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Cholesterol induces surface localization of polyphenols in model membranes thus enhancing vesicle stability against lysozyme, but reduces protection of distant double bonds from reactive-oxygen species

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ABSTRACT

The main scope of the present study was to analyze the membrane interaction of members of different classes of polyphenols, i. e. resveratrol, naringenin, epigallocatechin gallate and enterodiol, in model systems of different compositions and phase states. In addition, the possible association between membrane affinity and membrane protection against both lipid oxidation and bilayer-disruptive compounds was studied.

Gibbs monolayer experiments indicated that even though polyphenols showed poor surface activity, readily interacted with lipid films. Actually, a preferential interaction with expanded monolayers was observed, while condensed and cholesterol-containing monolayers decreased the affinity of these phenolic compounds. On the other hand, fluorescence anisotropy studies showed that polyphenols were able to modulate membrane order degree, but again this effect was dependent on the cholesterol concentration and membrane phase state. In fact, cholesterol induced a surface rather than deep into the hydrophobic core localization of phenolic compounds in the membranes.

In general, the polyphenolic molecules tested had a better antioxidant activity when they were allowed to get inserted into the bilayers, i.e. in cholesterol-free membranes. On the other hand, a membrane protective effect against bilayer permeabilizing activity of lysozyme, particularly in the presence of cholesterol, could be assessed. It can be hypothesized that phenolic compounds may protect membrane integrity by loosely covering the surface of lipid vesicles, once cholesterol push them off from the membrane hydrophobic core. However, this cholesterol-driven distribution may lead to a reduced antioxidant activity of linoleic acid double bonds.

Keywords: POLYPHENOLS, LANGMUIR MONOLAYERS, LIPOSOMES, FLUORESCENCE ANISOTROPY, LIPOSOME LEAKAGE, LIPID OXIDATION

1. Introduction

Polyphenols are secondary metabolites with phenolic rings in their structure which are produced mainly by plants and are involved in defense against ultraviolet radiation and pathogens. Actually, phenolic compounds may exhibit a wide range of properties depending on their particular structures. In fact, some of them are pigments such as the anthocyanins, others are related to food flavors. Nevertheless, polyphenols are most commonly associated to radical-scavenging capacity and to the binding of proteins, which has several consequences: the astringency perception, inhibition of several enzymes and formation of precipitates in beverages (Cheynier, 2005). Dietary polyphenols can be divided into several classes according to the number of rings and the structural elements connecting the phenolic rings. The main groups are: flavonoids, phenolic acids, phenolic alcohols, stilbenes and lignans (Dewick, 1995; Tsao, 2010). Among the most studied polyphenols, it can be cited epigallocatechin gallate, which is a major constituent of the green tea (Ramesh *et al.*, 2008), naringenin, that is a flavanone mainly found in tomatoes and citrus, specially grapefruits (Mir and Tiku, 2015) and resveratrol, the most studied polyphenol present in grapes (Das and Maulik, 2006). In addition, some polyphenols can be produced in animals by the action of intestinal bacteria such as the lignan enterodiol (Wang, 2002).

An important property of polyphenols is the ability to interact with membranes (Hendrich, 2006; Oteiza *et al.*, 2005). In this regard, Heindrich, in a comprehensive review, pointed out that phenolic compounds might interact mainly with the polar region of the phospholipids, although a deeper insertion may also occur, depending on flavonoid structures (Heindrich, 2006).

On one hand, it has been shown that polyphenols would interact with cell membranes in close contact to membrane proteins, in this way preventing lipid peroxidation and hemolysis (Blasa *et al.*, 2007; Grinberg *et al.*, 1997; Tedesco *et al.*, 2000). Han *et al.* (2006) also postulated a specific proteinaceous binding site common to all polyphenols in rat brain. On

the other hand, there is convincing evidence that polyphenols might interact not only with membrane-bound proteins but also with lipid membrane. By using various biophysical techniques, it was concluded that polyphenols may insert and modulate the properties of model membranes e.g. dipole potential, permeability, phase transition and thermotropic behavior (Huh *et al.*, 1996; Sarpietro *et al.*, 2007; Sroda *et al.*, 2008).

Pioneering work in our laboratory conclusively demonstrated that the activity of membrane-bound enzymes could be modified by changes in the membrane fluidity (Bloj *et al.*, 1973a, 1973b; Siñeriz *et al.*, 1973). In this regard, Meyer dos Santos *et al.* (2007) showed that the activity of P-glycoprotein, a transporter of the ABC type, is closely related to the physicochemical properties of the membrane. In the same trend, Takahashi *et al.* (2006) suggested that an alteration in the viscosity of the membrane could also impact ABCA1 activity. Interestingly, most polyphenols can increase membrane viscosity while others may exert the opposite effect (Tarahovsky *et al.*, 2008; Tsuchiya and Nagayama, 2008; Tsuchiya *et al.*, 2002). In a previous work, we demonstrated that resveratrol may enhance ATPase activity of reconstituted ABCG1 by modulating the fluidity of proteoliposome membrane (de Athayde Moncorvo Collado *et al.*, 2013). Patra *et al.* (2008) recently suggested that many of the positive effects of epigallocatechin gallate have their origin in the modification of the plasma membrane "rafts". Since the antioxidant activity is thought to be the main beneficial property of phenolic compounds, this work aimed to analyze the membrane interaction of different polyphenols and correlate it with the antioxidant effect displayed by them. Besides, the possible protection of bilayer integrity against membrane-active proteins was studied as well. The phenolic compounds used in this work are resveratrol, epigallocatechin gallate, naringenin and enterodiol; they are representative to the different classes of polyphenols and their chemical structure is shown in Figure 1.

2. Materials and methods

Chemicals: lysozyme and the polyphenols enterodiol, epigallocatechin-3-gallate (EGCG), naringenin and resveratrol were purchased from Sigma-Aldrich. Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), cholesterol (Cho) and linoleic acid (LA) were from Avanti Polar Lipids. 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH), 1,6 diphenyl-1,3,5-hexatriene (DPH) and calcein were purchased from Molecular Probes-Thermo. All other reagents were of the highest purity available.

Stock solutions of the four polyphenols and the fluorescent probes DPH and TMA-DPH were prepared in methanol and kept at -20 °C. Appropriate dilutions for experimental procedures were performed in the same solvent. Cho and phospholipids were kept in a nitrogen atmosphere, at -20 ºC as chloroform-methanol solutions in Teflon capped glass tubes. Concentration of phospholipids was routinely checked by the method of Ames (1966), while cholesterol concentration was assessed by the cholesterol oxidase method (Sigma).

Langmuir monolayer experiments: the surface activity of polyphenols was assayed in a NIMA 102M Langmuir microbalance (KSV NIMA, Finland) equipped with a custom made PTFE trough of 7 mL for constant area experiments. Methanolic stock solutions (1-10 mM) of polyphenols were injected into the 145 mM NaCl sub-phase under constant stirring and the surface pressure was measured with a Wilhelmy platinum plate, as a function of time and up to a constant value of surface pressure was reached, typically 15 min. The surface of exclusion of polyphenols from lipid interfaces was calculated by measuring the increment in surface pressure produced by the different polyphenols when injected into the subphase of lipid monolayers, standing at different surface pressure.

