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# Membrane effects of Cocoa Procyanidins in Liposomes and Jurkat T Cells

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**ABSTRACT**

We investigated the effects of the interaction between flavanols and related procyanidins (dimer to hexamer) with both cell and synthetic membranes, on bilayer fluidity and susceptibility to oxidation. Cocoa derived dimers (0.05 to 1 μg/ml) protected Jurkat T cells from AMVN-mediated oxidation and increased plasma membrane fluidity. These effects occurred in a concentration- and chain length-dependent manner. In liposomes, procyanidins prevented the $Fe^{2+}$-induced permeabilization of the membrane. Together, these results support the hypothesis that procyanidins could interact with the polar headgroup of lipids, increasing membrane fluidity and also, preventing the access of molecules that could affect membrane integrity.

**Key words:** Epicatechin, free radicals, lipid oxidation, membrane fluidity, membrane permeability, procyanidins.

**Abbreviations:** AMVN: 2,2'-azobis (2,4-dimethylvaleronitrile); $C_{11}$-BODIPY: 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid; CF: 5(6)-carboxyfluorescein; DCDCDHF: 5(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate; DPH: 1,6-diphenyl-1,3,5-hexatriene.

## INTRODUCTION

The consumption of plant-derived food has been associated with a reduction in the incidence of vascular disease and certain cancers (Steinmetz and Potter, 1996; Joshipura et al., 2001; Joffe and Robertson, 2001). The beneficial effects of some plants on human health have been attributed, in part, to the antioxidant action of a group of polyphenolic compounds, the flavonoids. Cocoa is particularly rich in a class of flavonoids, the flavan-3-ols, i.e. (+)-catechin and (-)-epicatechin, and in their derived oligomers, the procyanidins (Porter et al., 1991; Adamson et al., 1999; Lazarus et al., 1999) (Fig 1).

Among other properties, flavanols and procyanidins display antioxidant activity in both chemical and biological systems (Lotito and Fraga, 1998; Lotito et al., 2000; Rice-Evans, 2001). This antioxidant activity is usually explained based on the chemical structure of flavanols and procyanidins, which allows both free radical trapping and chelation of redox-active metals. Recently, we demonstrated that flavonoids and procyanidins can interact with lipids forming membranes through the adsorption to the polar lipids headgroup preventing oxidation (Verstraeten et al., 2003). Furthermore, we have shown that direct flavonoid-molecule interactions with DNA (Ottaviani et al., 2002) and certain proteins (Actis-Goretta et al., 2003; Mackenzie et al., 2004) can protect these macromolecules from oxidation at concentrations that can be physiologically relevant.

In the present work we investigated whether the adsorption of procyanidins to the membrane surface could lead to the alteration of two membrane physical properties: fluidity and permeability. We

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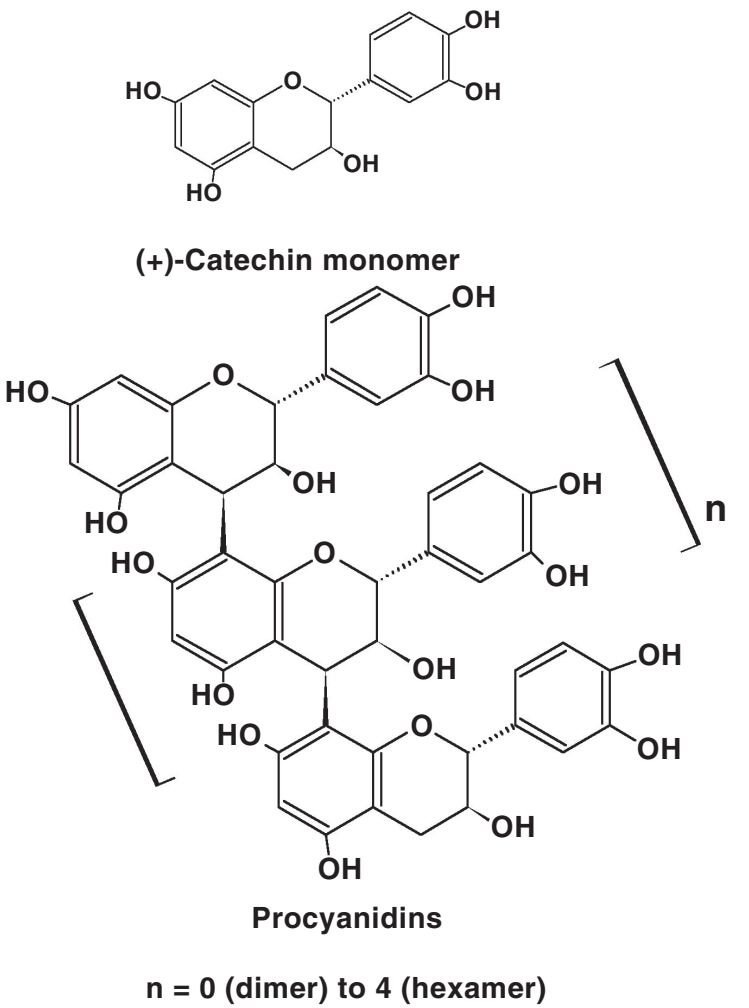


