ELSEVIER

International Journal of Pharmaceutics 124 (1995) 1–8

international journal of pharmaceutics

Research Papers

Flavonoid-biomembrane interactions: A calorimetric study on dipalmitoylphosphatidylcholine vesicles

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Received 25 October 1994; accepted 23 January 1995

Abstract

Flavonoids, a group of naturally occurring benzo-γ-derivatives, have been shown to possess several biological properties, many of which may be related, partially at least, to their capacity to penetrate into the cell membrane and so to affect membrane-dependent processes. In the present paper we report the results of an investigation by differential scanning calorimetry (DSC) on the effects of three flavonoids (quercetin, hesperetin and naringenin) upon the gel-to-liquid crystalline phase transition of model membranes constituted by L-α-dipalmitoylphosphatidylcholine (DPPC) vesicles. All tested flavonoids interacted with DPPC liposomes causing different shifts, towards lower values, of the T_m typical for DPPC multilayers; the enthalpy changes (ΔH), related to the calorimetric peak area, remained nearly constant. Furthermore, their effects on DPPC thermotropic behaviour were greater for high molar concentrations (up to a limit value different for each flavonoid) and were affected by the time. Several hypotheses (drug molecule aggregation into the model membrane, changes in the barrier function of the lipid bilayer, different drug structural features and conformation, possible formation of flavonoid-phospholipid complexes) are presented to explain the nature of the interaction between flavonoids and phospholipid vesicles.

Keywords: Flavonoid; Membrane; DSC; Liposome

1. Introduction

Biological membranes play an important role in drug transport, distribution, action, selectivity and toxicity (Seydel, 1991; Kaneko et al., 1994). Differential scanning calorimetry (DSC) represents a powerful and non-perturbing thermodynamic technique to characterize the thermotropic phase behaviour of lipid bilayers in liposomal structures, thus allowing convenient and sensitive determination of the interaction of drugs with artificial membranes. In fact, the presence of drug molecules in the ordered bilayer structure can modify the packing of lipid chains, depending on their amphipathic or lipophilic nature, causing variations in the transition temperature of the

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SSDI 0378-5173(95)00051-8

pure lipid and/or changes in enthalpy of chain melting (Cater et al., 1974; Papahadjopoulos et al., 1975; Sackmann, 1978; O'Learly et al., 1986; Jain, 1988).

Flavonoids, a group of naturally occurring benzo-γ-pyrone derivatives, have been shown to possess several biological properties (including antioxidant, hepatoprotective, antithrombotic, anti-inflammatory, bactericidal and antiviral activities), many of which may be related, partially at least, to their capacity to penetrate into the cell membrane and so to affect membrane-dependent processes, such as arachidonic acid metabolism, exocytotic histamine release, cyclic AMP phosphodiesterase activity and free radical-initiated lipoperoxidation (Gábor, 1987; Pathak et al., 1990; Middleton and Kandaswami, 1994). However, few data have been reported in literature concerning the flavonoid ability to perturb lipid bilayers (Price and Middleton, 1986; Ratty et al., 1988; Ikigai et al., 1993). In the present paper, we report the results of a systematic investigation by DSC on the effects of three flavonoids on the gel-to-liquid crystalline phase transition (the well known $L_\alpha - L_\beta$ phase transition) of L-α-dipalmitoylphosphatidylcholine (DPPC) liposomes as a model membrane. In addition, we attempted to clarify the nature of the interaction between flavonoids and phospholipid vesicles.

2. Materials and methods

2.1. Preparation of liposomes

Multilamellar liposomes were prepared in the presence and absence of flavonoids by the following procedure: chloroform-methanol stock solutions of DPPC (from 1:1 to 1:2, v/v depending on drug solubility) were mixed to obtain the chosen mole fractions (X_D) of drugs. The solvents were removed under a nitrogen flow in a rotoevaporator, and the resulting film was freeze-dried under vacuum to remove the residual solvents. Liposomes were obtained by adding to the film 50 mM Tris buffer (pH 7.4), then heating at a temperature above that of the gel-liquid crystalline phase transition (60°C) and vortexing three times for 1 min. The samples were shaken for 1 h in a water bath at 60°C to homogenize the liposomes.

