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The Interaction of Flavonoids with Membranes: Potential Determinant of Flavonoid Antioxidant Effects

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Twenty six phenolic substances including representatives of the families, flavanones, flavanols and procyanidins, flavonols, isoflavones, phenolic acids and phenylpropanones were investigated for their effects on lipid oxidation, membrane fluidity and membrane integrity. The incubation of synthetic phosphatidylcholine (PC) liposomes in the presence of these phenolics caused the following effects: (a) flavanols, their related procyanidins and flavonols were the most active preventing 2,2′-azo-bis (2,4-dimethylvaleronitrile) (AMVN)-induced 2-thiobarituric acid-reactive substances (TBARS) formation, inducing lipid ordering at the water-lipid interface, and preventing Triton X-100-induced membrane disruption; (b) all the studied compounds inhibited lipid oxidation induced by the water-soluble oxidant 2,2′-azo-bis (2-amidinopropane) (AAPH), and no family-related effects were observed. The protective effects of the studied phenolics on membranes were mainly associated to the hydrophilicity of the compounds, the degree of flavanol oligomerization, and the number of hydroxyl groups in the molecule. The present results support the hypothesis that the chemical structure of phenolics conditions their interactions with membranes. The interactions of flavonoids with the polar head groups of phospholipids, at the lipid–water interface of membranes, should be considered among the factors that contribute to their antioxidant effects.

Keywords: Flavonoids; Membranes; Lipid oxidation; Membrane physical properties; Antioxidants; Fluidity

Abbreviations: PC, phosphatidylcholine; AAPH, 2,2′-azo-bis (2-amidinopropane); AMVN, 2,2′-azo-bis (2,4 dimethylvaleronitrile); 6-AS, 6-(9-anthroyloxy)stearic acid; 16-AP, 16-(9-anthroyloxy)palmitic acid; C_6-NBD-PC, 2-(6-(7-nitrobenz-2 oxa-13 diazol-4-yl) amino) hexanoyl-1-hexadecanoyl-sn-3-phosphatidylcholine; 7,3′,4′-THIF, 7,3′,4′ trihydroxy-isoflavone

INTRODUCTION

Flavonoids are phenolic compounds present in plants and conserved in plant-derived foods. While the concept that flavonoids can provide positive health effects is gaining acceptance, there is a paucity of information regarding the mechanisms through which these compounds act.[1–4] Antioxidation, metal chelation, enzyme inhibition and gene regulation, are among the various mechanisms that are proposed to be involved in the beneficial health effects of flavonoids.[1]

Considering the existence in biological systems of domains or compartments with different hydrophilicity/hydrophobicity, it can be expected that flavonoids having a wide range of water–lipid solubility, would unevenly distribute in those domains in a way partially dictated by their chemical structure. Such distribution is particularly relevant to understand how tissue and cellular levels of flavonoids relate to their biological functions. For example, it is known that flavonoids can display antioxidant activity in numerous biological systems.[5–7] This antioxidant activity is usually determined at micromolar concentrations, when blood levels of flavonoids only reach such concentrations very transiently (2–4 h), after the consumption of certain flavonoid-rich foods.[8–10]

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Then, it is important to consider that the distribution of flavonoids in biological systems will depend, among other factors, on their relative hydrophilicity/hydrophobicity and on their interactions with particular macromolecules. These factors will determine the local concentration of flavonoids which will influence, among other factors, their capacity to regulate cellular events.

We have shown that (−)-epicatechin, (+)-catechin and their related procyanidins can adsorb to membranes through associations with the polar head-groups of phospholipids creating an environment rich in flavonoids.[11] Such enrichment could limit the access of oxidants to the bilayer and/or control the rate of propagation of free radical chain reactions occurring in the hydrophobic core of membranes.[11] Accordingly, it has been previously proposed that the location of flavonoids in the membrane could dictate their antioxidant capacity.[12]

In the present study, we studied the influence of flavonoid chemical structure on their capacity to interact with membranes and to prevent lipid oxidation. With this purpose we characterized, in liposomes, the effect of flavanones, flavanols and related procyanidins, flavonols, isoflavones, phenolic acids and phenylpropanones on: (a) lipid oxidation initiated by both, water- and lipid-soluble free radical generators; (b) membrane fluidity, determined at the water–lipid interface and in hydrophobic domains and (c) membrane disruption. We found that the most hydrophilic flavonoids can interact with the membrane surface providing protection against oxidant, as well as other external aggressors. Other major structure requirements for these effects are discussed.

MATERIALS AND METHODS

Materials

Flavanone, flavonol, eriodictyol, galangin and 7, 3′,4′ trihydroxyisoflavone (7, 3′,4′-THIF) were obtained from Indofine Chemical Co. (Hillsborough, NJ, USA); all the other phenolics were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA); dimer and hexamer from Cocoapro cocoa were purified[13,14] and supplied by Mars Inc. (Hackettstown, NJ, USA). Bovine brain PC was from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 6-(9-anthroyloxy)stearic acid (6-AS), 16-(9-anthroyloxy)palmitic acid (16-AP), octadecyl rhodamine B chloride and 2-(6-(7-nitrobenz-2 oxa-1,3 diazol-4-yl) amino) hexanoyl-1-hexadecanoyl-sn-3-phosphatidylcholine (C_6-NBD-PC) were from Molecular Probes Inc. (Eugene, OR, USA). 2,2′-Azobis (2,4-dimethylvaleronitrile) (AMVN) and 2,2′-azobis (2-amidinopropane) (AAPH) were obtained from Polysciences Inc. (Warrington, PA, USA). All other reagents were of the best quality available and were obtained from Sigma Chemical Co.

