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Relationship between the Antioxidant Capacity and Effect of Rosemary (*Rosmarinus officinalis* L.) Polyphenols on Membrane Phospholipid Order

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The antioxidant activity of rosemary (*Rosmarinus officinalis* L.) extracts is mainly due to phenolic abietane diterpenes and phenolic acids such as rosmarinic acid. In this study a comprehensive characterization of non-water-soluble and water-soluble extracts from rosemary was achieved by liquid chromatography coupled to electrospray and mass spectrometry. The antioxidant activity of these extracts and their respective major compounds (carnosic acid, carnosol, rosmadial, genkwanin, and rosmarinic acid) was analyzed and compared by using different in vitro systems. Whereas rosmarinic acid, carnosic acid, and carnosol exhibited similar antioxidant activity in a phospholipid membrane-free assay, carnosol behaved as an extremely potent antioxidant in a membrane-based assay (4−6 times stronger than the rest of the compounds). This differential antioxidant behavior suggests that factors other than the radical scavenging capability may be involved. All of the diterpenes induced severe effects on lipid order and packing of phospholipid model membranes. Rosmadial and carnosol decreased the number and/or mobility of water molecules located at the polar head group region of the membrane phospholipids as seen by Laurdan fluorescence spectroscopy. Carnosol also strongly enhanced lipid order at the hydrophobic core of the membrane. These effects throughout the bilayer correlated to the stronger antioxidant capacity of carnosol to inhibit lipid peroxidation. On the contrary, carnosic acid decreased membrane fluidity at deeper regions of the bilayer as measured by bilayer-to-micelle transition assay and self-quenching measurements by using octadecylrhodamine B. These effects may contribute to membrane stabilization and hindrance of radical propagation, which may cooperate with the electron donor ability of rosemary diterpenes in protecting the membranes against oxidative damage.

KEYWORDS: Antioxidant activity; TEAC; TBARS; rosemary; membrane fluidity; fluorescence polarization; diterpenes; carnosic acid; carnosol; rosmarinic acid; phospholipid membranes

INTRODUCTION

Increasing oxidative stress status of plasma and body tissues induced by reactive oxygen species (ROS) sometimes overcomes the endogenous antioxidant defense mechanisms of cells. ROS are generated both from endogenous sources, such as enzymatic activity, and from external stimuli such as UV radiation or xenobiotic exposure. An imbalance between pro-oxidant stimuli and antioxidant defenses may cause oxidative injury of biological molecules because it may be responsible for lipid peroxidation or protein and DNA modification (*1*, *2*). Some dietary phytochemicals such as carotenoids, α-tocopherol, or ascorbate are potent antioxidants that protect cells from oxidative damage and, therefore, considerable interest in naturally occurring antioxidants has emerged in the past 10 years. Among the herbal extracts reported to have antioxidative activity, rosemary is one of the most widely used and commercialized plant extracts not only as a culinary herb for flavoring but also as an antioxidant in processed foods and cosmetics (*3*, *4*).

Several studies have identified the compounds that are primarily responsible for the antioxidant properties of rosemary extracts both in lipophilic and in hydrophilic fractions obtained through several extraction methods (*5*, *6*) (**Figure 1**). The antioxidant activity of these extracts is due to the content of phenolic abietane diterpenes such as carnosic acid and its derivatives, carnosol, rosmadial, rosmanol, rosmanol isomers, and methyl carnosate. Moreover, the occurrence of other phenolic compounds such as flavonoids and phenolic acids, especially rosmarinic acid, also contributes to the bioactivity of this aromatic plant (*7*, *8*).

Some authors have proposed two different mechanisms for the antioxidant capacity of polyphenols (*9*, *10*). One is related to the antiradical scavenging capacity of compounds against superoxide ($^{\bullet-}O_2$) and hydroxyl ($^{\bullet}OH$) radicals, whereas the second one is assigned to the scavenging of lipoperoxyl ($ROO^{\bullet}$) radicals within the membrane environment, the latter being responsible for

