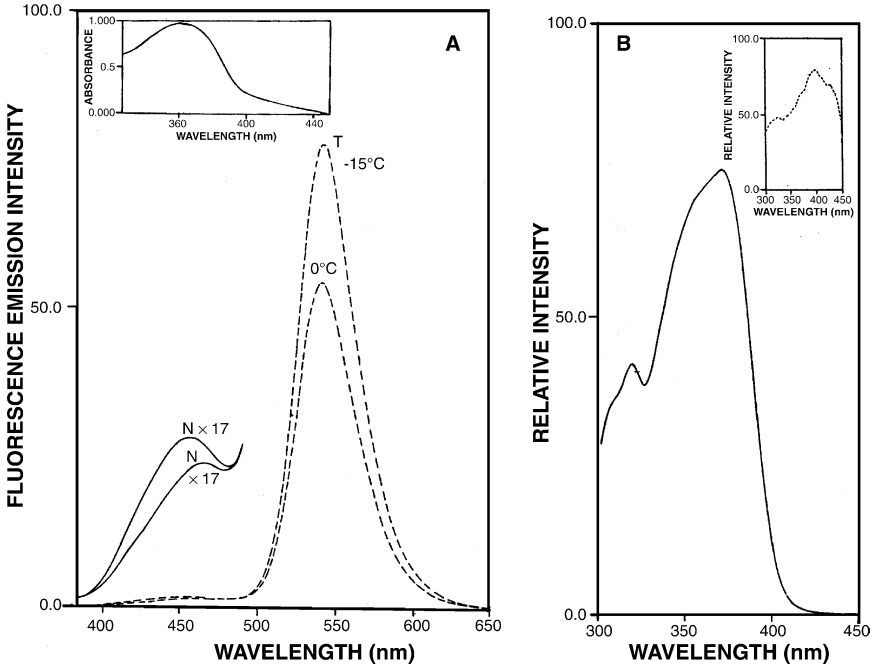

Fig. 1. (A) Fluorescence emission spectra (obtained using an excitation wavelength, $\lambda_{ex} = 370$ nm) of fisetin in egg lecithin liposomes measured above (0 °C) and below (−15 °C) thermotropic gel to liquid crystalline phase transition temperature (−12 °C) of the egg lecithin phospholipid and (B) Fluorescence excitation spectrum (monitored at emission wavelength, $\lambda_{em} = 580$ nm) of fisetin in egg lecithin liposomes. (Fig. 1A inset displays the absorption spectra of fisetin in egg lecithin liposome and Fig. 1B (inset) shows the fluorescence excitation spectrum of fisetin in liposomes monitored at emission wavelength, $\lambda_{em} = 460$ nm.)

We have also carried out a temperature dependence study of the fluorescence anisotropy (r) of fisetin in egg PC as well as DPPC liposomal membranes, to explore the potentiality and usefulness of this emission parameter for probing the structural changes in liposomes. The 'r' vs temperature plots (Fig. 2) show characteristic sigmoidal shape, revealing the thermotropic phase transition of the phospholipids from gel to liquid crystalline states. Phase transition temperature (T_m, 42 °C for DPPC and −12 °C for egg PC) values estimated from the midpoints of the sigmoidal shaped curves, are in good agreement with existing literature data based on different methods [7,30–32]. The anisotropy parameter serves as a useful indicator of the rigidity of the local environment of the fluorophore (fisetin). It shows zero or very low value in fluid solution where fluorophore can freely rotate. The significantly high values for the anisotropy (r) of fisetin we observe here (e.g. 'r' = 0.31 at −14 °C in egg PC liposomes, Fig. 2), together with the fact that this parameter is proving to be a sensitive monitor of the thermotropic phase transitions of the phospholipids, are consistent with the picture that the fisetin molecules are firmly incorporated in the membrane bilayers, and localized in motionally constrained sites.