Partition Coefficient ($P_{o/b}$) of Flavonoids and Related Compounds

To measure the $P_{o/b}$, flavonoids and related compounds (25–100 μM) were added to a mixture of 20 mM Tris–HCl, 140 mM NaCl buffer (pH 7.4) (presaturated with octan-1-ol overnight) and octan-1-ol (presaturated with the same buffer overnight) at a ratio 1:1 (v:v). Samples were vortexed for 30 s, incubated for 15 min at room temperature and centrifuged for 5 min at 1500*g*. The octan-1-ol and buffer phases were carefully separated, and aliquots were taken to determine the absorbance to estimate the concentration of the different compounds in both phases. The absorption spectra (240–700 nm) of the flavonoids and related compounds were previously obtained to assess the corresponding maximum absorption wavelength. The $P_{o/b}$ in the octan-1-ol/buffer system was calculated as $[C_o/C_b]$, where C_o is the concentration of the flavonoid or related compound in the octan-1-ol phase and C_b is the concentration of the flavonoid or related compound in the aqueous buffer phase.

Liposome Preparation

Phospholipid purity was checked by thin layer chromatography. PC dissolved in chloroform solution was brought to dryness under high vacuum in a Buchi rotavapor for 15 min and further exposed to a N_2 stream for 15 min. Dried phospholipids were resuspended (2.5 mg phospholipids/ml) in 20 mM Tris–HCl, 140 mM NaCl buffer (pH 7.4), vortexed for 1 min, and incubated at 45°C for 10 min under N_2. Small vesicles were obtained by three cycles of 45 s sonication in a Branson 250 Sonifier (Branson Ultrasonics Corp., Danbury, CT, USA) at 80 W and diluted to the final concentration stated in each experiment.

Evaluation of Lipid Oxidation

Liposomes (0.25 mg phospholipids/0.5 ml) were incubated at 37°C for 60 min in the presence of the flavonoids and related compounds at 5 μM concentration (monomer equivalents), and one of the following initiators: 10 mM AAPH or 10 mM AMVN. The stock solutions of the oxidants were prepared immediately before use. Incubations were stopped by the addition of 0.1 ml of 4% (w/v) butylated hydroxytoluene in ethanol. To evaluate lipid oxidation products, thiobarbituric acid-reactive substances (TBARS) were measured in the incubation mixtures.[15] TBARS determination in liposomes was considered as a reliable method to evaluate lipid oxidation since, in a pure lipid system, the only sources of TBARS are the products of decomposition of lipid hydroperoxides. The samples were added with 0.25 ml of 3% (w/v) sodium

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dodecyl sulphate. After mixing, 0.5 ml of 1% (w/v) 2-thiobarbituric acid in 0.05 M NaOH and 0.5 ml of 25% (v/v) HCl were added. Samples were vortexed and heated for 15 min in boiling water and TBARS were extracted in 2.5 ml of butan-1-ol. After centrifugation at 1000*g* for 10 min, the fluorescence of the butan-1-ol phase was measured at 535 nm ($\lambda_{excitation}$: 515 nm) in a Kontron SFM-25 spectrofluorometer (Kontron Instruments S.P.A., Milan, Italy). TBARS values were expressed as malondialdehyde equivalents.

Evaluation of Membrane Fluidity

The effects of flavonoids on membrane fluidity were evaluated using the fluorescent probes 6-AS and 16-AP.[16] PC liposomes (0.1 mM phospholipids) were treated with 6-AS or 16-AP to a final probe/lipid ratio of 1:500. Liposomes were then preincubated for 5 min at 37°C and, after the addition of 5 μM flavonoids or related compounds, samples were further incubated at 37°C for 2 min. Fluorescence polarization at 435 nm ($\lambda_{excitation}$: 384 nm) was measured at 37°C, and the fluorescence polarization (*P*) was calculated, as previously described.[17]

$$P = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + I_{\perp})}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities measured with the emission polarizer oriented parallel and normal to the excitation polarizer, respectively.

Evaluation of Membrane Disruption

PC liposomes (0.1 mM phospholipids) containing 3 mol % of the fluorescent probe octadecyl rhodamine,[18] were incubated at 25°C for 5 min, with or without the addition of 5 μM flavonoids or related phenolics. The concentration of the probe was enough to produce the self-quenching of its fluorescence. A 10 μl aliquot of a 1% (v/v) Triton X-100 was added, and after a 2 min incubation under continuous stirring, the fluorescence emission at 580 nm ($\lambda_{excitation}$: 560 nm) was recorded. The addition of the detergent was continued until the achievement of a constant fluorescence emission corresponding to the total disruption of the liposomes to micelles. Results are expressed as the concentration of detergent necessary to reach half of the maximal fluorescence (C_{50}).