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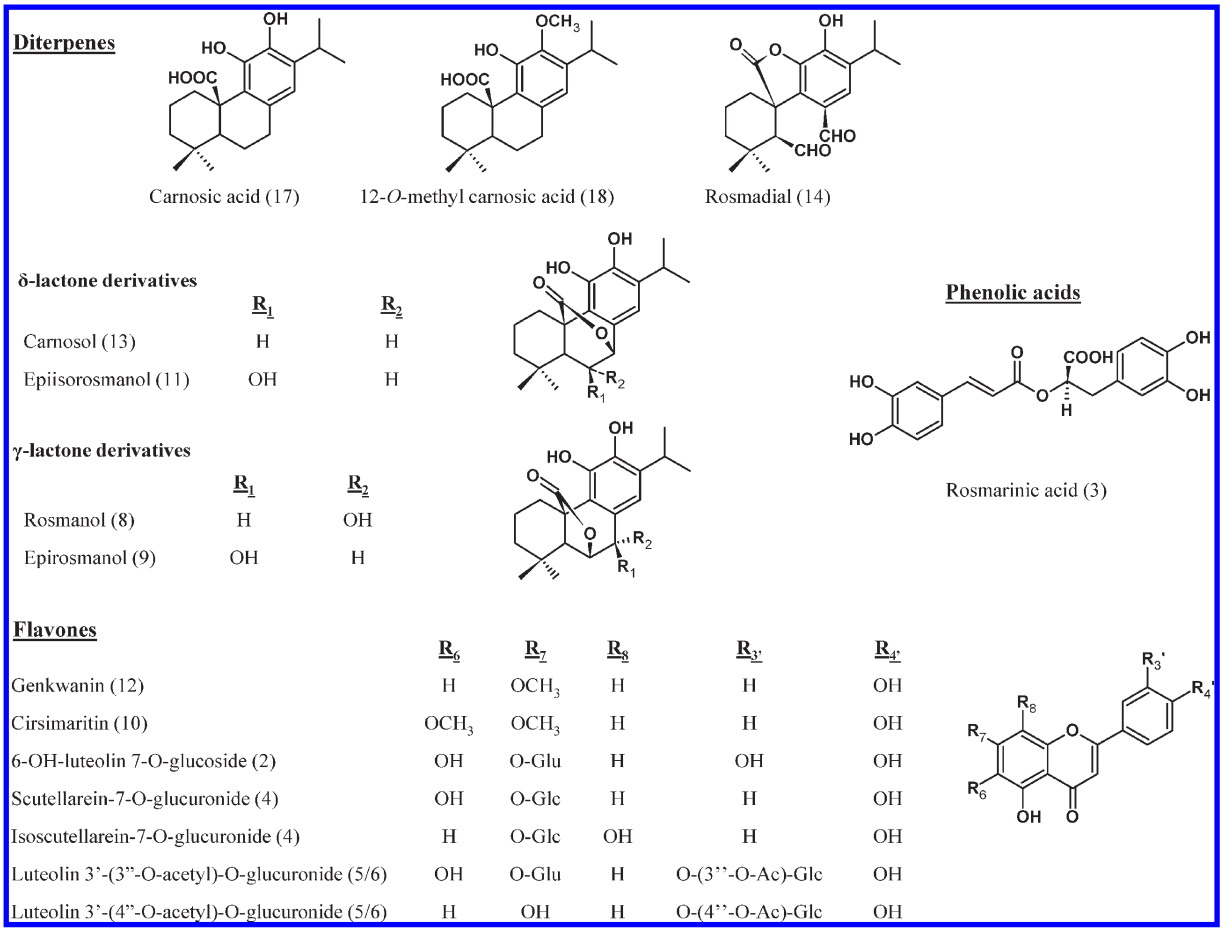

Figure 1. Chemical structures of the major phenolic compounds present in rosemary leaf extract.

chromosomal damage, lysosomal secretion, and inflammatory cell response.

Studies carried out using synthetic or naturally occurring phenolic compounds, either in model membranes or in cellular systems (*11*−*15*), have shown that membrane peroxidative damage could be prevented by lipophilic compounds having membrane-stabilizing effects. The preference of rosemary diterpenoids for lipophilic phases has been reported (*6*, *16*), suggesting that biological membranes might be one of their main targets, where they accumulate and exert their biological activity by altering physical membrane traits. We have previously shown that rosemary abietane diterpenes (*16*) as well as other plant compounds such as the diterpenoids, totarol and abietic acid (*17*, *18*), the galloylated catechins (*13*), anthraquinones (*19*), oleuropein (*20*), or the stilbene resveratrol (*21*) promoted significant perturbations of phospholipid membranes, which contribute to their biological effects. Therefore, the protective role of rosemary antioxidant compounds against peroxidative damage induced by free radicals could also be related to a more complex and membrane-related mechanism underlying their biological activity.