2.2. DSC

Aliquots of 120 μl of lipid aqueous dispersion (5 mg of lipid), pure or containing flavonoids, were transferred in a 160 μl DSC aluminium pan and submitted to DSC analysis. DSC was performed by using a Mettler TA 3000 system equipped with a DSC-30 cell and a TC-10 processor. The scan rate employed was 2°C/min over the temperature range 10–70°C. The sensitivity was 1.72 mW, and the reference pan was filled with Tris buffer solution. Palmitic acid was employed to calibrate the temperature scale and transition enthalpies (ΔH). Enthalpies were evaluated from the peak areas using the integration program of the TA processor, permitting the choice of different baselines and ranges of integration. For curves showing an ill-defined baseline, a fixed-arm planimeter was also employed. The areas calculated with these different methods lie within the experimental error (±5%).

To study the flavonoid-membrane interaction as function of the time, the samples underwent several scans, at 1 h intervals. The last scan was carried out 12 h after leaving the samples in the pan at a thermostated temperature (25°C).

After calorimetric scans, all samples were extracted from the pan and aliquots were used to determine the amount of phospholipid by phosphorus assay (Bartlett, 1959).

2.3. Flavonoid release from liposomes

A second series of experiments was carried out to evaluate the flavonoid transfer from lipid vesicles to aqueous medium surrounding liposomes. Samples of liposomes, containing hesperetin or naringenin ($X_D = 0.10$) and prepared as described above, were incubated at 25°C, under continuous mild shaking; at different times (1, 4, and 7 h) aliquots were withdrawn, opportunely diluted in 50 mM Tris buffer (pH 7.4) and filtered using a Millex-GS filter (0.22 μm; Waters-Millipore Corp., Milford, MA, USA) before injection

