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INTERACTION OF FLAVONOIDS WITH 1,1-DIPHENYL-2-PICRYLHYDRAZYL FREE RADICAL, LIPOSOMAL MEMBRANES AND SOYBEAN LIPOXYGENASE-1

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Abstract—The interaction of the antiperoxidative flavonoids namely, quercetin, quercetrin, rutin, myricetin, phloretin, phloridzin, catechin, morin and taxifolin with the 1,1,-diphenyl-2-picrylhydrazyl (DPPH) free radical was demonstrated. Flavonoid–DPPH interaction was looked at in the absence and presence of liposomes so as to reveal some information on bilayers. Perturbations in the lipid bilayers were monitored with the fluorescent probe, dansylhexadecylamine (DSHA). It was observed that the interaction of the flavonoids on the lipid bilayer occurred in the polar zone of the lipid bilayers. The flavonoids were able to scavenge free radicals and could do so in biomembranes. It is suggested that the DPPH free radical abstracts the phenolic hydrogen of the flavonoid molecule and that this could be the general mechanism of the scavenging action of the antiperoxidative flavonoids.

The effects of the flavonoids on soybean lipoxygenase-1 were investigated both in buffer and also in liposomal suspension. All the flavonoids studied showed inhibition of the enzyme in both systems but the inhibition was greater in the liposomal suspension. Quercetin was the most potent and it inhibited the lipoxygenase in the liposomal suspension by about 42% while the other flavonoids inhibited the enzyme by about 14–23%. We observed that the effect of myricetin and quercetin on the enzyme was pH dependent.

The flavonoids are a group of naturally occurring, low molecular weight benzo-γ-pyrone derivatives, ubiquitous in plants [1]. They serve multifunctions in plant physiology and are accessible to animals through their diet. Common food of plant origin contains from traces to several grams of flavonoids per kilogram fresh weight [1]. These plant constituents have been documented to exhibit a myriad of pharmacological effects on animal systems, some of which include anti-inflammatory and anti-allergic activity, RNA, DNA and protein synthesis, antiviral activity, lipid peroxidation, capillary fragility, catacholamine activity, tumor promotion and lymphocyte proliferative response [2–4].

Das and Ratty [5] have found several flavonoids that inhibit non-enzymatic lipid peroxidation. This antiperoxidative action of flavonoids led us to investigate as to whether these compounds could interact with free radicals. In this paper, we investigated the interaction of the antiperoxidative flavonoids with the 1,1,-diphenyl-2-picrylhydrazyl (DPPH) free radical. This is a very stable free radical used extensively in electron spin resonance studies. Flavonoid–DPPH interaction was looked at in the absence and presence of liposomes, entities which have been widely used as experimental working models of biological membranes. For investigations into the effects of surface active agents, it is advantageous to employ liposomes as they are stable, do not exhibit phase diagram changes and are comparable to intact cell systems. Our studies thus reveal information on the flavonoid-free radical interaction in the presence of bilayers. We also attempt to show the interaction between the flavonoids and lipid bilayers and the site of interaction. Perturbations in the lipid bilayer were monitored with the fluorescent probe, dansylhexadecylamine (DSHA). Fluorescent probes are used to reveal the proximity, rotational mobility and polarity of biomembranes and have been utilised to gain insight into membrane–ligand interactions [6–8].

We also investigated the effects of these flavonoids on soybean lipoxygenase-1 in the presence of liposomal membranes. Lipoxygenases catalyse the oxygenation of *cis*, *cis*-1, 4-pentadienes producing *cis*, *trans*-conjugated monohydroperoxydiene derivatives. The secondary products of lipoxygenase-catalysed reactions are important as cell-to-cell communicators. Nakadate *et al.* [9] have reported the involvement of lipoxygenase products in skin tumor production. Potent inhibitors of lipoxygenase could prove useful as anti-tumor and anti-inflammatory agents.