Monolayers of DPPC and DMPC standing at 20, 25 and 30 $mN.m^{-1}$ were studied for polyphenol adsorption from the subphase in order to assess the interaction of the different compounds with liquid condensed and liquid expanded monolayer phase, respectively. DPPC at low surface pressure was not studied as this lipid presents an isobaric monolayer

phase transition at around 10 $mN.m^{-1}$ at room temperature, which could interfere when surface pressure increments are intended to measure. Thus, experiments of polyphenol adsorption into lipid monolayers at surface pressures lower than 15 $mN.m^{-1}$ in the absence of cholesterol were carried out only with DMPC (Phillips and Chapman, 1968; Sabatini *et al.*, 2008). Furthermore, we aimed to determine the effect of the presence of cholesterol on the adsorption of polyphenols in both systems (X_{Cho} = 0.4). In cholesterol-containing monolayers there is a typical "ordered liquid" phase that has a high degree of mobility, similar to fluid phases but at the same time highly ordered hydrocarbon chains (Ipsen *et al.*, 1987; Sabatini *et al.*, 2008). Results are the average of at least five independent experiments.

Polyphenol-lipid bilayer partition: multilamellar vesicles (MLV) were prepared by drying organic solutions of lipids under a nitrogen stream, followed by resuspension in buffer (Tris-HCl 25 mM pH 7.4) and vortexing. Afterward, MLV were diluted to a final concentration of 100 µM and 1 µM of polyphenol was added. Mixtures were incubated at 37 ºC for 10 minutes and centrifuged at 10,000 x *g* for 15 minutes. Polyphenol concentration in the supernatant was estimated by the Folin method, with gallic acid as a standard (Singleton *et al.*, 1999). The phospholipid content of MLV was assessed by the method of Ames (1966) right after MLV preparation and at the end of the experiment from the MLV pellet. Controls without liposomes were routinely run. Percentage of polyphenol partition was calculated according to:

$$\%_{polyphenol\text{-}MLV} = [([polyphenol]_{added} - [polyphenol]_{supernatant}) / [polyphenol]_{added}] * 100.$$

Results are the average of at least three independent experiments made by triplicate.

Membrane order degree upon polyphenol-lipid bilayers interaction: polyphenol-bilayer interactions were evaluated by measuring fluorescence anisotropy of the fluorescent probes DPH and TMA-DPH.

The steady-state fluorescence anisotropy was determined in an ISS PC1 Photon Counting Spectrofluorimeter thermostatized by means of an external circulating bath (Cole Parmer), adjusting the excitation and emission wavelengths at 360 nm and 450 nm, respectively. No

significant fluorescence emission from the four polyphenols used was detected at the aforementioned excitation wavelength. Fluorescence anisotropy was calculated according to:

$$r = \frac{Iw - Ivh}{Iw + 2.Ivh}$$

where I_{vv} is the fluorescence intensity recorded with both the analyzing and the excitation beam polarizers vertically oriented, whereas I_{vh} is the fluorescence intensity recorded in crossed polarizers condition, in which excitation beam polarizer is vertically oriented whereas the emission channel is horizontally oriented. Briefly, DPPC or DPPC:Cho solutions were dried under nitrogen with the addition of either TMA-DPH or DPH in lipid:probe ratio of 1000:1. Lipids were resuspended with 20 mM Tris-HCl pH 7.4 buffer and vortexed in order to prepare MLV. Then, unilamellar liposomes of 100 nm diameter (LUV) were obtained by extrusion of these multilamellar vesicles through polycarbonate filters with the appropriate pore size (Millipore). Concentration of phospholipids in LUV was assessed after extrusion by the method of Ames (1966). Final lipid and polyphenol concentrations were adjusted to 20 and 0.2 μM, respectively. No significant changes in total fluorescence intensities of DPH and TMA-DPH were measured upon addition of polyphenols, at the working concentrations.

Protection against lipid oxidation: liposomes made of DPPC or DPPC:Cho containing 2% of linoleic acid (LA) were prepared as described above and were incubated at final concentration of 250 μM during 10 minutes in the presence or in the absence of 2.5 μM polyphenol (added from a methanolic concentrated solution) at either 30 ºC or 51 ºC i.e. 10 degrees under or above the main transition temperature of DPPC, respectively. Afterward, $CuSO_4$ and H_2O_2 were added at final concentrations of 0.1 and 2 mM respectively in order to trigger the production of reactive oxygen species by means of Fenton reaction, and the mixtures were further incubated for 30 minutes at either 30 or 51ºC. Then, deoxycholate was added at a final concentration of 1% and conjugated dienes levels were measured by absorbance at 233 nm. The final results were expressed as ratios relative to the untreated

controls, which represent the maximal oxidation.

Protection against membrane-active compounds: the protective effect of polyphenols against the lipid bilayer perturbing activity of lysozyme (Posse *et al.*, 1994) was assayed by studying permeability of lipid vesicles. Besides its hydrolytic activity, lysozyme induces the leakage of liposomal content owing its ability to bind membranes. Pre-incubation with polyphenols were carried out in order to know whether or not they can stabilize and protect membranes. Liposomes loaded with calcein at self-quenching concentrations were prepared by resuspending the dried lipid films in 20 mM Tris-HCl pH 7.4 buffer containing 50 mM calcein. LUV of 100 nm diameter were obtained by extrusion of MLV as described above. Non-encapsulated material was eliminated by gel filtration in Sephadex G-75 (Posse *et al.*, 1994) and liposome phospholipid concentration was measured after filtration by the method of Ames (1966). Phospholipids were adjusted to a final concentration of 20 µM, while polyphenols were added at a final concentration of 0.2 µM. Excitation and emission wavelengths were 490 and 520 nm respectively, and a 500 nm cutoff filter (Corning) was placed between the sample and the emission monochromator. Polyphenol showed no fluorescence emission in these conditions. The fluorescence scale was calibrated such that 100 % leakage corresponded to the calcein fluorescence in the presence of 0.1% Triton X-100. The measurements were carried out at either 30ºC and 51ºC in an ISS PC1 spectrofluorometer.

Miscellaneous: Statistics analysis and graphics were done by Prism GraphPad program.

3. Results

Polyphenols interact with lipid monolayers: as a first approach, adsorption isotherms of each polyphenol were carried out at air-water interfaces. Interestingly, only enterodiol showed a measurable surface activity since it was able to induce a final surface pressure (π) of 10 $mN.m^{-1}$ at a concentration of 40 µM. On the other hand, the other phenolic compounds under

study barely changed the system surface pressure, with final π of 2 $mM.m^{-1}$ at concentrations as high as 100 μM (Fig. 2).

Polyphenol adsorption at lipid monolayers assays were carried out at 30 μM of each polyphenol in the subphase. Based on the experimental points, exclusion curves were calculated for resveratrol (Fig. 3A), naringenin (Fig. 3B), EGCG (Fig. 3C) and enterodiol (Fig. 3D) in the four monolayers tested. Exclusion point values were calculated as the intersection of the experimental curves with the x-axis and represent the limit π each monolayer expelled polyphenol molecules out from the interface. As it can be observed in Fig. 3, polyphenols were able to show favorable interactions with the lipid monolayers, reducing the surface tension of the lipid interfaces at surface pressure values significantly higher than those attained by the pure polyphenols at the bare air/water interface. However, they were better adsorbed on monolayers at LE phase (DMPC, gray filled symbols) as compared to LC phase (DPPC, black filled symbols) at the same surface pressure. In fact, exclusion surface pressure of polyphenols in LE monolayers were around 50 $mN.m^{-1}$. The presence of Cho at X_{Cho}= 0.4 in DMPC monolayers (dashed gray lines, open symbols) was associated to substantial decrease in the exclusion surface pressures, which were closer to those observed in LC phase. Furthermore the presence of Cho in DPPC monolayers (dashed black lines, open symbols) did not significantly change the exclusion pressure values recorded with pure DPPC monolayers *i.e.* polyphenols interacted with both LC and "liquid-ordered" phases in a similar way. Considering the Δπ caused by polyphenols at each point, it can be concluded that naringenin and resveratrol caused the more pronounced changes at surface level while EGCG produced the most subtle changes in surface pressure. Also, EGCG showed lower exclusion pressures than the other polyphenols. The only exception was observed in DMPC monolayers, where the exclusion surface pressure for EGCG exceeded 50 $mN.m^{-1}$ (Fig. 3C, gray filled symbols). This somewhat unusual observation may be interpreted in concordance to Caturla *et al.* (2003), whom concluded that EGCG was able to

interdigitate between the molecules of DMPC in liquid-crystalline phase, thus forming an ordered structure, which stability might be enhanced due to the presence of this polyphenol.