**Figure 1.** Structure of catechin monomers and B type-procyanidins.

first investigated the ability of procyanidins to prevent AMVN-mediated oxidative damage in Jurkat T cells and to affect cell membrane fluidity. Finally, we characterized the effect of these oligomers on liposome membrane permeability both before and after membrane liposome oxidation with ferrous iron. Results indicate that after the adsorption of procyanidins to the plasma membrane, it becomes more fluid, effect that per se limits the propagation of lipid oxidation. In addition, procyanidins prevent the leakage of small molecules from vesicles, and help to maintain membrane integrity.

## METHODS

### *Materials*

Purified procyanidins (monomer to hexamer) from Cocoapro cocoa were purified and supplied by Mars Inc. (Hackettstown, NJ, USA). The purity of the fractions was 98.8, 99.0, 94.8, 95.4, 92.0, and 86.2 % for catechin monomers, dimers, trimers, tetramers, pentamers, and hexamer fractions, respectively. Bovine brain phosphatidyl serine (PS) and phosphatidyl choline (PC) were purchased from Avanti Polar Lipids (Alabaster, AL). The fluorescent probes 4,4-

difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid ($C_{11}$-BODIPY), 1,6-diphenyl-1,3,5-hexatriene (DPH), 5(6)-carboxyfluorescein (CF), and 5(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCDCDHF) were obtained from Molecular Probes Inc. (Eugene, OR). 2,2'-Azobis (2,4-dimethylvaleronitrile) (AMVN) was purchased from Polysciences Inc. (Warrington, PA). Jurkat T cells (human leukemia T cells) were obtained from the American Cell Type Culture Collection (Rockville, MD).

*Liposome preparation*

Phospholipids dissolved in chloroform were 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 mM phospholipids) in a 20 mM Tris buffer (pH 7.4) containing 140 mM NaCl, vortexed for 1 min, and incubated at 45°C for 10 min. Small vesicles were obtained by three cycles of 45 s sonication in a Branson 250 sonifier (Branson Ultrasonics Corp., Danbury, CT) at 80 W.

*Evaluation of cell oxidation*

Jurkat T cells (6 x $10^4$ cells) were suspended in 0.2 ml phosphate-buffered saline pH 7.4 (PBS) and preincubated for 5 min at 4°C in the presence of a dimers fraction (0.01 to 1 µg/ml). After the addition of 1 mM AMVN, cells were incubated for 60 min at 37°C with continuous shaking. Following a brief centrifugation, cells were incubated with 10 µM of DCDCDHF for 30 min at 37°C (Oteiza et al., 2000). Cells were centrifuged, and the pellet was suspended in PBS containing 0.1 % (v/v) Igepal, and incubated for 30 min at room temperature. The fluorescence of the samples (λ excitation: 475 nm, λ emission: 525 nm) was measured in a Kontron SFM-25 spectrofluorometer (Kontron Instruments SpA, Milan, Italy).

*Evaluation of membrane fluidity*

Jurkat T cells (6 x $10^4$ cells) were suspended in 0.3 µl of a 50 mM Hepes buffer (pH 7.4) containing 125 mM KCl and 3 µl of a 5 mM DPH stock solution in DMSO, and incubated at 37°C for 10 min. After incubation, the procyanidins (0.01 to 1 µg/ml) were added to the cell suspension, and were further incubated at 37°C for 30 min. Cell membrane fluidity was evaluated by the changes in DPH fluorescence polarization (λ excitation: 360 nm, λ emission: 450 nm) as previously described (Verstraeten et al., 2003).