EXPERIMENTAL

Chemicals. Dipalmitoyl-DL-phosphatidylcholine (DPPC) and DPPH were purchased from Sigma Chemical Co. (St. Louis, MO) and from Nakarai Chemicals Ltd. (Kyoto), respectively. Soybean lipoxygenase-1 was purchased from Serva Fienbiochemica GmbH & Co. (Tokyo) and the flavonoids, myricetin, quercetin, quercetrin, rutin, phlor-

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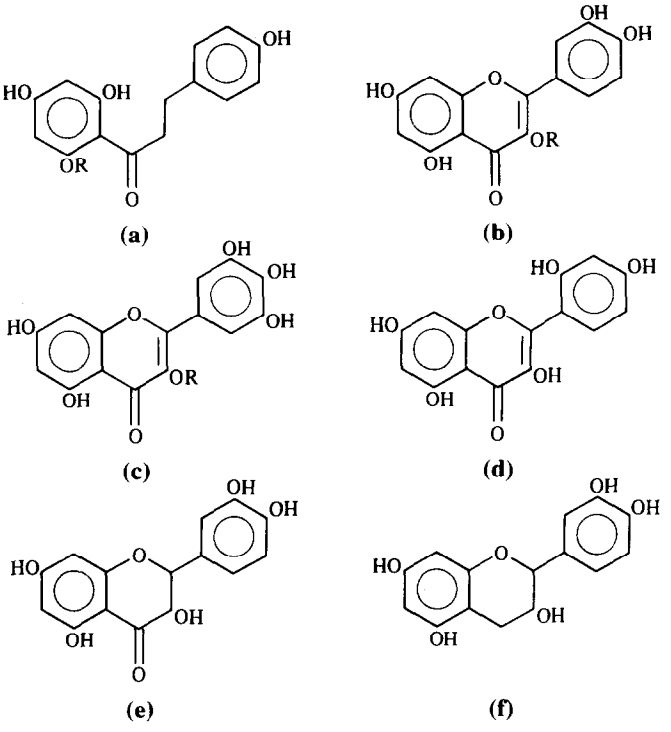

Fig. 1. Chemical structures of flavonoids used in this study. Chalcones: (a) phloretin (R=H), phloridzin (R=glucose); Flavanonol: (b) quercetin (R=H), quercetrin (R=rhamnose), rutin (R=rutinose); (c) myricetin (R=H), (d) morin; Flavanonol: (e) taxifolin (dihydroquercetin) and Flavanonol: (f) (+)-catechin.