Phenolic compounds partition into bilayers: partition of resveratrol, naringenin, EGCG and enterodiol in MLVs made of either DPPC or DPPC:Cho (6:4 molar ratio) was determined. Polyphenols were added to get a lipid:polyphenol ratio of 100:1 and samples were incubated 15 minutes at either 30ºC or 51ºC in order to test the interaction not only in gel phase but also in liquid-crystalline phase. Polyphenols were readily associated to DPPC MLVs regardless the temperature of incubation. In fact, 40 to 60% of polyphenols were partitioned into DPPC bilayers under the conditions tested. The only exception was enterodiol, 60% of which was found to be associated to membranes at 30ºC but only 20% at 51ºC (Table 1). Interestingly, the percentage of polyphenol partitioned to membranes dramatically decreased in all cases in the presence of 40% Cho. Furthermore, the membrane association was even lesser at 51ºC (Table 1). This is consistent with monolayers experiments, in which cholesterol would tend to exclude polyphenols from membranes.

Polyphenols modify membrane order degree of lipid bilayers: fluorescence anisotropy of the probes DPH and TMA-DPH was measured in DPPC and DPPC:Cho liposomes, in the absence and in the presence of the different polyphenols at 30 and 51ºC. These fluorescent probes are ordinarily used in model membranes, allowing to estimate the order degree of the bilayers. On one hand, DPH can be inserted parallel to the acyl chains of phospholipids or in the center of the membranes, thus labeling the hydrophobic core. On the other, TMA-DPH is a DPH derivative that probes the interfacial area owing its trimethylammonium moiety. Resveratrol and naringenin showed a disordering effect in gel phase, as estimated by DPH fluorescence anisotropy, with no significant changes in the liquid-crystalline phase (Fig. 4A). In contrast, EGCG and enterodiol significantly increased the membrane order of the liquid crystalline phase of DPPC liposomes, but they induced no change in the fluorescence anisotropy of DPH in gel phase (Fig. 4A). Interestingly, the effect of enterodiol could be

observed even at 1000:1 lipid:polyphenol ratio (not shown). These observations, *i.e.* that polyphenols are able to modify membrane order degree, can be drawn mainly from DPH assays since no significant changes were measured with TMA-DPH at either 30 or 51ºC (Fig 4B).

The presence of cholesterol dramatically altered the changes induced by polyphenols to DPPC liposomes. As a matter of fact, the most important effects were observed in the fluorescence anisotropy of TMA-DPH, which senses interfacial zone, rather than in the fluorescence anisotropy of DPH (Fig 4C vs Fig. 4D). Enterodiol, but particularly resveratrol decreased membrane order degree, while naringenin and EGCG showed only a modest disordering effect at 30ºC. These results would confirm that the presence of cholesterol might prevent phenolic compounds to reach the hydrophobic core of the bilayers. The only exceptions would be EGCG and enterodiol that did change DPH anisotropy but only at lipid:polyphenol ratios as high as 10:1 (results not shown).

Polyphenols display antioxidant activity in DPPC membranes: unilamellar liposomes of DPPC and DPPC:Cho were challenged with a membrane peroxidation assay by means of a Fenton reaction. On this regard, linolenic acid was added in order to increase the sensibility of the technique, but at a low final concentration (2 %) in order to minimize membrane order perturbation or aggregation (Hauser *et al.*, 1979).

As it can be seen in Fig. 5, the four polyphenols displayed antioxidant properties in DPPC membranes, with the only exception of EGCG, which was unable to protect membranes in liquid-crystalline phase (Fig. 5A). Interestingly, the presence of Cho impaired the antioxidant activity of EGCG at 30 ºC, whereas at 51 ºC, the only phenolic compound still active was naringenin. Indeed, the other three phenolic compounds could not prevent oxidative damage of liposomes by HO• and HOO• radicals in a cholesterol-induced “liquid ordered” state at 51 °C (Fig. 5B). The degree of oxidation was comparable in all cases, just slightly higher in the presence of Cho. In fact, absorbance values measured at 233 nm were consistently 5 to 7 %

higher in cholesterol-containing liposomes as compared to control free-cholesterol liposomes.

Polyphenols protect DPPC vesicles against membrane-active lysozyme: integrity of the bilayer permeability conferred by resveratrol, naringenin, enterodiol and EGCG against lysozyme was studied at 30ºC. Small unilamellar vesicles of DPPC and DPPC:Cho were prepared by sonication and loaded with calcein, as described in Materials and Methods. With the exception of resveratrol, all the polyphenols at a lipid:polyphenol ratio of 100:1 significantly reduced the leakage of calcein from DPPC liposomes induced by lysozyme (Fig. 6A). Cholesterol alone stabilized membranes since the release of calcein dropped 50 % as compared to vesicles prepared with pure DPPC. Moreover, the polyphenols tested protected even more the cholesterol-containing membranes against lysozyme. In fact, the total leakage of calcein in all cases was dramatically decreased. EGCG turned out to be the most efficient polyphenol. Disappointingly, the assay could not be carried out at 51°C as lysozyme did not induce any release of calcein at this temperature (de Athayde, unpublished results/supplementary material). Therefore, it was not possible to assess the protective effect of phenolic compounds in the liquid-crystalline state of liposomes.

4. Discussion

Even though the widespread occurrence of polyphenols in nature and their chemical composition, they still tend to be treated as a homogeneous group of molecules with similar properties and biological functions. However, most of the previous works have been carried out with one kind of polyphenol or within chemically related members of a family. As far as we know, this is one of the first studies involving members of the different classes of polyphenols (a flavonoid, both a flavonoid and phenolic acid derivate, a stilbene and an enterolignane). We undertook a comparative analysis of the membrane interaction of polyphenols of the mentioned classes in model systems and a correlation with their biological activities was

intended.

Langmuir monolayers have proven to be very useful systems for studying the interaction between lipid membranes and all kind of potentially membranous-active substances at the surface level (Brezesinski and Möhwald, 2003). Despite the difficulty in correlating monolayer surface pressures with the "lateral" pressures observed in biological systems, there is an agreement that the functional range of pressures would be in the order of 25-35 $mN.m^{-1}$ (Demel *et al.*, 1975; Fanani and Maggio, 1997 and 1998). As a matter of fact, it was recently described that resveratrol analogues interacted with DMPC monolayers by compression experiments complemented with differential scanning calorimetry assays (Sarpietro *et al.*, 2013). Accordingly, the polyphenols studied were capable of interacting with lipid monolayers, especially in LE phase. Actually, the four compounds tested did display surface activity when phospholipid monolayers were spread on the interface and at surface pressure values significantly higher than those attained in bare air/water interfaces, indicating significant interfacial stabilization as a consequence of favorable polyphenol/lipid interactions. The exclusion surface pressures of these phenolic compounds in monolayers in LC or in cholesterol-containing "liquid-ordered" phases were lower when compared to those observed in LE phase. Our results agree with Olas and Holmsen findings, who previously showed that resveratrol did interact with DPPC membranes, although they focused mainly in the role of the lipid charge rather than in the role of cholesterol in monolayers. Indeed, they concluded that resveratrol preferentially interacted with monolayers made of the anionic phospholipid phosphatidylserine rather than the zwitterionic phospholipids phosphatidylcholine and phosphatidylethanolamine (Olas and Holmsen, 2012).