*Evaluation of liposome permeability*

CF (0.1 M) was encapsulated into PC:PS liposomes as described (Verstraeten and Oteiza, 1995). Liposomes (30 µM phospholipids) were incubated for 5 min at 37°C in the presence of procyanidins (monomer to hexamer; at a concentration of 7.5 µg/ml), added with 25 µM ferrous iron, and incubated at 37°C for 90 min. Membrane permeability was evaluated based on a change in fluorescence as a function of the release of the probe into the medium (λ excitation: 490 nm, λ emission: 550 nm).

*Statistics*

One-way ANOVA followed by Dunnett's multiple comparison test and correlations were performed using routines available in StatView 5.0 (SAS Institute Inc., Cary, NC).

## RESULTS

*Evaluation of AMVN-mediated oxidation*

The capacity of the dimer fraction to inhibit AMVN-induced increase in cell oxidants was measured in Jurkat T cells. Similarly to that previously observed in liposomes (Verstraeten et al., 2003), the dimer fraction caused a marked inhibition of DCDCDHF fluorescence (Fig 2). The protective effect of the dimer depended on its concentration in the preincubation media, and was significant ($p < 0.01$) at concentrations as low as 0.05 µg/ml (86.1 nM).

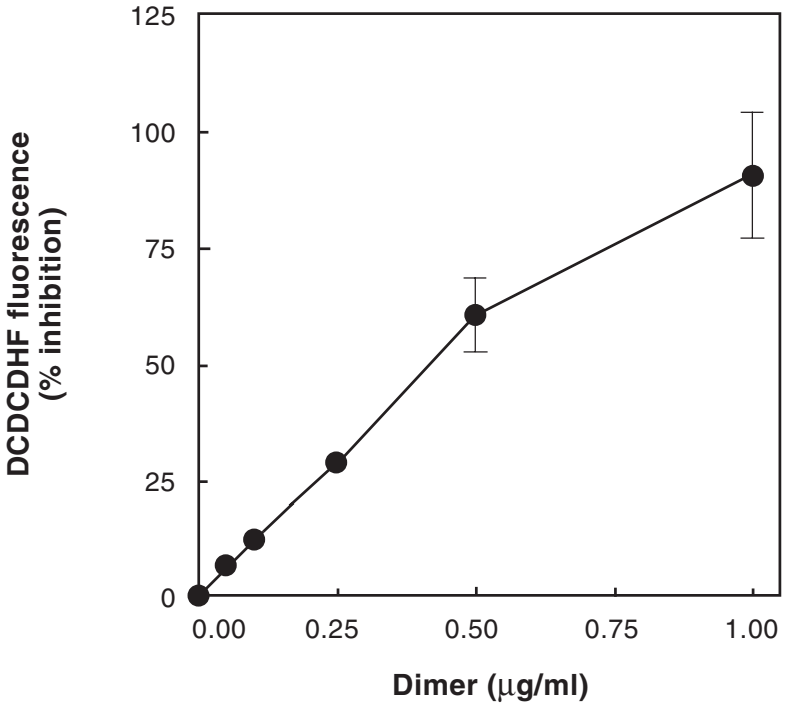


**Figure 2.** Effect of procyanidins on AMVN-mediated cell oxidation.
Jurkat T cells (6 x $10^4$ cells) were preincubated for 5 min at 4 °C in the absence or in the presence of the dimer fraction (0.01 to 1 μg/ml), and further incubated for 60 min in the presence of 1 mM AMVN. Intracellular levels of oxidant species were evaluated from the increase in DCDCDHF fluorescence, as described under Methods. Values are mean ± SEM of three independent experiments.

*Evaluation of membrane physical properties: membrane fluidity and permeability*

The capacity of the dimer and other procyanidin fractions to alter cell plasma membrane fluidity was investigated in Jurkat T cells. As depicted in Figure 3A, the dimer fraction caused a significant decrease in DPH fluorescence polarization, indicative of a higher membrane fluidity. The effect of the dimer on membrane fluidity depended on its concentration in the incubation media, and was significant ($p < 0.01$) at the lowest concentration tested (0.05 μg/ml). Similarly to the effect observed for the dimer fraction, the other procyanidin fractions, when evaluated at the same concentration (1 μg/ml), caused a significant increase in DPH fluorescence polarization. The magnitude of the effect on membrane fluidity was associated with the number of monomer units forming the procyanidin (Fig 3B).