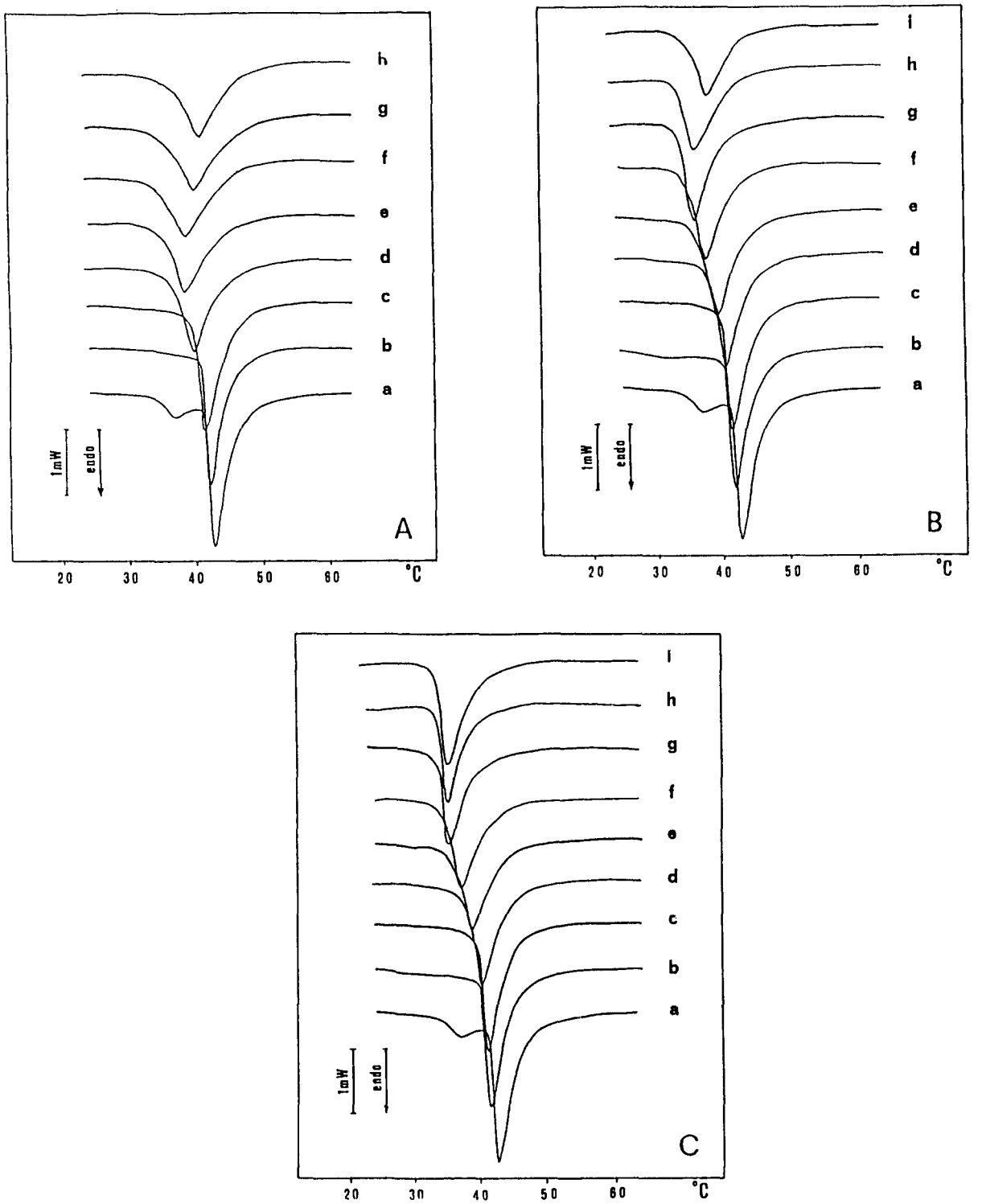

Fig. 1. Differential scanning calorimetry heating curves (referred to the first scan) of hydrated DPPC containing quercetin (A), hesperetin (B) or naringenin (C), at the following mole fractions: a = 0.000; b = 0.015; c = 0.030; d = 0.060; e = 0.090; f = 0.120; g = 0.180; h = 0.240; i = 0.330.

in a high-pressure liquid chromatograph with UV/visible detection (HPLC-UV/Vis) to evaluate the flavonoid amount in the aqueous medium.

The molar fraction used in this experiment ($X_D = 0.10$) was chosen because, at this drug concentration, hesperetin and naringenin begin to show different behaviour in the calorimetric scans. Unfortunately, we could not test quercetin because of its insolubility in water and in most solvents suitable for studies on liposomal preparations.

The HPLC apparatus consisted of a Varian 5000 system (Varian, Walnut Creek, CA, USA) equipped with a 20 μl loop and a Polychrom 3060 UV/Vis detector (Varian). Integration of the chromatographic peaks was achieved with a 4290 integrator (Varian, Walnut Creek, CA, USA). Chromatography was performed on a Pecosphere HS-5 HC ODS column (particle size, 10 μm; 15 cm × 4.6 mm i.d.; Perkin-Elmer, Norwalk, CT, USA). The mobile phase was methanol-water (45:55) containing 1% acetic acid. Detection was effected at 282 nm (hesperetin) or 287 nm (naringenin). The flow rate was set at 1.0 ml/min.

The results, expressed as mean ± S.D. of three experiments at least, were statistically analyzed by Student's t-test for paired data; statistical significance was accepted where $P < 0.05$.

2.4. Log capacity factor (log K')

Reverse-phase chromatographic retention times can be used to estimate oil/water partition coefficients; as reviewed by a number of authors (Braumann, 1986; Valko, 1987), a good correlation is found between log octanol/water partition coefficients and log K' using octadecyl silica columns. Log K' values for flavonoids were determined by high-pressure liquid chromatography with UV/visible detection (HPLC-UV/Vis), as previously described. Detection was effected at 220 nm (non-retained solvent peak), 254 nm (quercetin), 282 nm (hesperetin) or 287 nm (naringenin). Each flavonoid was dissolved in 100 ml of absolute methanol to give a final concentration of 10 μg/ml; samples were filtered prior to injection using a Millex HV13 filter (0.45 μm; Waters-Millipore Corp., Milford, MA, USA) and an aliquot (20 μl) was injected into the HPLC apparatus. Log K' values were calculated from the following relationship:

$$\log K' = \log \frac{T_r - T_0}{T_0}$$

where T_r is the retention time of the flavonoid peak and T_0 denotes the retention time of the non-retained solvent peak.