Evaluation of C_6-NBD-PC Incorporation into Membranes

PC liposomes (0.1 mM phospholipids) were incubated for 5 min at 25°C either in the absence or in the presence of 4 μM epicatechin hexamer. After incubation, the probe 2-(6-(7-nitrobenz-2 oxa-1,3 diazol-4-yl) amino) hexanoyl-1-hexadecanoyl-sn-3-phosphatidylcholine (C_6-NBD-PC) (5 mol % probe/lipid) was added to the samples. The incorporation of the probe into the bilayer was evaluated following the increase in fluorescence observed in an Olympus BX50 fluorescence microscope (Olympus Optical Co., Tokyo, Japan) after 0, 15 and 30 min of incubation at room temperature.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC, USA). Fisher least significance difference test was used to examine differences between group means. A *p* value <0.05 was considered statistically significant. Data are shown as mean ± SEM.

RESULTS AND DISCUSSION

Two possible relevant interactions between flavonoids and lipid bilayers are: (i) the adsorption on the membrane surface by the interaction of the hydrophilic flavonoids (or oligomers) with the polar head groups of lipids at the water–lipid interface and (ii) the partitioning of the flavonoid in the non-polar core of the membrane, associated with its hydrophobic nature. Besides the free radical reactivity dictated by their chemical structure,[19,20] the interactions of flavonoids with membranes could contribute to determinate their antioxidant capacity. Furthermore, as recently shown, flavonoid-membrane surface interactions could protect the integrity of lipid bilayer from disrupting agents.[11]

In the present work, we studied the interaction of a comprehensive number of flavonoids and related compounds (phenolic acids and phenylpropanones) (Table I) with liposome membranes. We first evaluated their capacity to inhibit lipid oxidation initiated by two oxidants, which act at aqueous or lipid domains. The antioxidant action of flavonoids was then related to their capacity to: (a) modulate membrane fluidity both, at the water–lipid interface and in the hydrophobic core of the bilayer and (b) protect membranes from detergent (Triton X-100)-induced membrane disruption. We selected 26 substances that, based on their chemical structure, belong to six flavonoid families.[21]

Partition of Flavonoids and Related Compounds in Hydrophilic/Hydrophobic Domains

The capacity of the studied compounds to distribute in hydrophilic/hydrophobic domains are rather heterogeneous as suggested by their capacity to partition in octan-1-ol/buffer, a system

TABLE I Structure of the compounds included in this study.

Structural formula	Compound	3	5	6	7	2′	3′	4′	5′
		Substitutions							
Flavanones	Flavanone	H	H	H	H	H	H	H	H
	Naringenin	H	OH	H	OH	H	H	OH	H
	Eriodictyol	H	OH	H	OH	H	OH	OH	H
	Hesperetin	H	OH	H	OH	H	OH	OMe	H
	Taxifolin	OH	OH	H	OH	H	OH	OH	H
Flavanols	Catechin	OH	OH	H	OH	H	OH	OH	H
	Epicatechin	OH	OH	H	OH	H	OH	OH	H
	Epigallocatechin	OH	OH	H	OH	H	OH	OH	OH
	Epicatechin gallate	GA*	OH	H	OH	H	OH	OH	H
	Epigallocatechin gallate	GA*	OH	H	OH	H	OH	OH	OH
Flavone/ols	Flavone	H	H	H	H	H	H	H	H
	Flavonol	OH	H	H	H	H	H	H	H
	Galangin	OH	OH	H	OH	H	H	H	H
	Kaempferol	OH	OH	H	OH	H	H	OH	H
	Morin	OH	OH	H	OH	OH	H	OH	H
	Quercetin	OH	OH	H	OH	H	OH	OH	H
	Myricetin	OH	OH	H	OH	H	OH	OH	OH
Isoflavones	Daidzein		H	H	OH	H	H	OH	H
	Genistein		OH	H	OH	H	H	OH	H
	7,3′,4′THIF		H	H	OH	H	OH	OH	H
				2		3	4		
Phenolic acids	Vanillic acid			H		OMe	OH		
	Protocatechuic acid			H		OH	OH		
Phenylpropanones	*trans*-Cinnamic acid			H		H	H		
	p-Coumaric acid			H		H	OH		

*GA: gallic group.

that is widely used to study the solubility of substances in hydrophilic/hydrophobic systems.[22] Since the pH of the water phase affects the partition coefficient of flavonoids in octan-1-ol/buffer,[23,24] we determined the $\log P_{o/b}$ at pH 7.4 and this pH was used in all the subsequent experiments. Considering the different families, flavonols and procyanidins showed the highest partition in the aqueous phase while flavanols, flavanones and isoflavones partitioned mostly in the hydrophobic phase (Fig. 1). Previous reports showed that the relative hydrophobicity, measured as the partition coefficient between water and olive oil, is slightly higher for the flavone than flavanones at the same hydroxylation degree.[23] Accordingly, we found a higher $P_{o/b}$ for quercetin compared to taxifolin. Similarly, in the absence of hydroxyl substitutes, flavone was 2-times more soluble in octan-1-ol than flavanone.