In bilayers, the four polyphenols under study readily partitioned into cholesterol-poor membranes, staying bound to lipid vesicles even after centrifugation. However, cholesterol dramatically decreased the polyphenol fraction associated to membranes. This is consistent with our observation that cholesterol would tend to exclude polyphenols from both lipid

monolayer and bilayer model systems. In this regard, Fadel *et al.* (2011) described that penetration of rosmarinic acid, another phenolic compound, in 1,2-dilinoleoyl-sn-glycero-3-phosphocholine monolayers was also partially impaired by cholesterol.

According to experiments carried out with fluorescent probes, we can postulate that resveratrol as well as naringenin would preferentially interact with the hydrophobic region of DPPC membranes in gel phase, which seems to agree with the bibliography (Arora *et al.*, 2000; Oteiza *et al.* 2005; Brittes *et al.*, 2012; Neves *et al.*, 2015).

Studies of resveratrol as membrane order modulator reported somewhat conflicting results, since in some cases resveratrol would appear to increase the acyl chains order in membranes made from mixtures of DPPC and POPC in the presence of amounts of cholesterol (Tsuchiya *et al.*, 2002) while in others resveratrol would make DPPC membranes more disordered (Brittes *et al.*, 2010). Our results are consistent with Brittes' findings. This seemingly opposite effect of resveratrol upon membranes was addressed recently and the studies concluded that this polyphenol might exert a dual role on their order degree depending its composition and lipid phase. In this regard, Wesolowska *et al.* (2009) reported that resveratrol might increase the hydration of DMPC and DPPC membranes (evidencing a more disordered state) on gel phase, and decrease it on liquid-crystalline phase (a more ordered state). Interestingly, this effect was observed both at the hydrophobic core and at the interfacial zone. Accordingly, Neves *et al.* (2015) concluded that resveratrol would stiffen egg PC membranes (rich in unsaturated lipids and therefore more fluid) and fluidify them in the presence of Cho and sphingomielyn (in which a highly ordered structure is induced).

In the same trend, naringenin was reported to be both an ordering molecule in SLPC membranes (Arora *et al.*, 2000) and a disordering agent of DPPC bilayers (Saija *et al.*, 1995). Nonetheless, in our hands naringenin only decrease the order of the hydrophobic chains in gel phase DPPC membranes.

On the other hand, EGCG and enterodiol showed the most significant ordering effect in the

hydrophobic core of DPPC membranes at liquid-crystalline phase. Nevertheless, they did not significantly modify the properties of gel phase. These findings are consistent with the results observed in monolayers in LE phase. EGCG affinity for membranes has been thoroughly observed and given its many hydroxyl groups and polar ester bonds, it is expected to interact mainly with the polar groups of the interfacial zone (Hashimoto *et al.*, 1999; Kajiya *et al.*, 2002; Sun *et al.*, 2009). However, it might be able to intercalate between phospholipids in liquid-crystalline phase exerting an ordering role, as was observed in DMPC membranes (Caturla *et al.*, 2003), which seems to agree with our results. Given the similar effect enterodiol showed, it can be proposed that this polyphenol might as well behave alike in liquid-crystalline phase.

Curiously, in our system, there was no significant changes induced by polyphenols in the hydrophobic core in the presence of cholesterol. The only exception was naringenin that did have an effect although only when added at a very high polyphenol:lipid ratio (not shown), indicating a possible exclusion by cholesterol. On the other hand, resveratrol and enterodiol did cause a significant disordering effect on the interfacial zone of cholesterol-containing membranes. These results are in agreement with those found in monolayers.

It has been suggested that the regulation of the order degree of membranes by phenolic compounds would be similar to that exerted by cholesterol (Heindrich, 2006). As a consequence, the presence of cholesterol at these levels would prevent further modulation of membrane order by polyphenols, at least in the hydrophobic core (Tsuchiya *et al.*, 2002).

The antioxidant activity of polyphenols both upon lipids and membranes has been mainly ascribed to its ability to both scavenge reactive oxygen and nitrogen species and to chelate redox-active metals (Bors *et al.*, 1990; van Acker *et al.*, 1998). Resveratrol has been reported to be a chelator of metalic ions such as Cu^{2+}, thus being an efficient antioxidant when such ions are involved in the formation of reactive oxidant species. Flavonoids and some of their derivatives or polymers, on the other hand, seem to be better scavengers of oxidant radicals

than chelators (Belguendouz *et al.*, 1997; Frémont *et al.*, 1999). While antioxidant properties have been reported regarding enterodiol, little has been discussed about its mechanism of action in membranes, although a role as radical scavenger is generally accepted (Kitts *et al.*, 1999; Hu *et al.*, 2007).

Our results on the membrane protection by the different classes of polyphenols against oxidative damage showed a moderate antioxidant capacity, strongly modulated by the presence of cholesterol in the membranes.

The dependence of the cholesterol content and the antioxidant capacities of the phenolic compounds seemed to be correlated, at least partially, to the partition of polyphenols into lipid vesicles, as shown in Table 1. As the association of polyphenols to membranes decreased with cholesterol content, the antioxidant activity was consequently lower. The only phenolic compound still able to partially protect cholesterol-containing membranes from reactive oxygen species at 51 °C was naringenin, as it was the only polyphenol still able to get associated to bilayers in this condition. On the other hand, no clear correlation between membrane association and the antioxidant capability could be assessed for EGCG, which might be due to a relatively poor antioxidant activity in this system. In this regard, a recent work reviewed and compared this polyphenol antioxidant activity and concluded that EGCG may act as pro-oxidant as well, depending on the working conditions (Kim *et al.*, 2014).

The consequences of the presence of cholesterol on the physical characteristics of the membranes may have dual effects on its peroxidation by oxidant agents. Firstly, the fluidity of the membrane affects the diffusion of the intermediary species of the peroxidation reactions. Thus, a more fluid environment will lead to a faster propagation but will also enhance the termination. The opposite effect will be observed in more rigid systems (Schnitzer *et al.*, 2006). Also, the stiffening effect on membranes above the phase transition temperature would prevent the access of soluble radicals to peroxidable chemical groups within the membrane (Mazari et al, 2010; Mosca *et al.*, 2011). Nevertheless, this could also prevent the

access of radical scavengers into the membrane, decreasing the antioxidant effect thereof (Gutierrez *et al.*, 2003). It is interesting to note that, as lipid peroxides accumulate, an increasing stiffening effect may be affecting further peroxidation (Ayuyan and Cohen, 2006). Overall, the prevalence of either effect will depend on each particular system. Finally, considering the conditions of these assays we assume that cholesterol was not peroxidized, as LA is far more susceptible to undergo peroxidation, and did not contribute to conjugated diene readings this way (Llijana *et al.*, 1986; Schnitzer *et al.*, 2006).