To investigate whether procyanidins can prevent the ferrous iron-mediated increase in membrane permeability, we used PC:PS liposomes containing the probe CF that were preincubated at 37°C for 5 min in the presence of the procyanidin fractions at 7.5 μg/ml concentration. Procyanidins did not induce per se the leakage of the probe (data not shown). After the preincubation, lipid damage was triggered by the addition of 25 μM ferrous iron, and incubation at 37°C. In the absence of procyanidin, the addition of ferrous iron caused a time- and concentration-dependent release of the entrapped probe (Insert to Fig 4). When the hexamer fraction was assayed, the release of CF was prevented ($p < 0.01$) (Insert to Fig 4). This protective effect was also produced by preincubation in the presence of the other procyanidins. An association between the release of CF and the number of monomer units forming each procyanidin was observed, increasing the protective effect with the number of monomer units in the molecule (Fig 4).

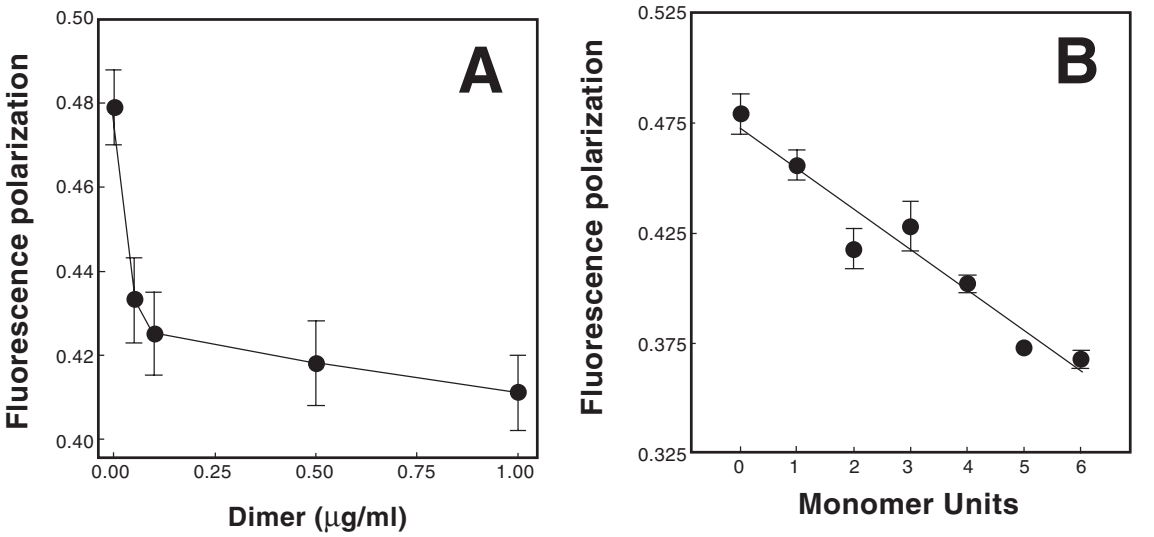


**Figure 3.** Effect of procyanidins on Jurkat T cells plasma membrane fluidity.
Jurkat T cells (6 x $10^4$ cells) containing 15 nmol DPH, were incubated at 37°C in the presence of procyanidins (monomer to hexamer), and plasma membrane fluidity was evaluated after 30 min of incubation. (A) Effect of the dimer fraction (0.01 to 1 μg/ml) on DPH fluorescence polarization. (B) Association between the fluorescence polarization of the probe and the number of monomer units present in the procyanidins at 1 μg/ml concentration. Values are mean ± SEM of four independent experiments.