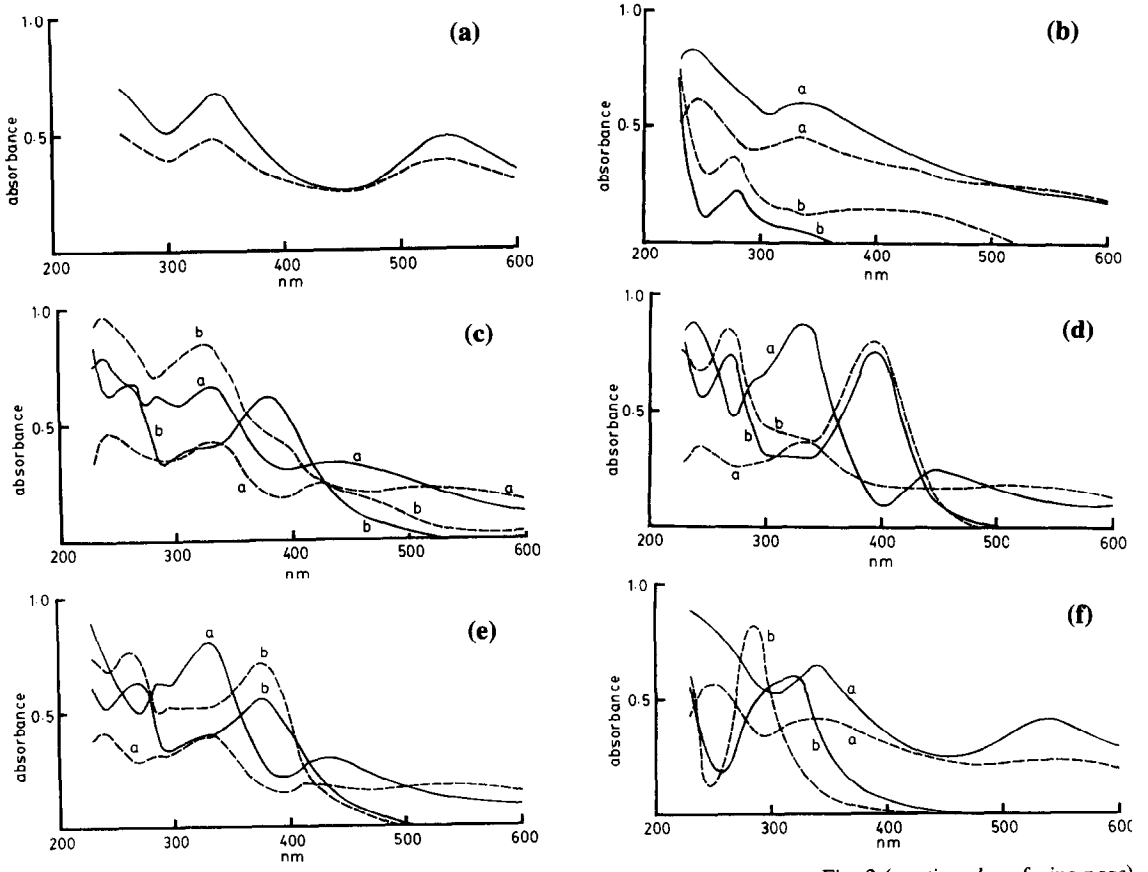

Fig. 2 (*continued on facing page*)