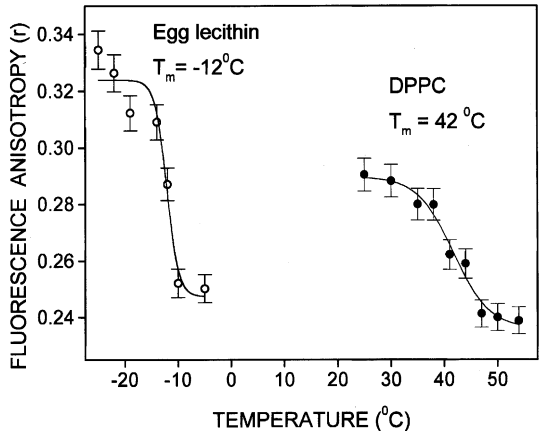

Fig. 2. Variation of steady-state fluorescence anisotropy (r) of ESPT fluorescence of fisetin in egg lecithin (open circles) and DPPC (solid circles) liposomes as a function of temperature. $\lambda_{ex} = 370$ nm; $\lambda_{em} = 535$ nm.

A comparative study was next performed on the oxidation index (OI, $A_{232\,nm}/A_{210\,nm}$) of egg lecithin liposomes in the presence as well as absence of fisetin, which were found to be 1.14 and 2.66, respectively. The antioxidant nature of fisetin is clearly evident from the decrease in oxidation index in presence of fisetin in the unsaturated liposomes [29]. In Fig. 3, we present a comparison of the production of MDA as estimated spectrophotometrically by the absorption intensity monitored at its spectral maximum (534 nm) in auto (air) as well as induced (in presence of Fe^{2+}) states [29], both in presence and absence of fisetin. From this study, we infer that fisetin inhibits LP upto 50% in egg PC liposomal membranes.

The BDE values and torsion angles (ψ, between C3–C2–C1′–C2′) for the radicals were calculated by AM1 and PM3 methods and are listed in Table 1. Ball and stick models of fisetin and its radicals along with their respective heat of formation (H_f) values are given in Fig. 4. It is found that the 3-OH fisetin radical has the lowest calculated BDE value followed by the 3′-OH and 4′-OH fisetin radicals while the corresponding value of the 7-OH fisetin radical is predicted to be the highest. A lower BDE value is usually attributed to a

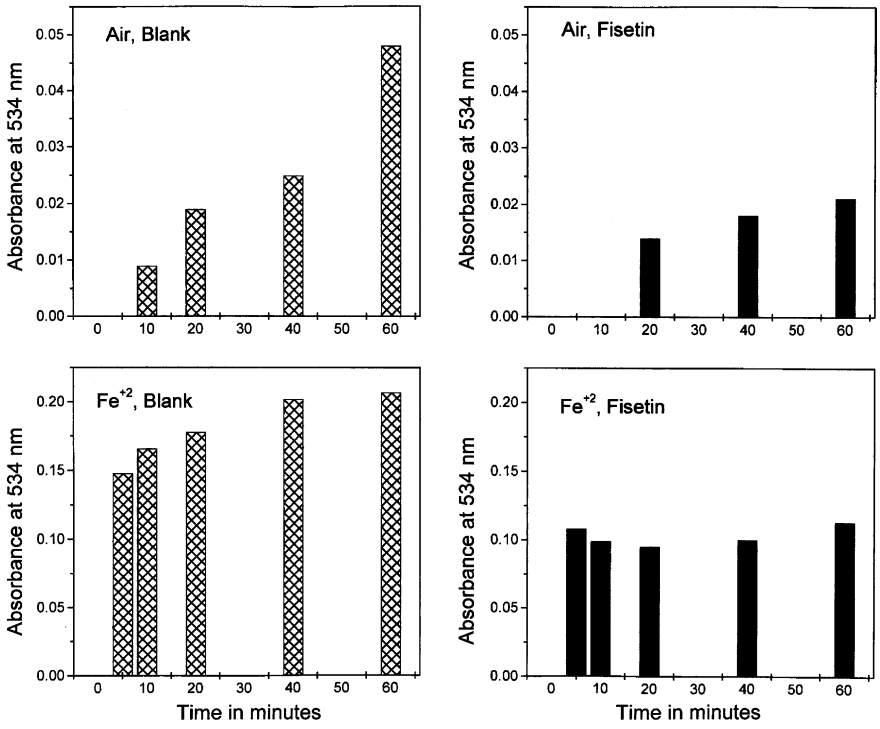

Fig. 3. A comparative study of the lipid peroxidation (LP) of egg lecithin liposomes in presence and absence of fisetin. The measurements were made after various incubation times (in min) of oxidation, both in air and Fe^{+2} induced states. The extent of LP was monitored by the amount of malondialdehyde (MDA) produced using TBARS assay. This procedure is based on measuring the absorbance of the stable product (formed between MDA and thiobarbituric acid (TBA) in the aqueous phase) at 534 nm (where this product has its absorption maximum).