2.5. Drugs used

Quercetin dihydrate, hesperetin and naringenin (analytical grade) were purchased from Extrasynthèse (Genay, France), methanol and chloroform from Sigma-Aldrich S.r.l. (Milan, Italy). Synthetic DPPC was obtained from Fluka Chemical Co. (Buchs, Switzerland); solutions of the lipid were chromatographically pure as assessed by two-dimensional thin-layer chromatography.

3. Results and discussion

In Fig. 1 we report the calorimetric heating curves of DPPC liposomes in the presence of different mole fractions of the three flavonoids examined. All drugs interacted with DPPC liposomes causing different shifts of the T_m typical for DPPC multilayers towards lower values; the enthalpy changes (ΔH), related to the calorimetric peak area, remained nearly constant (data not shown).

The interaction observed between flavonoids and DPPC liposomes may be largely explained in terms of a fluidifying effect due to the introduction of lipophilic molecules into the ordered structure of the lipid bilayer (Jain, 1988; Castelli et al., 1989, 1991). In fact, drug molecules act as a spacer in such a structure, causing a destabilization of the lipid mosaic with a decrease in the T_m of the gel to liquid-crystal phase transition. The negligible variation in the ΔH can be due to a superficial interaction between drugs and lipids, at the level of polar heads of DPPC, since