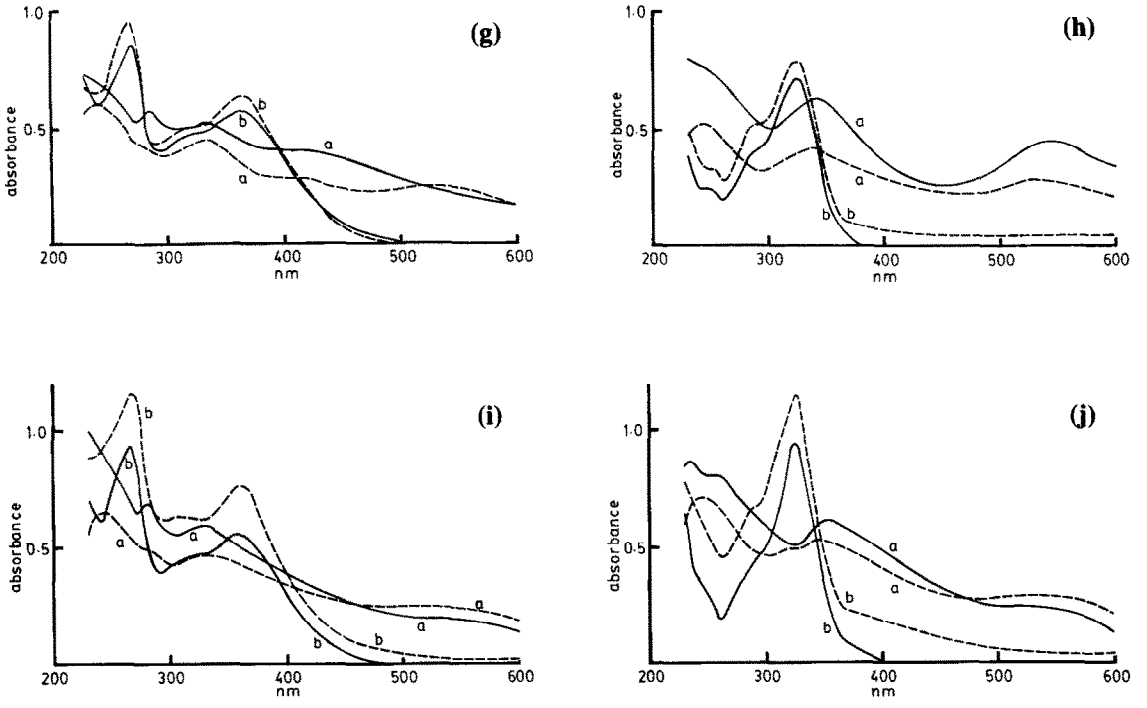

Fig. 2. (a) Absorption spectra of DPPH in buffer and in DPPC liposomal suspension. Solid line denotes the spectrum in buffer mixture (Tris–HCl/NaCl), pH 7.4 and the broken line denotes the spectrum in DPPC liposomal suspension (1 mM phospholipid), pH 7.4. The absorption spectra of DPPH in the different media were recorded after an incubation period of 30 min. The absorption maxima for the solid line occurred at 340 and 540 nm whilst that for the broken line occurred at 335 and 535 nm. (b) Absorption spectra of DPPH and catechin. a ----, DPPH spectrum in buffer and in the presence of catechin; a ———, DPPH spectrum in liposomal suspensions (catechin was present in both the reference and test cuvettes, see text); b ----, catechin spectrum in buffer solution; b ———, catechin spectrum in liposomal suspension (DPPH was present in both the references and test cuvettes, see text). (c) Absorption spectra of DPPH and myricetin. a ----, DPPH spectrum in buffer and in the presence of myricetin; a ———, DPPH spectrum in liposomal suspension (myricetin was present in both the reference and test cuvettes, see text); b ----, myricetin spectrum in buffer solution; b ———, myricetin spectrum in liposomal suspension (DPPH was present in both the reference and test cuvettes, see text). (d) Absorption spectrum of DPPH and morin. a ----, DPPH spectrum in buffer and in the presence of morin; a ———, DPPH spectrum in liposomal suspension (morin was present in both the reference and test cuvettes, see text); b ----, morin spectrum in buffer solution; b ———, morin spectrum in liposomal suspension (DPPH was present in both the reference and test cuvettes, see text). (e) Absorption spectra of DPPH and quercetin. a ----, DPPH spectrum in buffer and in the presence of quercetin; a ———, DPPH spectrum in liposomal suspension (quercetin was present in both the reference and test cuvettes, see text); b ----, quercetin spectrum in buffer solution; b ———, quercetin spectrum in liposomal suspension (DPPH was present in both the reference and test cuvettes, see text). (f) Absorption spectra of DPPH and phloretin. a ----, DPPH spectrum in buffer and in the presence of phloretin; a ———, DPPH spectrum in liposomal suspension (phloretin was present in both the reference and test cuvettes, see text); b ----, phloretin spectrum in buffer solution; b ———, phloretin spectrum in liposomal suspension (DPPH was present in both the reference and test cuvettes, see text). (g) Absorption spectra of DPPH and rutin. a ----, DPPH spectrum in buffer and in the presence of rutin; a ———, DPPH spectrum in liposomal suspension (rutin was present in both the reference and test cuvettes, see text); b ----, rutin spectrum in buffer solution; b ———, rutin spectrum in liposomal suspension (DPPH was present in both the reference and test cuvettes, see text). (h) Absorption spectra of DPPH and phloridzin. a ----, DPPH spectrum in buffer and in the presence of phloridzin; a ———, DPPH spectrum in liposomal suspension (phloridzin was present in both the reference and test cuvettes, see text); b ----, phloridzin spectrum in buffer solution; b ———, phloridzin spectrum in liposomal suspension (DPPH was present in both the reference and test cuvettes, see text). (i) Absorption spectra of DPPH and quercetrin. a ----, DPPH spectrum in buffer and in the presence of quercetrin; a ———, DPPH spectrum in liposomal suspension (quercetrin was present in both the reference and test cuvettes, see text); b ----, quercetrin spectrum in buffer solution; b ———, quercetrin spectrum in liposomal suspension (DPPH was present in both the reference and test cuvettes, see text). (j) Absorption spectra of DPPH and taxifolin. a ----, DPPH spectrum in buffer and in the presence of taxifolin; a ———, DPPH spectrum in liposomal suspension (taxifolin was present in both the reference and test cuvettes, see text). b ----, taxifolin spectrum in buffer solution; b ———, taxifolin spectrum in liposomal suspension (DPPH was present in both the reference and test cuvettes, see text).

etin, phloridzin, catechin, morin and taxifolin (dihydroquercetin) were purchased from Sarsyntex Lab. (France). All other reagents used were of analytical grade. Stock solutions of flavonoids and DPPH were made up in absolute ethanol and tetrahydroxyfuran, respectively.

Preparation of DPPC liposomes. About 30 mg of DPPC was dissolved into 4.0 ml chloroform in a round-bottom flask. The chloroform solvent was then removed by rotary evaporation under vacuum for about 2 hr. This resulted in the formation of a thin film of lipid at the bottom of the flask. The film of DPPC was swollen by 4.0 ml of 0.2 M Tris–base/0.2 M sodium chloride buffer, pH 7.4, by vortex mixing with glass beads present. The turbid dispersed suspension was collected in a sonication tube and subjected to ultrasonic irradiation by a probe-type titanium tipped Tomy UR-200P sonicator at 50 W, 55–60° and for 30 min (1 min of sonication followed by 30 sec interval). Ultrasonic irradiation was carried out under oxygen-free nitrogen atmosphere.