Table 1
Calculated BDE and torsion angle (ψ, between C3–C2–C1′–C2′) values of the optimized fisetin radicals (RO·)

Radical	BDE (kcal/mol)	Torsion angle, ψ (in deg)
3-OH	16.77	+179.701
3′-OH	19.73	+179.61
4′-OH	20.34	+179.714
7-OH	27.74	+179.645

higher ability to donate a hydrogen atom from the hydroxyl group and thereby scavenge free radicals [14]. Our results indicate that the hydroxyl groups in the 3, 3′, 4′ positions of fisetin are more effective in scavenging free radicals than the 7-OH group on the A-ring. Thus the C and B rings of fisetin appear to play a more important role in comparison to the A-ring in scavenging free radicals and imparting antioxidant property to the molecule.

In conclusion, this research exemplifies the usefulness of the intrinsic fluorescence properties of natural flavonols to characterize their binding sites in liposomal membranes. Fluorescence emission and excitation profiles along with fluorescence anisotropy data clearly indicate that fisetin molecules are localised and rigidly bound around the interfacial region (between the polar head and hydrophobic tail) of the egg PC liposomes. This region is freely accessible to the approaching free radicals and serves as the reaction site for the antioxidant activity of the membrane-bound fisetin molecules. The experimental as well as theoretical studies on the antioxidant properties of fisetin indicate that this plant flavonoid has considerable potentiality for use as a therapeutic agent against free radical-mediated diseases.

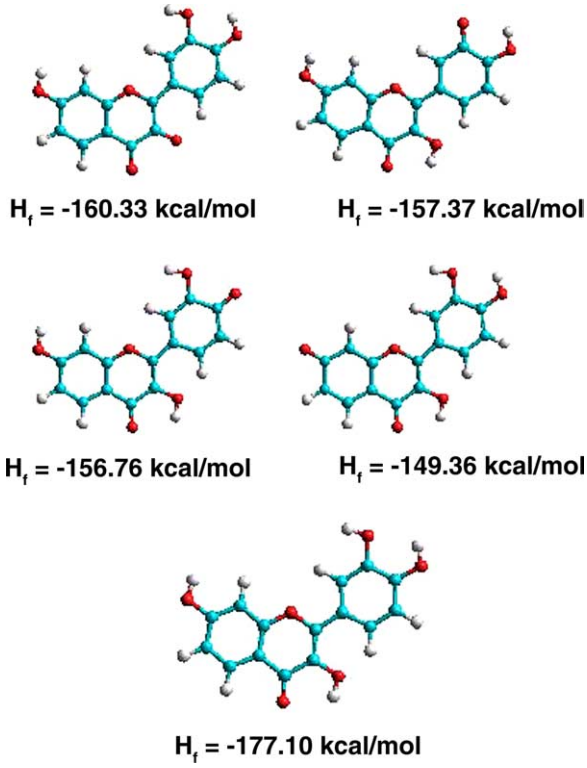

Fig. 4. Ball and stick representations of the optimized structures of fisetin and its different radicals along with their respective heat of formation (H_f) values. The optimized structures were obtained by energy minimization and heats of formation were estimated for these optimized structures using semi empirical quantum chemical methods implemented in the HYPERCHEM 7.5 software package.

Furthermore, we can foresee the possibility of future extension of our fluorescence approach, described herein, to a series of naturally occurring flavonols varying in the OH substitution positions (on the A and B rings) for exploring the role of their sites of solubilisation in the membrane in modulating their efficiencies as antioxidants.

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