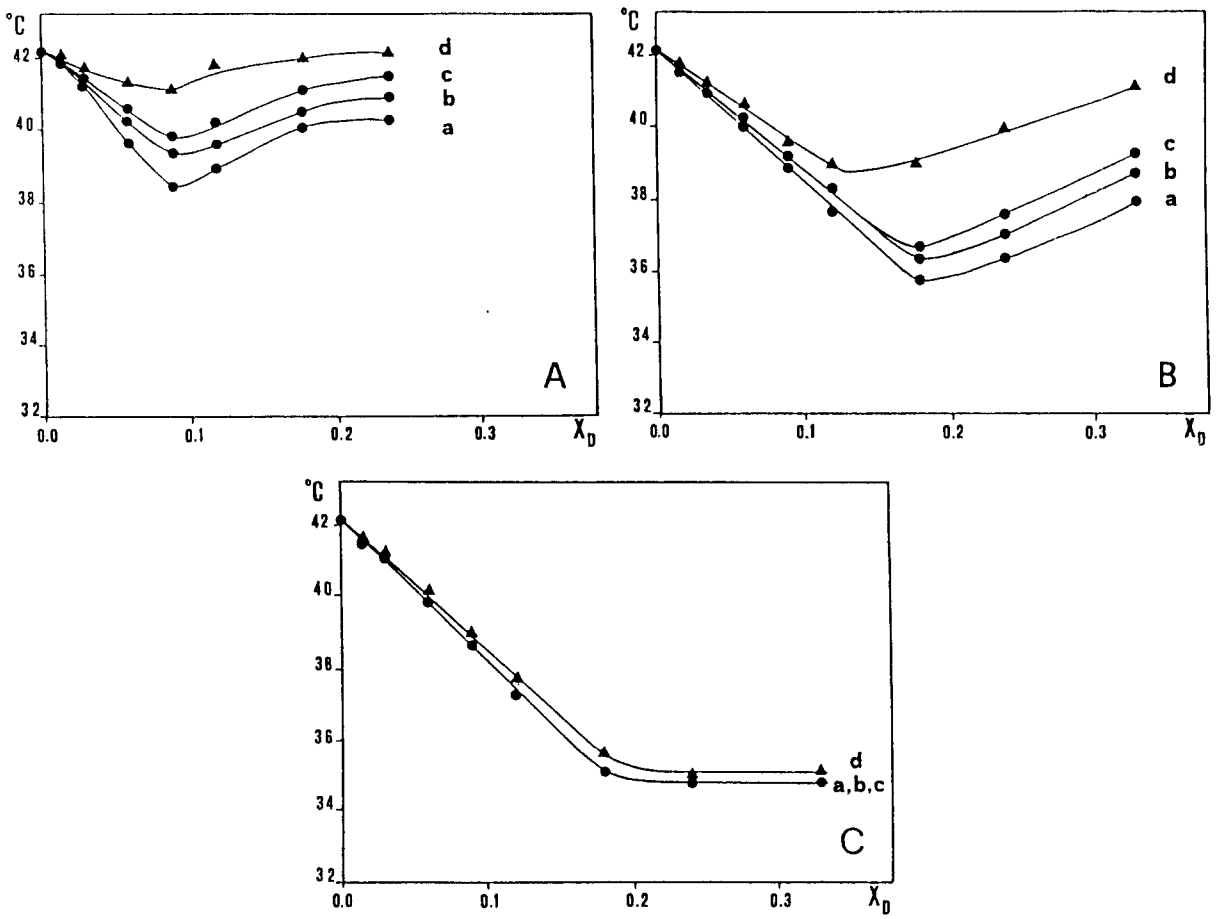

Fig. 2. Transition temperature (T_m, °C) values (average of at least four different samples), in heating mode, of hydrated DPPC containing quercetin (A), hesperetin (B) or naringenin (C), as function of their mole fractions. The curves are referred to the calorimetric scan at 1 h (a), 2 h (b), 3 h (c) and 12 h (d).

Table 1
Main transition peak temperature (T_m, °C) of DPPC dispersions at different molar fractions of quercetin, hesperetin and naringenin, referred to the first (1 h) scan and the last (12 h) scan

Molar fraction	T_m (°C)					
	1 h			12 h		
	Quercetin	Hesperetin	Naringenin	Quercetin	Hesperetin	Naringenin
0.000	42.2	42.2	42.2	42.2	42.2	42.2
0.015	41.7	41.3	41.1	41.9	41.5	41.2
0.030	41.1	40.9	41.0	41.6	41.1	41.1
0.060	39.6	40.0	40.1	40.1	40.6	40.2
0.090	38.3	39.0	38.5	41.0	39.4	38.9
0.120	38.8	37.5	37.0	41.7	38.7	37.4
0.180	40.0	35.6	34.9	41.9	39.2	35.5
0.240	40.1	36.3	34.7	42.0	39.8	34.9
0.330	–	37.8	34.9	–	41.0	35.0

molecules can interact with lipids in liposomes intercalating between the flexible acyl chains of lipids as 'interstitial impurities', causing T_m variations, but no ΔH changes (Jorgensen et al., 1991), according to the temperature depression of melting point for ideal solutions (Guggenheim, 1952; Lee, 1977).

However, the T_m shift elicited by these three different compounds followed a similar trend only for low molar fractions ($X_D = 0.03–0.09$), but a different behaviour was observed at higher flavonoid concentrations. In fact, as shown in Fig. 2C, naringenin induced a concentration-dependent perturbation of the ordered lipid structure until $X_D = 0.18$; at higher drug concentrations the T_m values remained practically constant. Instead, quercetin and hesperetin produced a negative shift of T_m values until $X_D = 0.09$ and 0.18, respectively, and then the T_m went again to higher values (Fig. 2A and B).

Also, when the T_m variations were studied as a function of time (see Table 1 and Fig. 2), some differences were found between the three tested flavonoids. In fact, the liposomes charged with naringenin showed a nearly reproducible calorimetric peak also after remaining 12 h at room temperature; in contrast, when the vesicles charged with quercetin or hesperetin were examined, a time-dependent decrease in the T_m shift was observed, particularly at high X_D values.

This behaviour leads us to make some considerations concerning the drug distribution into model membranes. Firstly, it is known that, at temperatures below the T_m, drug molecules are less soluble in the rigid structure of the gel phase so that they can aggregate (Lee, 1977). Thus, at high concentrations, flavonoid molecules may be supposed to aggregate with each other and therefore to segregate into isle-like clusters (Jain, 1988); the result of such a process is the subtraction of drug molecules from the homogeneous lipid-drug dispersion, causing an increase of T_m toward the values shown when the ordered lipid lamellar structure of pure DPPC liposomes is not perturbed by the drug. Secondly, the T_m increase might be caused by the capacity of flavonoids to leave the lipidic membranes with time, transferring in the aqueous medium.