With regard to the above-mentioned, there were two main aims of this study: first, to determine the composition and to evaluate the antioxidant activity of two rosemary extracts (one hydrophilic and another hydrophobic) and the major compounds from these extracts (**Figure 1**) both in a membrane-free and in a membrane-based system; second, to study the perturbation of membrane physical properties by these molecules in terms of lipid order and membrane packing by means of several fluorescence methods including steady state anisotropy of DPH probes, self-quenching properties of octadecylrhodamine B (C18-Rh), and generalized polarization of Laurdan.

MATERIALS AND METHODS

Reagents. Synthetic lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and L-α-phosphatidylcholine tissue-derived from egg yolk (EYPC) were obtained from Avanti Polar Lipids (Birmingham, AL). Stock solutions were prepared in chloroform/methanol (1:1) and stored at −20 °C. Sigma-Aldrich Corp. (St. Louis, MO) supplied the following reagents for antioxidant assays: 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 1,1,3,3-tetraethoxypropane (TEP), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), thiobarbituric acid (TBA), and butylated hydroxytoluene (2,[6]-di-*tert*-butyl-*p*-cresol) (BHT). Molecular Probes (Eugene, OR) supplied the fluorescent probes 3-(4-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (PA-DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), octadecylrhodamine B chloride (C18-Rh), and 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan). Stock solutions of DPH probes and Laurdan were prepared in *N*,*N*′-dimethylformamide (Merck KGaA, Darmstadt, Germany), whereas C18-Rh stock was diluted in ethanol. All fluorescent probes stock solutions were stored at −20 °C in the dark. Water-soluble and non-water-soluble commercial powdered extracts of rosemary leaves (*Rosmarinus officinalis* L.) were kindly provided by Monteloeder, S.L. (Elche, Spain). The approximate content of the major compounds (w/w) determined by HPLC-DAD in the extracts used were 8% of rosmarinic acid in the water-soluble extract and 10% carnosol, 5% carnosic acid, 6% rosmadial, and 6% genkwanin in the non-water-soluble extract. All other reagents were of analytical, spectroscopic, or chromatographic reagent grade and were obtained from Merck KGaA. Double-distilled water and deionized water were used throughout this work.

Semipreparative HPLC. Solutions of rosemary water-soluble extracts at a concentration of 30 mg/mL were prepared in double-distilled water, centrifuged at room temperature, and filtered through a 0.45 μm nylon filter. Additionally, rosemary lipophilic extracts were dissolved in dimethyl sulfoxide (100 mg/mL final concentration) and prepared as

described for the water-soluble extract. Ten milliliters of each filtered supernatant was injected into a self-packed preparative reverse phase column LiChrospher 100 RP-18, 15 μm, 250 × 25 mm i.d. (Merck KGaA). Preparative elution was performed using a chromatographic method adapted from a previously reported one (*22*): (A) acetonitrile/water/acetic acid (15:84:1) and (B) methanol as a mobile phase and a linear gradient from 0 to 100% of solvent B for 60 min at a flow rate of 39 mL/min. The purification was carried out using a WellChrom preparative HPLC system (Merck-Knauer, Berlin, Germany). Every peak was collected according to its retention time, and the purity of each fraction was checked by analytical HPLC-DAD-ESI-MS/MS as described below. Fractions were considered to contain almost pure compounds (≥95% purity) when these accounted for >95% as determined by diode array and the base peak chromatogram obtained by MS/MS. Fractions containing only pure compounds were pooled, solvent exchanged to pure methanol through the use of semipreparative Prevail C_{18} cartridges (Alltech Associates Inc., IL), and then evaporated to dryness by rotatory evaporation. Last traces of solvent were removed by lyophilization for 48 h, and after that, the purified compound was weighted and subjected to HPLC analysis to check for possible degradation.