Effects of Flavonoids and Related Compounds on AAPH- and AMVN-induced Lipid Oxidation

One of the mechanisms that could be involved in the beneficial effects of flavonoids on human health is their capacity to act as antioxidants both *in vitro* and *in vivo*.[7] This antioxidant activity has been mainly attributed to the capacity of flavonoids to scavenge oxygen and nitrogen active species[25] and to their redox-active metals-chelating ability.[26] The relevant chemical characteristics of flavonoids that contribute to the antioxidant/oxidant scavenging activity are: (a) the 3′,4′ hydroxyl (catechol) group in the B ring; (b) the 2,3 double bond in conjugation with a 4-oxo

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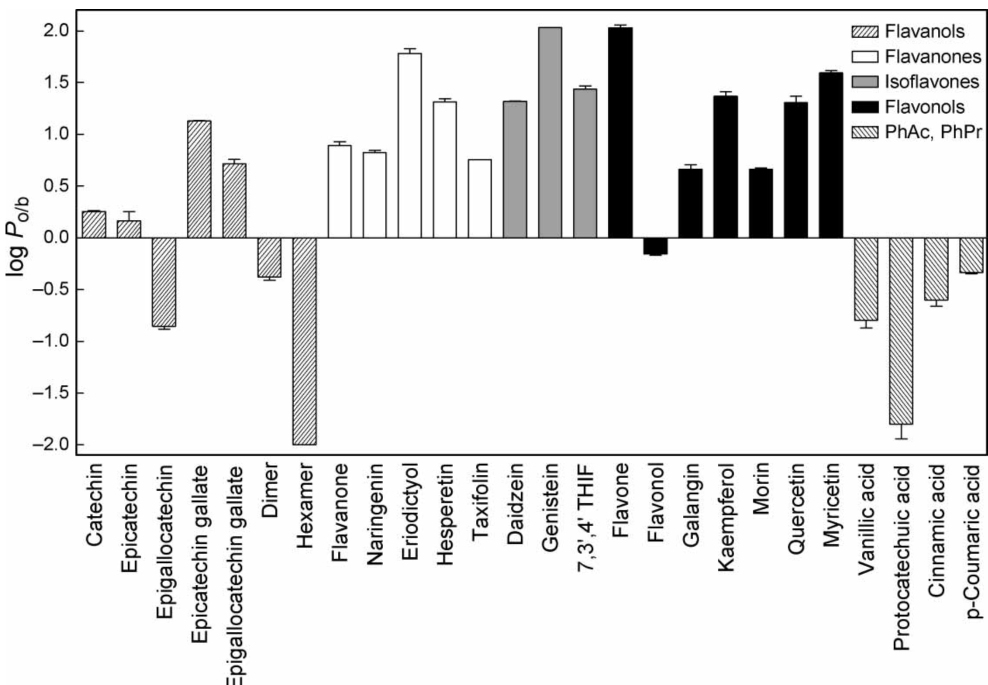

FIGURE 1 Partition coefficient ($P_{o/b}$) of flavonoids and related compounds. $P_{o/b}$ was measured in octan-1-ol/buffer system as described in the "Materials and Methods" section. Results are shown as mean ± SEM of four independent experiments. PhAc, phenolic acids; PhPr, phenylpropanones.

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group in the C ring and (c) the presence of hydroxyl groups in positions 3 and 5.[25,27,28] We propose that the ability of flavonoids to interact with the membrane polar head groups, increasing their local concentration at the water–lipid interface of membranes, could be one factor leading to a higher antioxidant capacity.

To evaluate the antioxidant action of the studied flavonoids and related compounds, we used a model of liposome oxidation induced by one of these two azo-compounds: AAPH, which generates radicals in hydrophilic domains and AMVN, which acts in hydrophobic domains. Both azo-compounds decompose at 37°C at a constant rate generating, in the presence of O_2, peroxyl radicals. Figure 2 shows the capacity of the studied phenolics to inhibit AAPH- and AMVN-induced TBARS formation (Fig. 2A and B, respectively). All the tested compounds at 5 μM concentration exerted an antioxidant effect, measured as the inhibition of TBARS formation, when the initiator was AAPH. None of the flavonoid families showed a particularly higher protection against AAPH-induced TBARS formation (Fig. 2A). However, quercetin, the only flavonoid that satisfied the three above mentioned structural characteristics, was the most effective inhibiting AAPH-initiated TBARS formation.

When lipid oxidation was initiated with the hydrophobic radical generator AMVN, not all the assessed flavonoids showed an antioxidant effect. Flavanols and their related procyanidins showed the highest antioxidant effect (Fig. 2B). For flavonols, the high antioxidant effect could be due to the fact that most of them meet the above mentioned three structural characteristics that contribute to flavonoid antioxidant capacity. However, this cannot solely explain the antioxidant effects observed for flavanols. For these compounds, the number of hydroxyl groups or other characteristics unrelated to free radical stabilization seems to further define their antioxidant effect. Among the other flavonoid families, only the compounds with a 3′,4′-catechol group in the B-ring (the flavanones eriodictyol and taxifolin, the isoflavone 7,3′,4′ THIF and protocatechuic acid) had a significant antioxidant capacity in AMVN-induced oxidation. The relevance of this 3′,4′-catechol group is confirmed by the finding that its methylation in hesperetin and vanillic acid leads to a marked decrease in their antioxidant capacity when compared to their corresponding non-methylated forms (eriodictyol and protocatechuic acid). Non-phenolic compounds (flavone and cinnamic acid), as well as coumaric acid did not protect liposomes from AMVN-induced oxidation.