Table 2
Flavonoid concentrations (% of the employed molar fraction) in the aqueous medium surrounding DPPC vesicles at different times

Time (h)	Hesperetin	Naringenin
1	27.9 ± 0.7	33.5 ± 1.5
4	31.3 ± 0.7 [a]	34.8 ± 0.8
7	35.3 ± 4.1 [a]	30.3 ± 1.2

[a] $P < 0.05$ vs 1 h.
Each point is the mean ± S.D. of three experiments at least.

The different behaviour of naringenin and hesperetin, observed when the flavonoid concentration in the aqueous medium surrounding liposomes was evaluated at different times from the beginning of the experiment, has allowed us to discriminate between the two hypotheses suggested above. In fact, as shown in Table 2, there was no evident change in naringenin concentration in the aqueous medium during the entire experimental period; conversely, we observed a slight but significant and time-dependent increase in hesperetin levels in the medium surrounding DPPC vesicles.

These results seem to demonstrate that hesperetin (as well as, perhaps, quercetin) alters the barrier function of lipid bilayers, thus transferring from the DPPC vesicles to the aqueous medium. Conversely, no change in membrane permeability appears to occur when liposomes are charged with naringenin. Similarly, Ikigai and co-workers (1993) reported that catechins can damage lipid bilayers. Of course, we cannot exclude that a (perhaps concomitant) aggregation of quercetin molecules causes, partially at least, the observed time- and concentration-dependent thermotropic phase behaviour.

Several factors should modulate the interaction of flavonoids with lipids in the model membrane. For example, the presence of different substituents in the backbone structure of drugs has been clearly demonstrated to modulate their incorporation or interaction with lipids in model membranes (Castelli and Valencia, 1989). The flavonoids used in the present study have been chosen according to the following structural char-

acteristics: (1) the *o*-dihydroxy (catechol) structure of the B ring; (2) the 2,3-double bond in conjugation with a 4-oxo function; and (3) the presence of 7-, 5- and 3-hydroxyl groups. Quercetin presents these three structural features, whereas naringenin and hesperetin were devoid of the second and naringenin presents only one hydroxyl group on the B ring. Thus, the existence of a structure-dependent relationship in flavonoid perturbing effects on the thermotropic behaviour of lipid membranes might be suggested. In addition, unlike hesperetin and naringenin, quercetin is able to assume a planar conformation, as calculated by using the program Hyperchem™ Release 2 for Windows (Autodesk, Inc.); this can explain why quercetin shows, at low concentrations (up to $X_D = 0.09$), a deeper interaction with DPPC membranes than hesperetin and naringenin. Finally, the values of the log K' calculated for naringenin, quercetin and hesperetin were, respectively, 0.458, 0.510 and 0.530. Flavonoids are known to anchor through chemical bonds to the polar head of main phospholipids, so forming reversible physico-chemical complexes (Bombardelli and Spetta, 1991); thus, one can speculate that, also depending on their liposolubility, there is a relationship between flavonoid interaction with model membranes and formation of flavonoid-phospholipid complexes.

The different modification of the fluidity of the model membrane may be a critical factor in determining many functions of cell membranes and the activity of molecules embedded in them. For example, we have previously demonstrated that the flavonoid ability to interact with biomembranes is, together with their redox properties, a fundamental requisite for the expression of their antioxidant activity (Saija et al., 1995). Besides, an increase in lipid fluidity in arterial and venous biomembranes seems one of the mechanisms responsible for flavonoid-induced enhancement in their resistence (Ivanov et al., 1992), and the biological activity of tea catechins is attributable to their capability to damage lipid bilayers (Ikigai et al., 1993). However, further experiments are needed to increase our understanding of the relationship between flavonoid-membrane interactions and their biological activity.

Acknowledgements

This research was partially supported by Italian M.U.R.S.T. (60% and 40%). The authors would like to thank Professor N.A. Mancini (CUMEC) for his kind permission to use the Mettler TA 3000 system.

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