To sum up, the assumption that cholesterol would exclude polyphenols from the hydrophobic core of membranes, as may be inferred from the results described above, might account at least partially for this decrease in antioxidant activity when comparing DPPC to DPPC:Cho vesicles.

A series of interesting works involving flavanols and their derivatives and polymers, reviewed by Verstraeten *et al.* (2015) strongly indicate that the exclusion proposed in our work should not prevent the scavenging capabilities of polyphenols. For instance, a better antioxidant activity was demonstrated when flavonoids got adsorbed in the interfacial zone of the membrane (Saija *et al.*, 1995).

Contrasting results about polyphenols and membranes are not infrequent in the literature. In fact, the wide variety of membrane models, polyphenol concentrations and techniques used are believed to account for these discrepancies, as pointed out by Selvaraj *et al.* (2015). As a matter of fact, one of the first studies that comprehensively analyzed this subject supported the idea that surface localization is positively linked to anti-oxidant activity (Erlejman *et al*. 2004). They observed that surface localization of phenolic compounds was associated to a reduced membrane fluidity and the immediate consequence of that was an enhanced anti-oxidant activity. These authors used bovine brain PC for their experiments. Brain PC may contain high levels of polyunsaturated fatty acids that have double bonds close to the interfacial zone. To name two of them, docosahexaenoic acid, with a first double bond in

position 4 and eicosapentaenoic acid with a first double bond in position 5. However, in our study, we used linoleic acid, that has two double bonds: one in position 9 and the other in position 12. These double bonds are away from the phospholipid bilayer interface, hence polyphenols forced to stay on the membrane surface may not be able to efficiently protect them from reactive-oxygen species. On the contrary, polyphenols at the surface will preserve better double bonds that are closer to their positions such as those found in fatty acids from brain PC. Another important difference with that study is that Erlejman et al. used 2,2 0 -azo-bis (2,4-dimethylvaleronitrile) and 2,2 0 -azo-bis (2-amidinopropane) as initiators, while we started the oxidation by a Fenton reaction.

The modulation of membrane properties by the different polyphenols showed to be important for another interesting, albeit less explored, biological activity of ploy phenols i.e. a membrane stabilizing effect. Indeed, the presence of naringenin, enterodiol and EGCG significantly reduced the lysozyme-induced bilayer permeabilization. It was already reported that EGCG enhanced the stability of liposomes at low concentrations, presumably because its surface localization on liposomes (Nakayama *et al.*, 2000). However, one of the most important properties of EGCG, its bactericidal effect, was explained by the induction of membrane leakage, although at concentrations clearly higher than those tested in our study and only in the presence of phosphatidylethanolamine, i. e., in bacterial-like model systems (Ikigai *et al.*, 1993). A more recent study with egg PC giant vesicles seems to agree that the stabilizing and leakage-inducing effects of EGCG upon membranes are indeed dependent on its concentration (Tamba *et al.*, 2007). Curiously, resveratrol did not show any protection of the bilayer integrity in cholesterol-free DPPC membranes. However, in the presence of cholesterol, which is a membrane stabilizer itself, the four polyphenols stabilized even more the membranes against the action of lysozyme. Since cholesterol seemed to exclude polyphenols from the core of membranes, they would be able to interact at the surface level with lipid bilayers. As a consequence, they might be more efficient in preventing lysozyme

from interacting with membranes in this way. Another plausible explanation would be that polyphenols may interact and inhibit lysozyme as a membrane-disrupting protein. However, no indication of direct interaction between polyphenols and lysozyme was measured. Actually, the tryptophan fluorescence spectrum of the protein did not change in the presence of any of the polyphenols tested (Fig. 7). Hen egg lysozyme has 6 Trp residues (at positions 28, 62, 63, 108, 111 and 123), although most of the lysozyme emission is due to Trp-62 and Trp-108, which are located at the active site (Imoto *et al.*, 1972), as a consequence of self quenching by energy transfer between the other Trp residues.

Therefore, these phenolic compounds would protect membranes by interacting with phospholipid bilayers and not by a direct inactivation of lysozyme. Indeed, the presence of cholesterol in membranes would enhance the membrane-protective activity of polyphenols by inducing a surface localization, away from the lipid hydrophobic core.

5. Conclusions

Polyphenols of four different chemical families readily interacted with the membrane model systems tested i.e. monolayers and liposomes, showing preferential association with cholesterol-poor membranes as well as disordered/fluid membranes.

Cholesterol enhanced polyphenol-mediated membrane protection against lysozyme by favoring a surface localization of phenolic compounds. On the other hand, linoleic acid double bonds were less protected from reactive-oxygen species when polyphenols were driven to the surface by cholesterol. As a corollary, phenolic compounds will be better fatty acid antioxidants if they are located closer to the double bonds.

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Figure legends

Figure 1. Chemical structure of the polyphenols resveratrol (Resv, A), naringenin (Nar, B), epigallocateccin gallate (EGCG, C) and enterodiol (Ed, D).

Figure 2. Surface-activity (Gibbs monolayers) of resveratrol (Resv, ○), naringenin (Nar, □), epigallocatechin gallate (EGCG, ◊) and enterodiol (Ed, △) at the water/air interphase. Polyphenols were injected into the subphase and surface pressure was recorded after 15 minutes. The curves (sigmoidal curve fit was performed using GraphPad Prism) are representative of three independent experiments.

Figure 3. Exclusion curves of Resv (A), Nar (B), EGCG (C) and Ed (D) in monolayers. DMPC was used to get LE phase (gray, filled symbols) and DPPC for LC phase (black, filled symbols). DMPC:Cho monolayers are denoted with dashed gray lines and open symbols, while DPPC:Cho monolayers are shown in dashed black lines and open symbols. Each point was determined by triplicate, the shown values are mean ± standard deviation. Regression analysis was performed using GraphPad Prism software ($P < 0.001$).

Figure 4. Membrane order determination of DPPC LUVs (panels A and B) and DPPC:Cho LUVs -at molar ratio of 6:4- (panels C and D) by fluorescence anisotropy of DPH (A and C) and TMA-DPH (B and D), recorded at 30 and 51°C. Assays were carried out in the absence (white) or presence of polyphenols Resv, Nar, EGCG and Ed (in gray scale, from lighter to darker respectively) at lipid:polyphenol ratio of 100:1. Values shown are mean ± standard deviation of 10 measures and are representative of three independent assays.

Figure 5. Relative oxidation of liposomes made of DPPC:LA at molar ratio of 100:2 (panel A) and DPPC:Cho:LA at molar ratio of 60:40:2 (panel B) in the absence (white) and in the presence of the polyphenols Resv, Nar, EGCG and Ed (in gray scale, from lighter to darker respectively) in a lipid:polyphenol molar ratio of 100: 1. Relative oxidation was assessed by measuring conjugated dienes (λ = 233 nm). ANOVA and Dunnet's multiple comparisons test (compared to Control groups) were performed using GraphPad Prism, which assigned a score from * to *** to significantly different groups (α = 0.01).

Figure 6. Relative leakage of encapsulated calcein induced by lysozyme in DPPC (panel A) and DPPC:Cho (6:4) liposomes (panel B) in the absence (white) and presence of polyphenols Resv, Nar, EGCG and Ed (in gray scale, from lighter to darker respectively) in a lipid:polyphenol ratio of 100:1 at 30°C. ANOVA and Dunnet's multiple comparisons test (compared to Control groups) were performed using GraphPad Prism; significantly different values compared to the corresponding control group are indicated by * (α = 0.01).