Effects of Flavonoids and Related Compounds on Membrane Fluidity

The consequences of the interactions between flavonoids and related compounds, and liposome membranes related to change membrane fluidity were evaluated through their capacity to affect lipid ordering using the probes 6-AS and 16-AP. The anthroyloxy group in probes 6-AS and 16-AP,

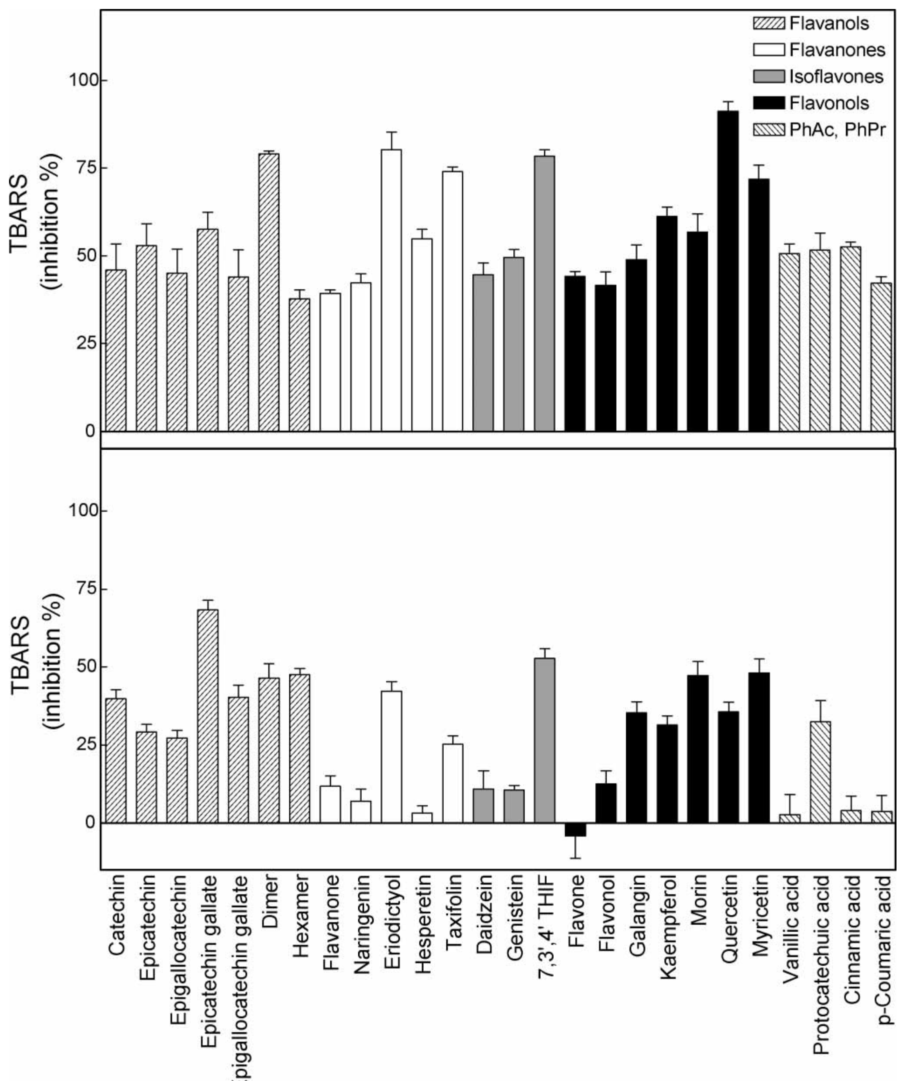

FIGURE 2 Effects of flavonoids and related compounds on AAPH- or AMVN-induced lipid oxidation. Liposomes were incubated for 60 min at 37°C in the absence or the presence of 5 μM flavonoids or related compounds and (A) 10 mM AAPH or (B) 10 mM AMVN. Lipid oxidation was evaluated as TBARS and determined as described in "Materials and Methods" section. Percentage of inhibition is referred to the maximal TBARS formation determined in the absence of added flavonoids or related compounds. Results are shown as mean ± SEM of four independent experiments. PhAc, phenolic acids; PhPr, phenylpropanones.

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senses the fluidity of its close environment and is located about 7 and 18 Å from the membrane surface, respectively.[29] Figure 3A and B, show the changes induced by the flavonoids and related compounds at 5 μM concentration in the fluorescence polarization of both, 6-AS and 16-AP. Flavanols, procyanidins and flavonols caused the largest effects on the fluorescence polarization of the probes, then increasing lipid ordering at two depths in the bilayer. Accordingly, green tea catechin and related metabolites, assayed at higher concentrations than those used in the current study, also decreased membrane fluidity at different depths in the bilayer.[30] No significant correlations were observed between the $\log P_{o/b}$ and the changes in fluorescence polarization for 6-AS or 16-AP when all the tested compounds were considered.