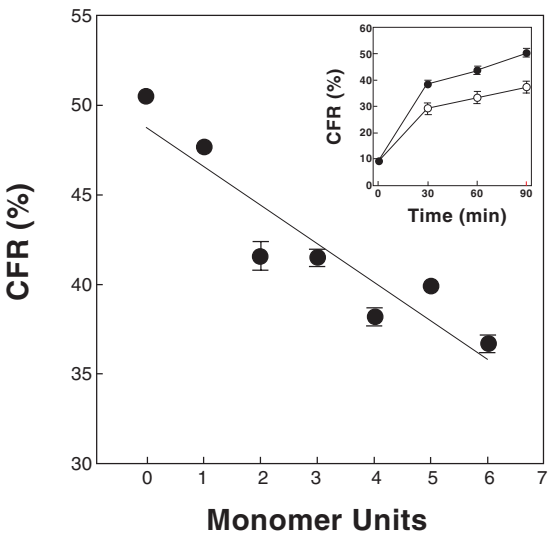


**Figure 4.** Effect of procyanidins on $Fe^{2+}$-mediated membrane permeabilization.
PC:PS liposomes containing the fluorescent probe CF encapsulated in self-quenching conditions (0.1 M) were incubated for 5 min at 37°C in the absence or presence of procyanidins (monomer to hexamer; 7.5 μg/ml). After incubation, lipid oxidation was triggered by addition of 25 μM $Fe^{2+}$. Membrane permeability was evaluated from the release of the probe (CFR) after 90 min of incubation. Results are shown as mean ± SEM of four independent experiments.
*Insert*: Kinetics of CF release. Liposomes were incubated in the absence ( ● ) or presence of the hexamer fraction (◯), and CF release was started by addition of 25 μM $Fe^{2+}$.

## DISCUSSION

Flavanols and procyanidins are rather water soluble molecules that can interact with the polar headgroups of phospholipids forming membranes. As a consequence of these interactions, we propose that procyanidins could limit the incorporation of certain deleterious molecules that could affect membrane integrity and function.

In previous studies, we demonstrated that flavanols and procyanidins act as antioxidants protecting liposomes against different oxidants (Lotito et al., 2000). The protective effect of procyanidins showed a dependence on the procyanidin chain length that was related to the type of oxidant used (Lotito et al., 2000). Using a pro-oxidant that generates radicals in the lipid phase (AMVN), we observed that the antioxidant action of the procyanidins in liposomes was positively associated to the number of monomer units (Verstraeten et al., 2003). Consistently, we observed that in Jurkat T cells the dimer fraction decreased the AMVN-dependent accumulation of intracellular oxidants. Then, it is possible to conclude that the protective effect of procyanidins not only occurs in syntethic membranes, but also in biological ones. This protection could be associated with either the incorporation of dimers or other procyanidins into the cells (Mackenzie et al., 2004) or to an adsorption on the cell surface. Given the short time of cell preincubation in the presence of the dimer, it is quite possible that the adsorption of the procyanidin to the cell surface is the prevalent mechanism.

The interaction between the phospholipid headgroups and the procyanidins was associated with a lower incorporation of hydrophobic molecules into the lipid bilayer. The fact that the protective effect of procyanidins was correlated with the number of monomer units present in the oligomers indicated that not only the total amount of monomer units, but also the tridimensional structure of the molecule and the monomers' disposition on the membrane contributed to the procyanidin-membrane interactions.

The possibility that the interaction of the procyanidins with cell membrane components could result in a variation of membrane fluidity was investigated. It is accepted that, when membranes become less fluid, they are more prone to be oxidized due to potential increases in the propagation rate. Conversely, an increase in membrane fluidity leads to a lower extent of lipid oxidation (Cervato et al., 1988; McLean and Hagaman, 1992; Verstraeten et al., 1997). For the experiments presented in this work, we used the fluorescent probe DPH. Based on the fact that this molecule is evenly distributed in the bilayer, it responds to global changes in membrane fluidity by altering its rotational motion and, hence, its fluorescence. When Jurkat T cells were incubated in the presence of the different procyanidin fractions, a significant increase in membrane fluidity was observed. By contrast, in PC:PS liposomes, flavanols and procyanidins (dimer to hexamer) did not affect the fluidity of the bilayer (Verstraeten et al., 2003). The finding of a higher fluidity in cell plasma membrane due to procyanidins suggests that this property is not a consequence of the interaction procyanidin/lipid, but of a more complex interaction, that could involve proteins. It is also possible that the physiological characteristics of the cell membranes allow a different interaction with the procyanidins. It is worthwhile to note that *in vivo*, the alteration of membrane fluidity could not only result in a decrease of lipid oxidation rates, but it could also modulate the functionality of membrane-associated enzymes (Davis et al., 1976; Muriel and Sandoval, 2000; Whiting et al., 2000), the intracellular transport (Eze and McElhaney, 1987; Tomassoni et al., 1999), and membrane receptors (Conforti et al., 1990; Kuo et al., 1990).