Figure 7. Tryptophan fluorescence spectra of lysozyme (2 μM) in the absence (‡) or presence of 0.2 μM Resv (○), Nar (□), EGCG (◊) and Ed (Δ). Excitation wavelength was set at 290 nm. Spectra are representative of three independent assays.

Table 1. Percentage of polyphenols Resv, Nar, EGCG and Ed partitioned to DPPC and DPPC:Cho -at molar ratio of 6:4- MLVs after 15 minutes incubation at 30 and 51° C. Values are representative of three independent experiments, with deviations in the order of 5 to 10%.

Supplementary Figure 1. Relative leakage of encapsulated calcein in DPPC SUVs as a

function of temperature, normalized to the maximum measured value (corresponding to 35 ° C). Assays were carried out in triplicate, the values in the graph correspond to mean ± SD.

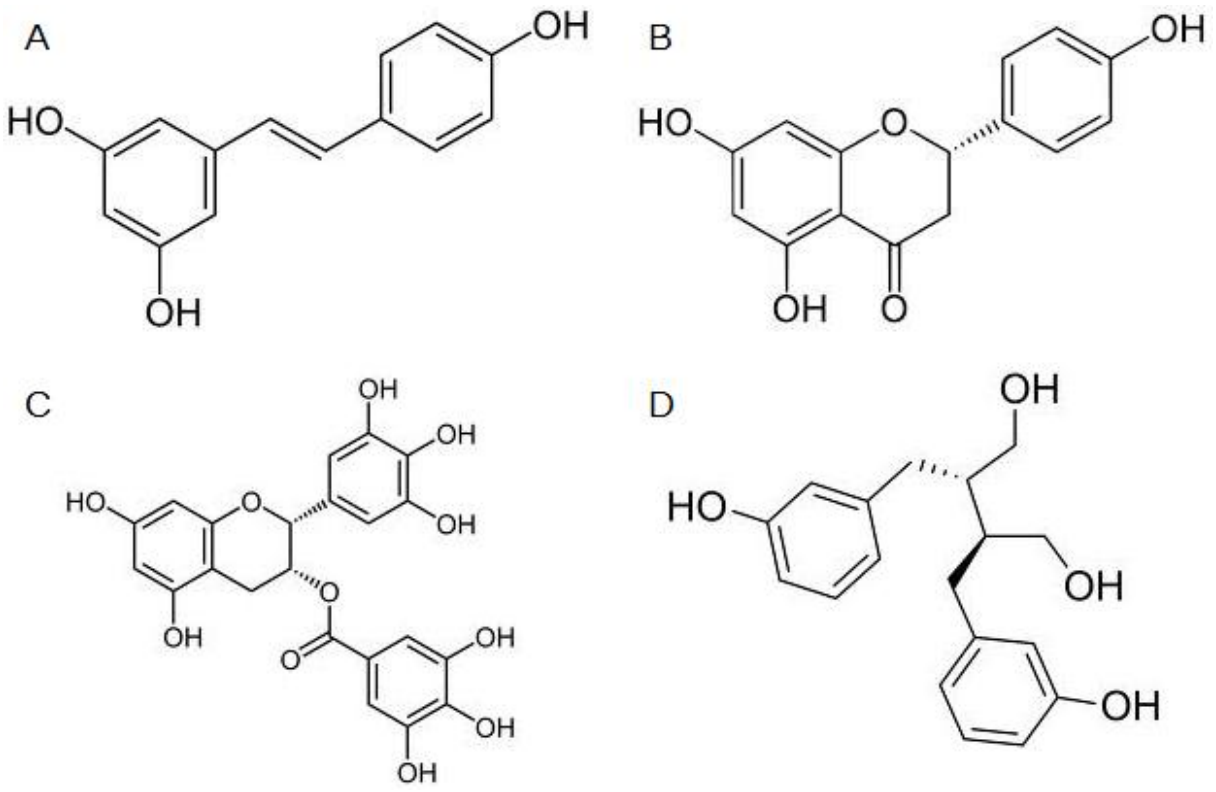


Fig. 1

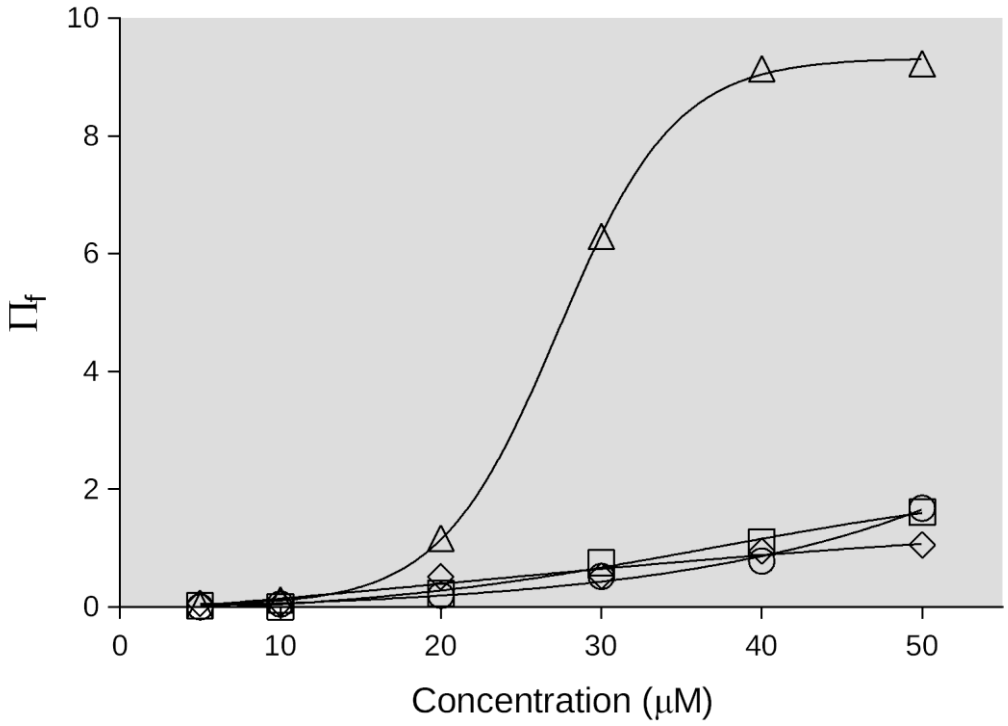


Fig. 2

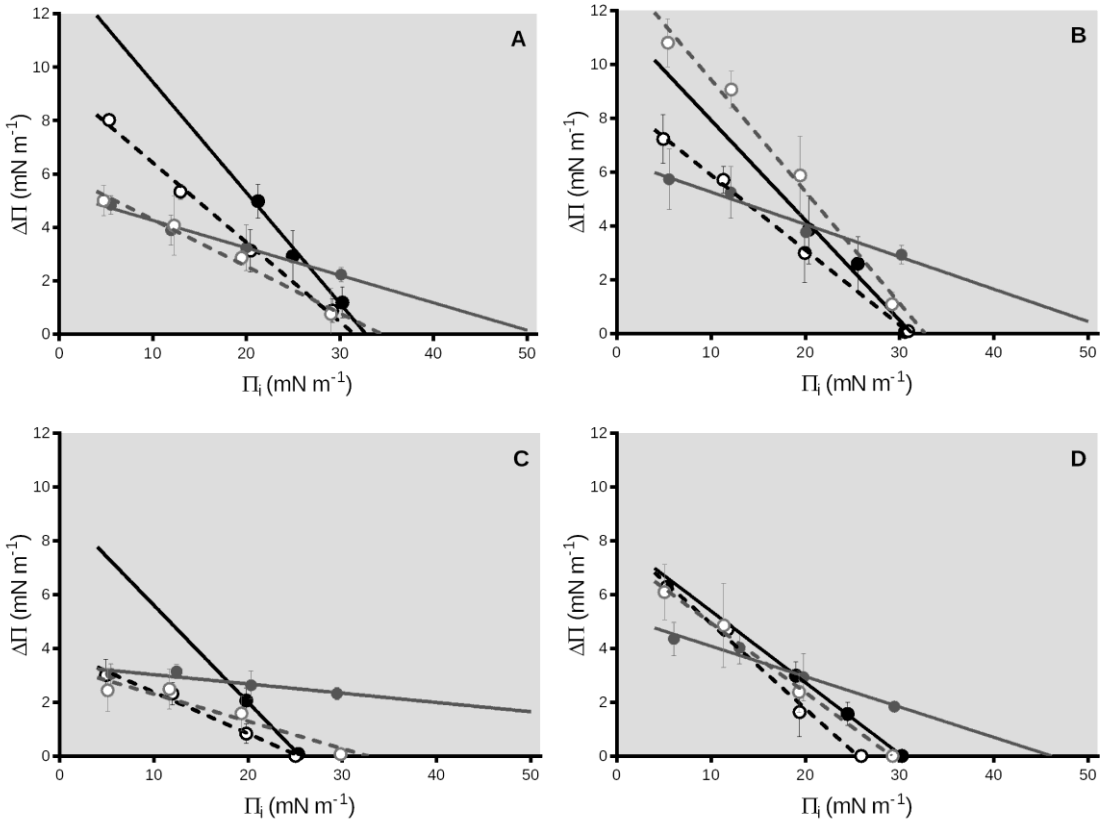

A
B
C
D
ΔΠ (mN m-1)
Πi (mN m-1)

Fig. 3

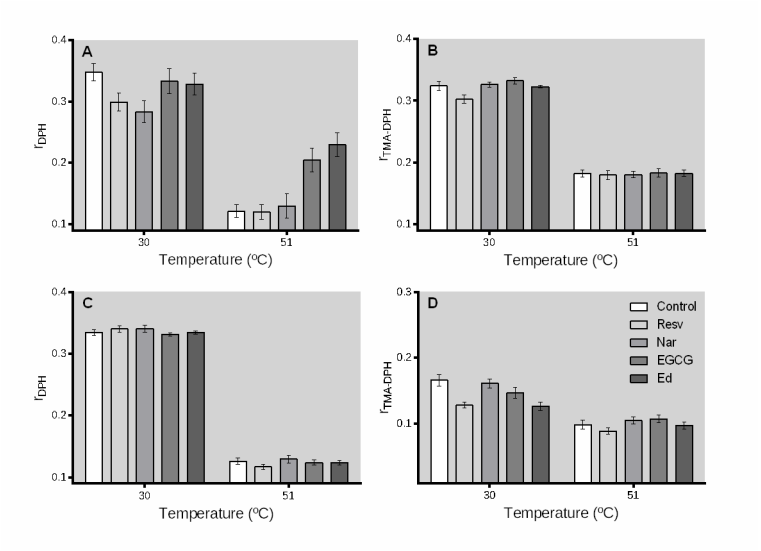

A
B
C
D
rDPH
rTMA-DPH
Temperature (ºC)
Control
Resv
Nar
EGCG
Ed

Fig. 4

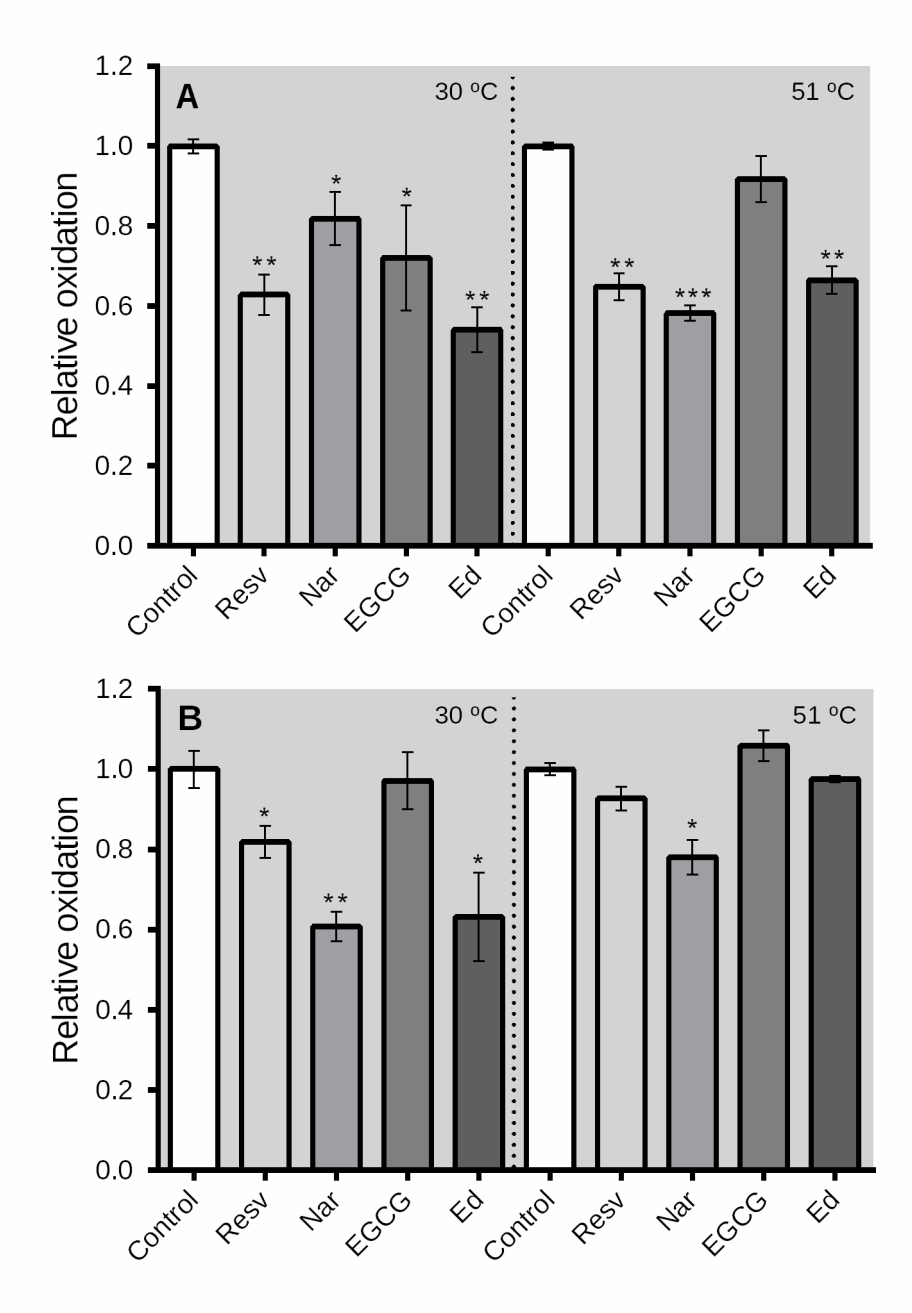

A
30 °C
51 °C
Relative oxidation
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Resv
Nar
EGCG
Ed
B
30 °C
51 °C
Relative oxidation