Other approaches have been used to characterize the interaction of flavonoids with membrane surfaces. For example, Olilla *et al.*[31] used a PC-coated HPLC column, and measured for different flavonoids, the retention delay as a parameter of surface interaction. Using that methodology, a positive correlation was found between the number of hydroxyl groups in flavonoids and the retention delay. The affinity of flavonoids for PC liposomes was also evaluated by measuring their capacity to quench the fluorescence of the membrane probe diphenylhexatriene.[23] The affinity of the flavonols morin and quercetin for liposomes was markedly higher than that of the flavanones pinocembrin, naringenin,

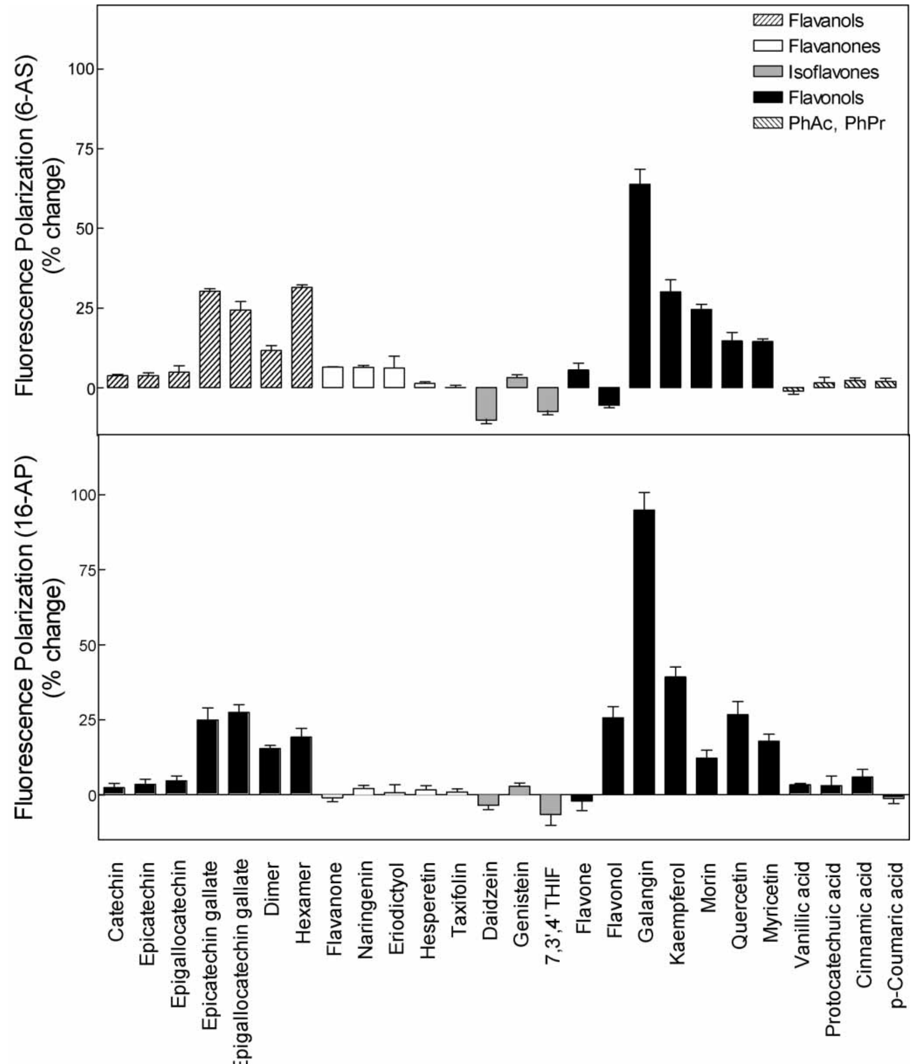

FIGURE 3 Effects of flavonoids and related compounds on membrane fluidity. Liposomes containing the fluorescent probes (A) 6 AS or (B) 6 AP were incubated with 5 μM flavonoids at 37°C. The fluorescence polarization was measured as described in "Materials and Methods" section. Percentage of inhibition is referred to the fluorescence polarization determined in the absence of added flavonoids or related compounds. Results are shown as mean ± SEM of four independent experiments. PhAc, phenolic acids; PhPr, phenylpropanones.

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eriodictyol and hesperetin,[23] this was attributed to the planar three-dimensional structure of flavonols compared to a tilted configuration of flavanones. Similarly, we found that changes in 6-AS polarization were higher for morin and quercetin (24.6 and 14.7%, respectively) compared to naringenin, eriodictyol and hesperetin (6.4, 6.1 and 1.3%, respectively).

The capacity of flavonoids to interact with the water–lipid interface of membranes has been also associated to the induction of changes in membrane surface potential. We recently showed that membrane surface potential correlates with the number of monomer units in procyanidins.[11] Using data taken from Verstraeten *et al.*[11] a significant correlation ($r: 0.99$, $p < 0.001$) was found between the capacities of catechin, epicatechin and derived dimeric and hexameric procyanidins to induce changes in membrane surface potential and 6-AS fluorescence polarization.