The irradiated solution was assayed for phospholipid using the phospholipid test kit (Wako Pure Chemicals Industries Ltd., Osaka) based on Allen's procedure [10]. The DPPC liposomal suspension was then diluted to the desired concentration with the Tris/NaCl buffer, pH 7.4, and irradiated in a water bath sonicator for 1–2 min. There are several advantages to using DPPC to prepare liposomes. Small, sonicated DPPC vesicles, below the phase transition temperature, fuse to form vesicles at 700 Å which further fuse to form vesicles with a size of 950 Å. Such vesicles have been documented to be unilamellar, spherical and stable for about six months [11–14]. From gel chromatographic and electron microscopic data (not presented), we found that the liposomal suspension produced by the procedure described above consisted mostly of single, unilamellar vesicles with a diameter of about 700 Å.

DPPH–flavonoid interaction. The absorption spectra of DPPH in the presence of various flavonoids in Tris/NaCl buffer, pH 7.4, and in liposomal suspensions were determined. The chemical structures of the flavonoids used in this study are presented in Fig. 1. The absorption spectra of the flavonoids in these reaction mixtures were also determined. The Hitachi 220A spectrophotometer was used in these determinations which were carried out at 25°. Stock solutions (10 mM) of DPPH and flavonoids were used. The liposomal suspensions were made up to a phospholipid concentration of 1.0 mM. The reaction mixtures used in obtaining the DPPH spectra in the presence of the different flavonoids contained 15 μl of flavonoid solution and 15 μl of DPPH or tetrahydrofuran made up to 3.0 ml with buffer or liposomal suspension. The reaction mixtures used to obtain the absorption spectra of the flavonoids comprised 15 μl of DPPH solution and 15 μl of flavonoid solution or absolute alcohol and made up to 3.0 ml with either buffer or liposomal suspension. The effect of 0.05 mM flavonoid solution on the absorbance at 540 nm of 0.05 mM DPPH was also studied.

Lipid bilayer–flavonoid interaction. Flavonoid stock solutions (10 mM) were used. The DSHA fluorescent probe used in this study was prepared as described by Iwamoto and Sunamoto [6]. The liposomal suspensions contained 1.0 mM phospholipid. Five millilitres of the DPPC liposomal suspension was mixed with 25 μl of 0.1 mM DSHA and subjected to ultrasonic irradiation at 50 W for 5 min at 55–60°. The mixture was then diluted to 0.05 mM phospholipid. The resulting mixture contained a DSHA to DPPC ratio of 1 : 200. Three millilitres of the DSHA–DPPC liposomal suspension was pre-incubated at 25° for 13 min after which 15 μl of either 10 mM DPPH or 10 mM flavonoid solution was added. The mixture was mixed thoroughly and incubated at the same temperature for a further 17 min. The fluorescence polarisation or p value of the DSHA probe was monitored on a Union Giken fluorescence polarisation spectrophotometer ES-501S using a sharp cut-filter Y46 (Hoya Glass Works, Tokyo) of which cell compartment was connected to a Komatsu-Yamamoto Coolnics Model CTR-120. A Sord Microcomputer M200 Mark II system was adapted to control the measurement conditions and data collection.

Effects of flavonoids on soybean lipoxygenase-1. Stock solutions (10 mM) in absolute alcohol of the respective flavonoids listed in Fig. 1 were prepared. Their effects were investigated in 0.01 M Tris-base/0.1 M sodium chloride buffer, pH 9.0 and in DPPC liposomal suspensions containing 1.0 mM phospholipid. 0.05 mM linoleic acid substrate and 0.03 mM flavonoid solution were mixed with 3.0 ml of either buffer or liposomal suspension in clean quartz cuvettes. Both the test and reference cuvettes contained the same reagents. Two mUnits of soybean lipoxygenase-1 was then added to the test cuvettes, mixed well and the enzyme activity assayed by monitoring the absorbance at 234 nm for 5 min at 25°. Controls were set up and these did not contain any flavonoid.