HPLC-DAD-ESI-MS/MS Analysis. The LC-MS system consisted of an Agilent LC 1100 series (Agilent Technologies, Inc., Palo Alto, CA) controlled by Chemstation software. The HPLC instrument was coupled to an Esquire 3000+ (Bruker Daltonics, GmbH, Germany) mass spectrometer equipped with an ESI source and ion trap mass analyzer. A Merck LiChrospher 100 RP-18, 5 μm, 125 × 4 mm (i.d.) column with a LiChroCART 4-4 guard column, containing the same stationary phase (Merck KGaA) was used for analytical purposes. The mobile phase was the same as described previously for semipreparative HPLC but using a linear gradient from 0 to 100% B in 30 min, followed by 5 min of isocratic 100% B and returning to initial conditions for column re-equilibration. The flow rate was 1 mL min^{-1}. DAD detection was performed from 200 to 600 nm, and chromatogram traces at 320 and 285 nm were recorded for water-soluble and non-water-soluble compound detection, respectively. The electrospray ionization source was operated in negative mode to generate $[M - H]^-$ ions using the following conditions: desolvation temperature was set at 300 °C; dry gas (nitrogen) and nebulizer were set at 10 L min^{-1} and 60 psi, respectively. MS and MS/MS spectra were acquired over 100−1000 amu at 10 ms, and the capillary voltage was 4.0 kV.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay. The TEAC assay was performed as described elsewhere (*23*). ABTS radical cation ($ABTS^{\bullet+}$) was first produced by incubating ABTS stock solution (7 mM) with 2.45 mM potassium persulfate for 12−24 h at room temperature. For the study of phenolic compounds the $ABTS^{\bullet+}$ solution was diluted with ethanol or water (lipophilic and hydrophilic assay respectively) to an absorbance of 0.70 (±0.02) at 734 nm (approximately 45 μM). For the photometric assay 1 mL of the $ABTS^{\bullet+}$ solution and 100 μL of antioxidant solution were mixed for 45 s and measured immediately after 1 min at 734 nm. A calibration curve was prepared with different concentrations of Trolox (0−10 μM). Results were expressed in millimoles of Trolox per gram of extract or compound on a dry basis.

Thiobarbituric Acid-Reactive Substances (TBARS) Assay. The oxidation of lipid vesicles was induced by a free radical generator (AAPH), and the capacity of rosemary extracts and compounds to inhibit the formation of lipid oxidation products was measured by TBARS assay, as previously described (*24*). Small unilamellar vesicles (SUVs) were prepared by sonication of multilamellar vesicles (MLVs) of egg yolk phosphatidylcholine (EYPC) during six cycles of 30 s on ice (Sonifier 250, Branson Co., CT) and then diluted to a final concentration of 0.76 mM with Tris buffer (140 mM NaCl, 20 mM Tris, pH 7.4). Vesicle solutions containing AAPH but no added antioxidant were used as 100% lipid oxidation control, whereas samples containing just lipid vesicles (no AAPH or antioxidant added) were used as 0% lipid oxidation control. All of the samples were submitted to identical treatment as follows. One milliliter of SUVs solution was incubated for 10 min at 37 °C with rosemary antioxidants, and AAPH was added (10 mM final concentration) afterward to induce the lipid peroxidation reaction. The reaction mixture was incubated at 37 °C for 60 min with vortexing for 15 s every 10 min and quenched by the addition of 200 μL of BHT (4% w/v in ethanol) and freeze−thawing the samples at −80 °C. The colorimetric reaction with thiobarbituric acid was carried out by adding 250 μL of sodium dodecyl sulfate (3% w/v), 500 μL of TBA (1% w/v), and 500 μL of 7 mM HCl to the samples and incubating at 95 °C for 15 min. The TBA reactive species (mainly malondialdehyde) were then extracted with 2 mL of 1-butanol, and the fluorescence was measured in microplate reader FluoStar Galaxy (BMG-Labtechnologies, Offenburg, Germany), using an excitation filter of 500 nm and an emission filter of 530 nm. A calibration curve was performed with different amounts of MDA (0−3.2 nmol) previously obtained by acidic hydrolysis of 1,1′,3,3′-tetraethoxypropane (TEP).