Effects of Flavonoids on the Liposome Bilayer Transition to Micelle

The capacity of flavonoids and related compounds to protect membranes from disruption was characterized using a model of transition of liposome bilayer to micelles induced by the non-ionic detergent Triton X-100. This transition was assessed using the fluorescent probe octadecyl rhodamine which is incorporated into the liposomes at a concentration of the probe that causes the self-quenching of its fluorescence.[18] The incorporation of the detergent within membrane phospholipids leads to an increase in the distance between the fluorophore molecules,

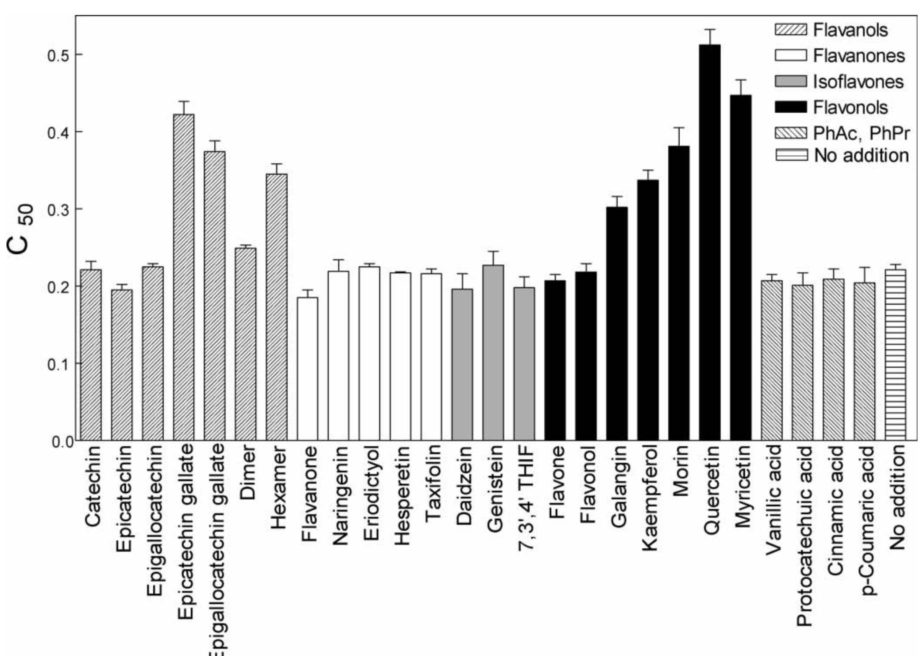

FIGURE 4 Effects of flavonoids and related compounds on membrane disruption. Liposomes containing the fluorescent probe octadecyl rhodamine were incubated at 25°C in the absence or presence of 5 μM flavonoid or related compounds. After incubation, Triton X-100 (0–1.5 mM) was added and the increase in fluorescence emission was recorded as described in the "Materials and Methods" section. Results (C_{50} of Triton X-100) are shown as mean ± SEM of four independent experiments. PhAc, phenolic acids; PhPr, phenylpropanones.

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leading to an increase in fluorescence emission associated to the transition liposome to micelle. Maximal fluorescence is achieved after the total disruption of the bilayer.[32] When assayed at 5 μM concentration, flavanols and flavonols were the only flavonoids that protected liposome membranes from Triton X-100-induced disruption (Fig. 4). By considering the differences found for three flavonoids from different families with the same number and distribution of hydroxyl groups, taxifolin, catechin and quercetin, it can be inferred that the 2,3 double bond in the C-ring is relevant for the protection of membranes from disruption. Taxifolin had no effect while quercetin was significantly more active than catechin in affecting 6-AS fluorescence polarization (5.7 vs. 14.7% change, for catechin and quercetin, respectively) and preventing liposome disruption (4.7 vs. 81% change in C_{50} for catechin and quercetin, respectively). Considering the tested compounds within the isoflavone family, the presence of the B ring in position 3, could confer a more hydrophobic character to the compounds that probably led to a lack of effect on detergent-induced liposome disruption. For example, comparing genistein with kaempferol which have the same distribution of hydroxyl groups, genistein had no significant effects neither on 6-AS fluorescence polarization nor on membrane protection against Triton X-100- or AMVN-induced oxidation, while kaempferol induced a 30% change in 6-AS fluorescence polarization and protected the bilayer against disruption and AMVN-induced oxidation. Among the flavanols, the presence of a gallate residue at position 3 in epicatechin gallate and epigallocatechin gallate, determined a marked increase in the anti-disruption effect compared to epicatechin and epigallocatechin. As previously described,[11] the degree of flavanol polymerization is a relevant determinant in the prevention of Triton X-100-induced liposome disruption. Dimeric and hexameric procyanidins had a protective effect while their monomer, epicatechin, did not prevent membrane disruption. Among the assayed flavonols and flavanols, the protective effect was associated with the total number of hydroxyl groups present in the molecule. A significant regression was found between the number of hydroxyl groups in the flavanols or flavonols and C_{50} ($r = 0.91$, $p < 0.004$; and $r = 0.87$, $p < 0.05$ for flavanols and flavonols, respectively).