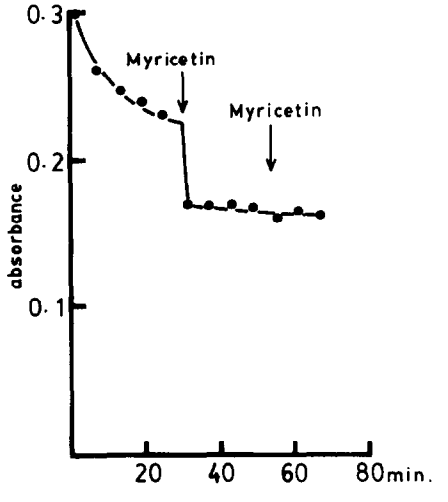

Fig. 3. Effect of myricetin on the absorbance of DPPH at 540 nm over time. The absorbance was monitored for about 50 min in DPPC liposomal suspension at 37°. Concentration of DPPH was 5×10^{-5} M and of DPPC liposomes, 1 mM phospholipid. Any further addition of myricetin did not result in any further decrease in absorbance. The percentage decrease in absorbance by myricetin was found to be 24%.

Table 1a. Reaction mixtures to obtain the absorption spectra of DPPH

Reagent	Volume in cuvette reference	test
THF	15 μl	—
DPPH solution (10 mM)	—	15 μl
Flavonoid solution (10 mM)	15 μl	15 μl
Buffer mixture of DPPC liposomal solution (1.0 mM phospholipid)	2.97 ml	2.97 ml

Table 1b. Reaction mixtures to obtain the absorption spectra of flavonoids

Reagent	Volume in cuvette reference	test
DPPH solution (10 mM)	15 μl	15 μl
Absolute ethanol	15 μl	—
Flavonoid solution (10 mM)	—	15 μl
Buffer mixture or DPPC liposomal solution (1.0 mM phospholipid)	2.97 ml	2.97 ml

Table 1c. Effect of flavonoids on the decrease in absorbance at 540 nm

Flavonoid	Decrease in absorbance buffer	liposomes	% Decrease in absorbance buffer	liposomes
(+)-Catechin	0.197	0.079	43	23
Quercetin	0.320	0.129	70	38
Quercetrin	0.325	0.093	71	27
Rutin	0.301	0.127	66	37
Taxifolin	0.263	0.074	57	22
Phloretin	0.086	0.124	19	36
Phloridzin	0.028	0.044	6	13
Myricetin	0.300	0.155	65	34
Morin	0.312	0.157	68	46

RESULTS AND DISCUSSION

DPPH–flavonoid interaction

Absorption spectral studies on the interaction between flavonoids and the DPPH free radical showed that the spectrum of the latter was altered in the presence of the flavonoids (Fig. 2b–j). This alteration was observed in both the buffer and the liposomal suspension and was indicative of interaction between DPPH and flavonoid compounds. Figure 2a shows the absorption spectra of the flavonoid solutions in buffer and in liposomal suspension with DPPH present. The spectra of the flavonoids did not alter in the liposomal suspension with DPPH present when compared to their spectra in the buffer mixture without any DPPH. The flavonoid molecule was suggested to occur in the aqueous phase of the liposomal suspension and was interacting with the DPPH molecule, which being hydrophobic, would be located in the lipid bilayer. The DPPH absorption spectrum was sensitive to this interaction but not the flavonoid absorption spectrum. For all the flavonoids studied, the absorption maximum at 540 nm for DPPH was reduced in

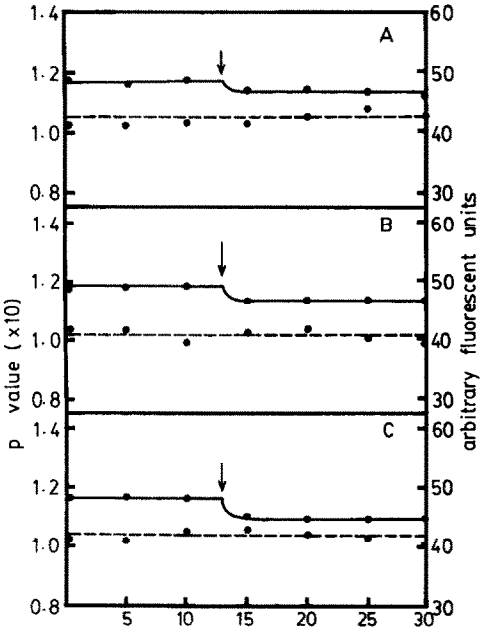

Fig. 4. Effect of (A) phloretin, (B) rutin and (C) myricetin on the *p* value and fluorescent intensity of the DSHA probe at 25°. The broken lines denote the effect of the flavonoids on the *p* value and the solid lines denote the effect of the flavonoids in the fluorescent intensity. The arrow indicates the point of application of the flavonoids.