Concerning the physiological relevance of these procyanidin-cell interactions in humans, it is important to stress that both the monomer and dimer fractions were detected in blood after the consumption of a food rich in flavonoids (Rein et al., 2000; Wang et al., 2000; Holt et al., 2002). This presence makes feasible an interaction of the monomers and dimers with circulating cells. On the contrary, considering that the bioavailability of higher molecular weight procyanidins is negligible in plasma, their

effect could be relevant in the oral cavity and the upper gastrointestinal tract. A pharmacological action of higher molecular weight procyanidins is also possible and it needs further research.

As a result of lipid oxidation, the geometry of lipids could be affected, leading to the formation of unspecific pores that allow the leakage of certain molecules. The possibility that the procyanidins could reduce the increased permeability of the bilayer due to lipid oxidation was investigated using liposomes oxidized with AMVN or ferrous iron. When AMVN was used, the total release of CF was achieved almost immediately after its addition, even at low AMVN concentrations. Thus, the oxidation that was triggered using ferrous iron as an initiator led to a time-dependent release of the probe. The preincubation in the presence of procyanidins significantly reduced the effect of ferrous iron on liposome permeability. The finding of a protective effect of the procyanidins on membrane permeability by lipid oxidation, is in agreement with a previous report from Zhu et al. (2002), showing a decrease in free radical-mediated hemolysis in erythrocytes from rats fed with a procyanidin-rich meal. Taken together, in addition to the mechanisms currently accepted for the protective effects of procyanidins (free radical trapping and metal chelation), these compounds could interact with membranes at two other levels (Fig 5): a) increasing membrane fluidity, and thus reducing the rate of lipid oxidation; and/or b) limiting the access of certain molecules that need to reach the hydrophobic region of the membrane to exert their deleterious effects. This mechanism could be mediated by the hydrogen bonding to the polar lipids headgroup, as well as to proteins located at the cell surface. Through these kinds of interactions we suggest that procyanidins help maintain the integrity of the membranes, preventing the access of hydrophobic molecules, including those that affect membrane rheology and/or induce oxidative damage to cell components.

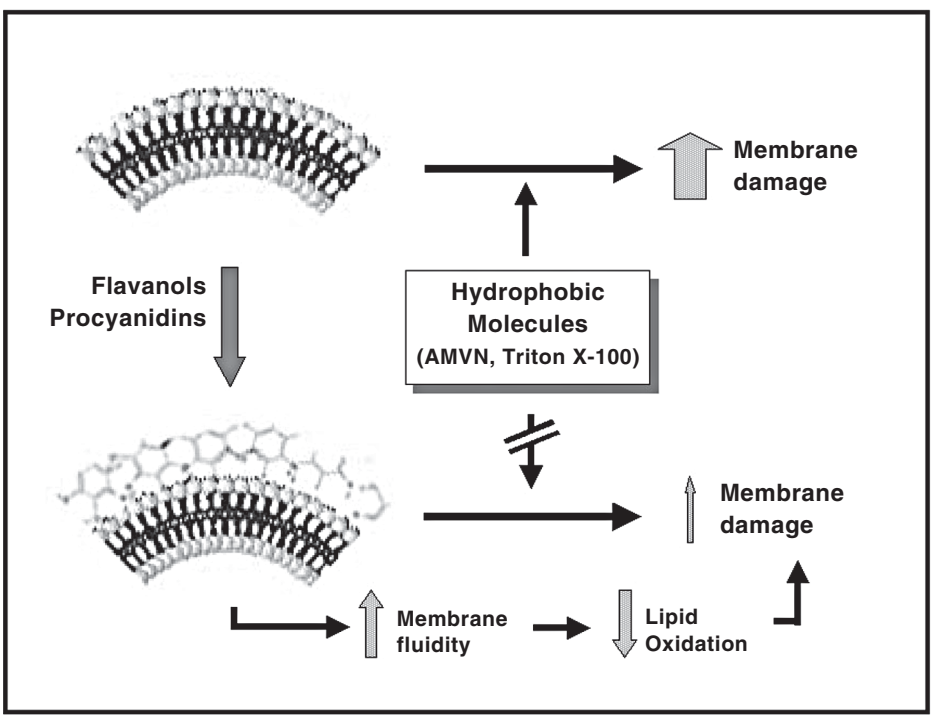


**Figure 5.** Membrane-procyanidins interaction and changes in membrane damage.

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