The above results show a dependence of the protective role of flavonoids on detergent-induced liposome disruption, with the number of hydroxyl groups in the molecule. This dependence suggests that the formation of hydrogen bonds between hydroxyl groups in flavonoids and the polar head groups of lipids can be particularly relevant for the interactions of flavonoids with the membrane surface at the water–lipid interface. Accordingly, the surface interaction (retention delay) of different flavonoids with a column coated with phospholipids (DPPC) was positively correlated with the number of hydroxyl groups in the flavonoids.[31]

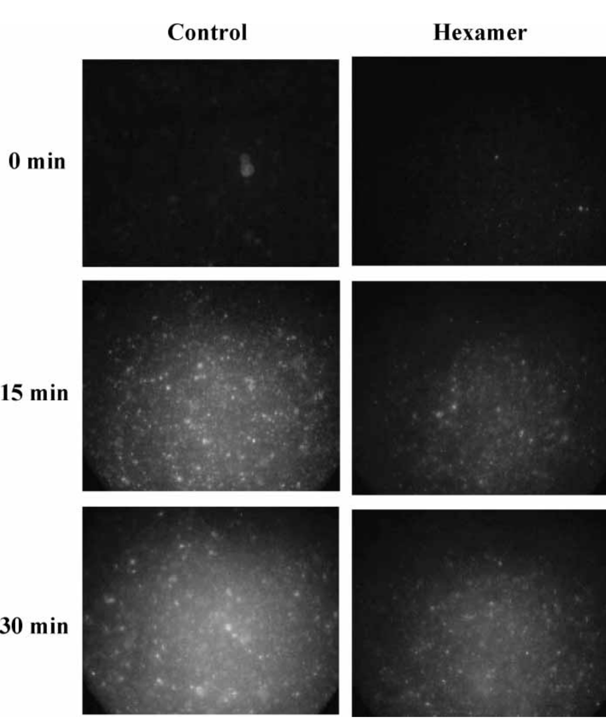

FIGURE 5 Effect of epicatechin hexamer on C_6-NBD-PC incorporation into liposome membranes. Liposomes were incubated at 25°C for 5 min either in the absence (control) or in the presence of 25 μM epicatechin hexamer (hexamer). After incubation, 5 mol % of the fluorescent probe C_6-NBD-PC was added to the samples and the kinetics of the incorporation of the probe into the membrane was visualized by fluorescence microscopy. The photographs correspond to a representative experiment.

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When all the assayed compounds were included in the statistics, changes in 6-AS fluorescence polarization and C_{50} were significantly correlated ($r : 0.64$, $p < 0.001$). For the flavanols and related procyanidins, the correlation between their capacity to induce changes in 6-AS fluorescence polarization and C_{50} for membrane disruption protection showed a remarkable adjustment ($r : 0.95$, $p < 0.001$). These findings support the hypothesis that the interaction of flavanols and procyanidins with the water–lipid interface of membranes can protect the bilayers from external aggressors.[11]

To further assess the above described protective action of flavanols on membrane integrity, the incorporation of a phospholipid probe, (C_6-NBD-PC) in PC liposomes was tested without or with pre-treatment of the liposomes with hexameric procyanidins. The incorporation of C_6-NBD-PC was markedly retarded in the liposomes preincubated with the hexamer (Fig. 5), suggesting that the procyanidin is interacting with the membrane surface, partially impeding the access of the probe.

A positive and significant correlation was observed between the capacity of the flavonoids to inhibit AMVN-induced TBARS formation and both, to prevent Triton X-100-induced membrane disruption ($r : 0.74$, $p < 0.003$) and to change 6-AS fluorescence polarization ($r : 0.50$, $p < 0.01$). However, the mentioned parameters did not correlate when the initiator of the oxidation was AAPH ($r : 0.28$, $p = 0.15$). Such different behaviour between the two initiators could be explained by considering both the efficiency of flavonoids for trapping radicals, which is determinant when the radicals are generated in the aqueous media (AAPH), and the interaction of flavonoids with membranes. The later would be more relevant to the antioxidant capacity of flavonoids when the radicals are generated in the lipid phase (AMVN). Another important finding that support this hypothesis, is the positive and significant correlation found between the capacity of flavanones, isoflavones, phenolic acids and phenylpropanones to inhibit AAPH- and AMVN-induced lipid oxidation ($r : 0.80$, $p < 0.002$) while these two parameters did not correlate for flavanols and related procyanidins and flavonols.

The possibility that a flavonoid-membrane interaction could be one mechanism involved in their antioxidant activity is also supported by *in vivo* experiments.[33] Erythrocytes isolated from the cocoa extract-fed animals showed a reduced susceptibility to free radical-mediated haemolysis[33] suggesting that flavonoids could be adsorbed to the erythrocyte membrane preventing oxidation.

CONCLUDING REMARKS

The results presented in this work stress the importance of considering not only the chemical structure of flavonoids *per se*, but also the nature of the interactions between these molecules and membranes, when estimating flavonoid potential antioxidant capacity. Even when the different flavonoid families were similarly effective protecting lipids from water-soluble oxidants, significant differences among the families were found in the protective capacity against lipid-soluble oxidant-induced damage. The more hydrophilic flavonoids could establish hydrogen bonding with the polar head groups of phospholipids at the water–lipid interface of membranes. Consequently, flavonoids may provide a level of protection for the bilayer from external and internal aggressors (i.e. oxidants) contributing to preserve the structure and function of biological membranes. The capacity of flavonoids to interact with the membrane at the water–lipid interface should be considered as another contributing factor to the antioxidant capacity of flavonoids.

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