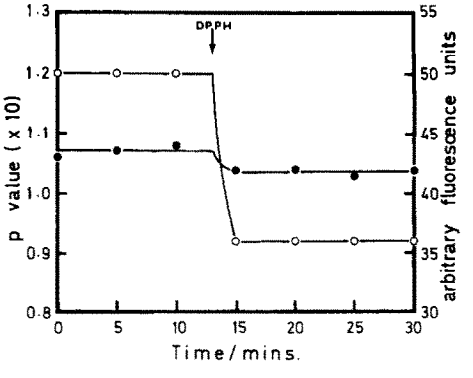

Fig. 5. Effect of DPPH on the *p* value and fluorescent intensity of the DSHA probe at 25°: ●, effect of DPPH on the *p* value; ○, effect of DPPH on the fluorescent intensity.

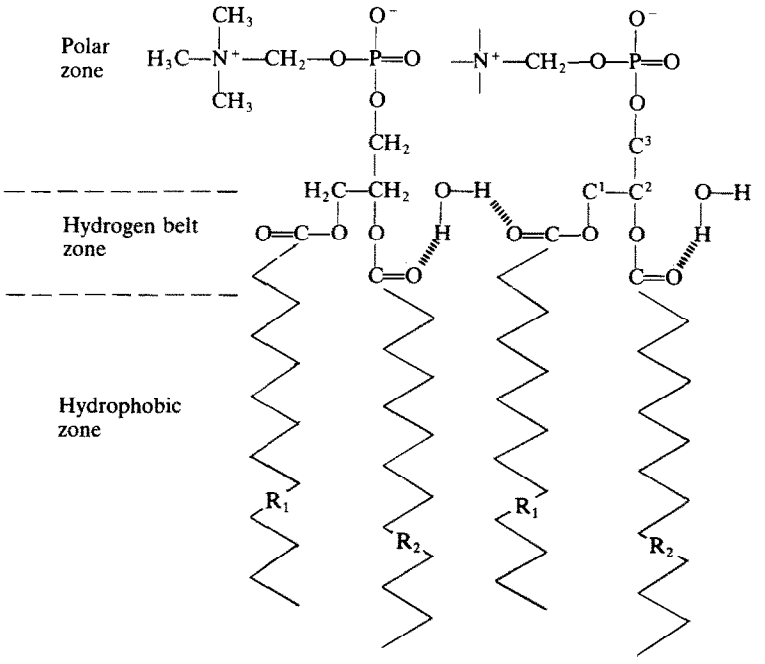

Fig. 6. Structure of dipalmitoylphosphatidylcholine DPPC. The figure shows two molecules of DPPC linked in the hydrogen belt zone via water. R_1 and R_2 denote palmitic acid.

the presence of flavonoid. This decrease in absorbance was found to occur within 1 min of the addition of flavonoid as illustrated with myricetin in liposomal suspension in Fig. 3. The percentage decrease in absorbance effected by the various flavonoids is presented in Table 1c. Considering the chemical structures of the electron-donating flavonoids and the electron-accepting DPPH, it was suspected that a charge transfer complex was formed between the two species. A decrease in the absorbance was indicative of the loss of the DPPH free radical species. Flavonoids could thus scavenge free radicals and could do so in biomembranes. It is strongly suggested that the DPPH free radical abstracts the phenolic hydrogen of the flavonoid molecule and this could be the general mechanism of the scavenging action of the antiperoxidative flavonoids. Phloretin and phloridzin were observed to be more active in decreasing the DPPH absorbance in liposomal suspensions than in the buffer. These two flavonoids can rotate around the aliphatic moiety to afford a linear structure and thus engage the DPPH molecule more easily in the highly ordered lipid membranes than in the aqueous buffer.