Fig. 5

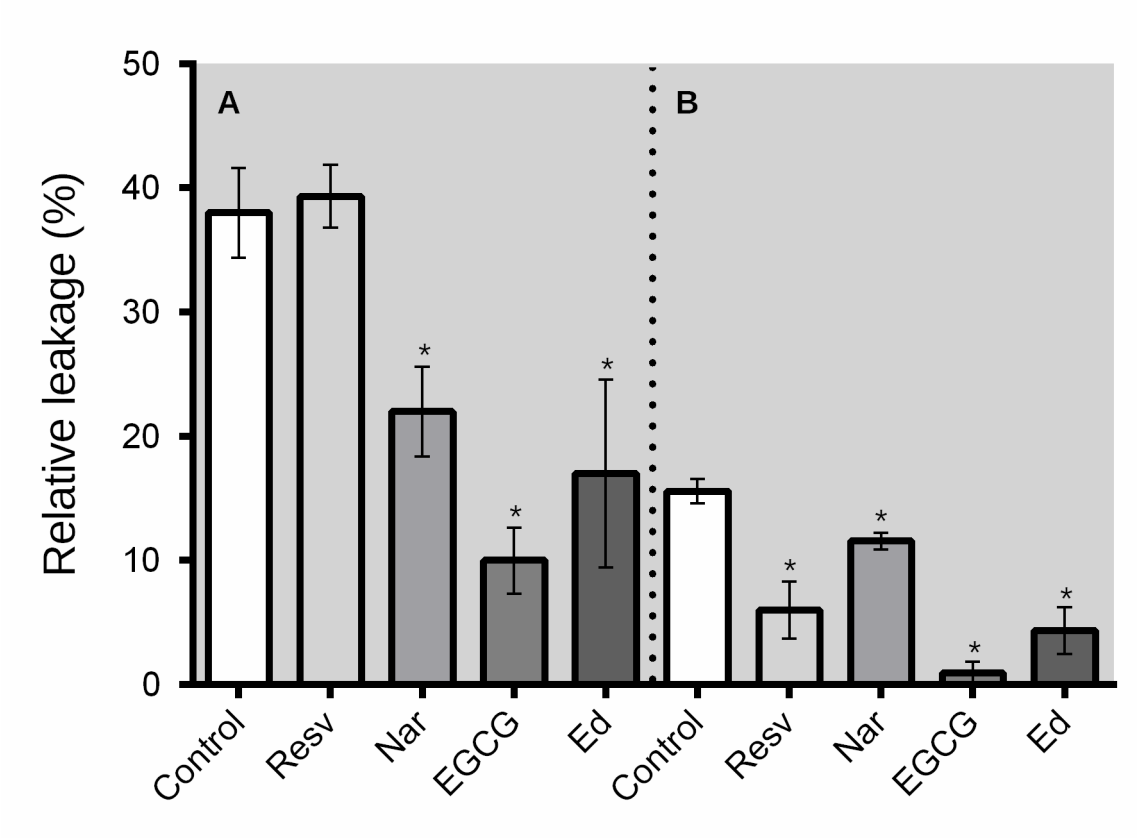

Relative leakage (%)
50
40
30
20
10
0
A
B
*
Control
Resv
Nar
EGCG
Ed
Control
Resv
Nar
EGCG
Ed

Fig. 6

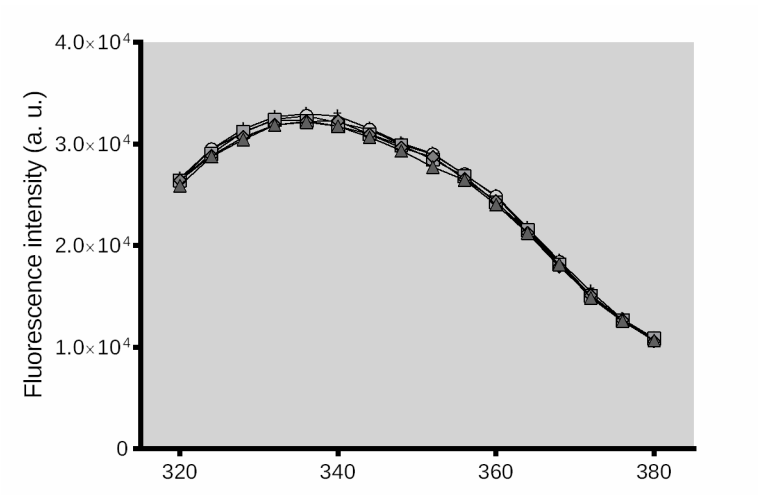
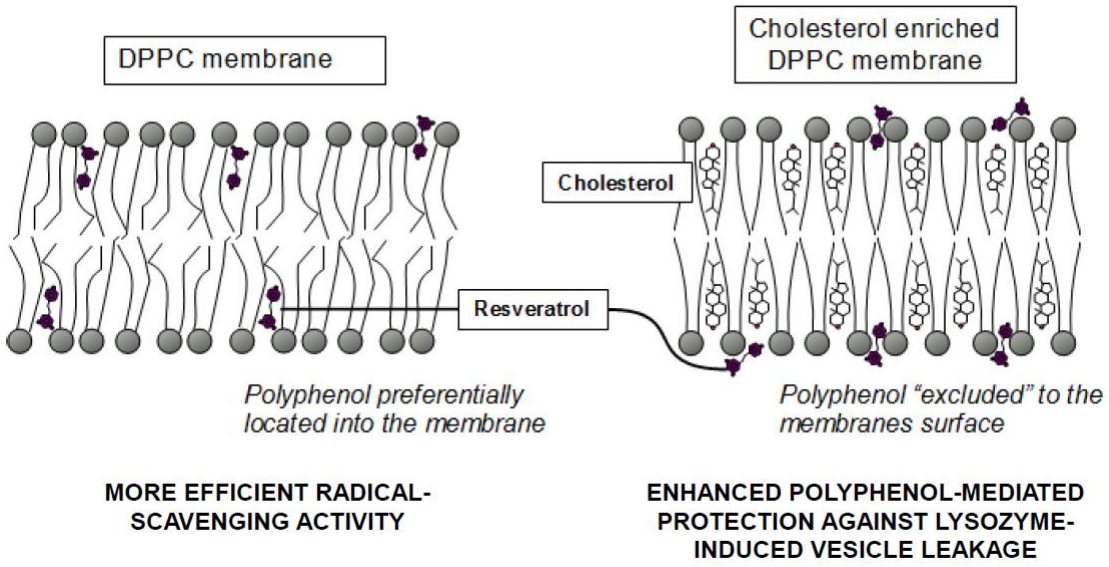

Fluorescence intensity (a. u.)
4.0×10⁴
3.0×10⁴
2.0×10⁴
1.0×10⁴
0
320
340
360
380

Fig. 7



Graphical Abstract

	DPPC		DPPC:CH 6:4	
	30° C	**51° C**	**30° C**	**51° C**
Resveratrol	45,2%	39,7%	2,8%	1,4%
Naringenin	56,4%	60,0%	12,7%	12,3%
EGCG	64,3%	58,7%	18,4%	1,7%
Enterodiol	60,0%	19,4%	20,0%	5,5%

Highlights

1. The four polyphenols in study favorably interacted with lipid mono and bilayers.
2. Cholesterol prevented a deep penetration of phenolic compounds into membranes.
3. Polyphenol proximity to reactive bis-allylic groups improved antioxidant efficiency.
4. Surface localization enhanced protection against lysozyme-induced membrane leakage.
5. Scavenging activity: Nar>Resv~Ed>EGCG; bilayer stabilization: EGCG>Nar~Ed>Resv.