Lipid bilayer flavonoid interaction

The DSHA fluorescent probe used in these studies is located in the polar regions close to the surface of the membrane surface [6, 15, 16]. Thus, the fluorescence depolarisation of this probe would reflect perturbations in its microenvironment close to the membrane surface. However, we found that the flavonoids did not cause any fluorescence depolarisation of the DSHA probe at 25°. They did cause a slight decrease in the fluorescence intensity of the probe, as illustrated in Fig. 4 with the flavonoids, phloretin, rutin and myricetin. DPPH, on the other hand, caused a slight decrease in the p value and a larger decrease in the fluorescent intensity of the DSHA probe (Fig. 5). DPPH has been documented to lie in the "hydrogen belt" zone of bilayers [17] and when it permeates into the bilayer, the fluidity

Table 2. Effect of flavonoids on soybean lipoxygenase-1

Flavonoid	% Inhibition of absorbance at 234 nm buffer	liposome	Rate after 5 min incubation in liposomes
Control	0	0	1.6 ($\times 10^{-3} s^{-1}$)
(+)-Catechin	10	22	1.3
Quercetin	25	42	0.6
Quercetrin	8	17	1.5
Rutin	7	19	1.4
Taxifolin	2	23	1.4
Myricetin	11	14	1.3
Morin	10	15	1.5
Phloretin	10	19	1.4
Phloridzin	4	17	1.3

Table 3. Effect of flavonoids on soybean lipoxygenase-1 at pHs 7.4 and 9.0

Flavonoid	% Inhibition after 5 min incubation		Rate of enzyme activity after 5 min incubation	
	pH 7.4	pH 9.0	pH 7.4	pH 9.0
Control	0	0	0.8	1.7
Quercetin	86	29	0.1	0.8
Myricetin	32	9	0.6	1.6

of the bilayer slightly increases (resulting in the decrease of p value) and the apparent micropolarity around the probe decreases (causing a decrease in the fluorescence emission intensity) [6]. Thus, flavonoids do not penetrate the lipid bilayer enough to affect the DSHA probe. We found that the fluorescence emission maxima of the flavonoids could indicate that the microenvironment of the flavonoids changed from a polar (buffer) to a less polar one on the lipid bilayer. We postulate that since flavonoids do not penetrate into the bilayer, the interaction of the flavonoids on the lipid bilayer occurs in the polar zone of the lipid bilayers (Fig. 6).

Effect of flavonoids on soybean lipoxygenase-1

The effects of flavonoids on soybean lipoxygenase-1 were investigated in buffer and in liposomal suspension. The linoleic acid substrate in the latter system would be intercalated within the DPPC lipid bilayer. All the flavonoids studied inhibited the enzyme in both systems but the inhibition was greater in the liposomal suspension (Table 2). In aqueous buffer, the linoleic acid substrate could form micelles, making it inaccessible to the enzyme. The absorbance at 234 nm measured the extent of conjugated diene formation from linoleic acid catalysed by the lipoxygenase. Quercetin inhibited the lipoxygenase in the liposomal suspension by about 42% while the other flavonoids inhibited the enzyme by about 14–23% (Table 2). The control did not contain any flavonoid. Quercetin was found to be the most potent inhibitor of soybean lipoxygenase-1. It was also observed that quercetin inhibited the rate of enzyme activity after 5 min incubation by about 60% compared to the 5–19% inhibition by the other flavonoids.

Takahama [19] showed that quercetin inhibited soybean lipoxygenase-1 and suggested that this inhibition may be due to the reduction of the linoleic acid radical by quercetin that is formed during the lipoxygenase activity. We found that the inhibition of the enzyme by the flavonoids was not related to their antioxidant activity. We had found that myricetin was the most potent of the antiperoxidative flavonoids studied here [5] but quercetin was the most potent inhibitor of lipoxygenase. Our finding is analogous to the report by Reddana *et al.* [20], who showed that the inhibition of 5-lipoxygenase by vitamin E is not related to its antioxidant function but relates to a strong interaction between vitamin E and the enzyme. We suggest that the inhibition of soybean lipoxygenase-1 by quercetin is due to its structural specificity for the enzyme.

During our studies with lipoxygenase, we observed that the effects of myricetin and quercetin on the enzyme was pH dependent. Table 3 shows that at pH 7.4, the flavonoids inhibited the enzyme to a greater extent than when the effect was studied at pH 9.0. This finding suggests that the pharmacological action of flavonoids could be pH dependent.

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