Steady-State Fluorescence Anisotropy. The steady-state fluorescence anisotropy, $\langle r \rangle$, of the probes was measured in an SLM-8000C spectrofluorometer as previously described (*13*). Chloroform/methanol solutions containing 0.1 mM total phospholipid (DMPC) and the appropriate amount of phenolic diterpenes (carnosol, rosmadial, and carnosic acid), the phenolic acid rosmarinic acid, or the flavone genkwanin were dried under a stream of oxygen-free N_2, and the last traces of solvent were removed by keeping the samples under high vacuum for >3 h. MLVs were formed by incubating the dried lipid on 1 mL of buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 mM EDTA buffer) for 15 min, at a temperature above the gel to liquid-crystalline transition, 30 °C, with occasional vortexing followed by three freeze−thaw cycles. Aliquots of DPH anionic and cationic derivatives, that is, PA-DPH and TMA-DPH, respectively, in *N*,*N*′-dimethylformamide were directly added into the lipid dispersion to obtain a probe/lipid molar ratio of 1:500. Samples were incubated for 1 h above the gel to liquid-crystalline phase transition temperature (30 °C), and measurements were taken immediately thereafter. The measurements were taken at a constant rate of 0.25 °C/min over the range from 12 to 40 °C. The samples were stabilized at the desired temperature, and measurements were performed with continuous mixing. The experimental steady-state anisotropy $\langle r \rangle$ was defined by

$$\langle r \rangle = \frac{(I_{VV} - GI_{VH})}{(I_{VV} + 2GI_{VH})} \quad (1)$$

where the *G* factor, accounting for differential polarization sensitivity, was determined by measuring the polarized components of fluorescence of the probe with horizontally polarized excitation ($G = I_{HV}/I_{HH}$). The vertically and horizontally polarized emission intensities, elicited by vertically polarized excitation, were corrected for background scattering by subtracting the corresponding polarized intensities of a phospholipid preparation lacking probes. Samples were excited at 360 nm (slit width, 1 nm), and fluorescence emission was recorded at 430 nm (slit width, 4 nm). The measurements were made with Glan-Thompson calcite prism polarizer (SLM Instruments, Inc., IL), and background intensities due to the lipid vesicles were always taken into account.

Control fluorescence experiments were carried out just by measuring the nonpolarized fluorescence intensity of the probes incorporated in DMPC membranes after the addition of increasing concentrations of rosemary isolated compounds. Fluorescence measurements of four independent replicates for each treatment were performed using an identical system as described above.

Octadecylrhodamine B Self-Quenching. The effect of rosemary antioxidant compounds on membrane resistance to solubilization was measured by monitoring the bilayer-to-micelle transition of DMPC model membranes induced by a nonionic detergent such as Triton X-100 (*25*). Octadecylrhodamine B (C18-Rh) fluorescence is quenched when incorporated into membranes at high concentrations (1−10% molar). However, its fluorescence increases when bilayer-to-micelle transition takes place due to detergent addition.

SUVs of DMPC were employed as model membranes and prepared by sonication of MLVs formed as previously described. The final concentration of phospholipid was adjusted to 0.1 mM, and aliquots of C18-Rh were directly added into the lipid dispersion to a molar ratio of 1.8%. Samples were then incubated for 1 h above the gel to liquid-crystalline phase transition temperature (30 °C). The corresponding amounts (0, 2, 5, 10, and 20% molar) of rosemary isolated compounds were added to the C18-Rh-labeled liposomes suspension, and fluorescence measurements were taken immediately thereafter, adding increasing concentrations of detergent. Stock solutions of Triton X-100 at 1 or 10% (v/v) prepared in distilled water were used for that purpose. The fluorescence increase [IF (%)] of C18-Rh (λ_{exc}, 560 nm; λ_{em}, 580 nm) was expressed as

$$\text{IF (\%)} = (I_i - I_0)/(I_{max} - I_0) \times 100 \quad (2)$$

where I_0 is the initial fluorescence value, I_i is the fluorescence value after each TX-100 addition, and I_{max} corresponds to the maximum dilution of the fluorescent probe (fluorescence plateau). IC_{50} values for TX-100, which are the detergent concentrations needed to complete 50% of the bilayer-to-micelle transition, were obtained by plotting IF (%) versus detergent concentration.

The effect of rosemary compounds on the packing order of membrane phospholipids was also determined by using the self-quenching fluorescence properties of octadecylrhodamine B (*26*). For this purpose, the probe's emission spectrum and fluorescence intensity were recorded after addition of increasing concentrations of rosemary compounds until no changes in fluorescence values were observed. The increase of the fluorescent probe self-quenching was calculated as follows:

$$Q\,(\%) = [(I_{max} - I_i)/I_{max}] \times 100 \quad (3)$$

where I_i is the fluorescence value at each addition of the compound and I_{max} is the fluorescence value when the C18-Rh is completely released after addition of an excess of TX-100. Fluorescence measurements were carried out at 37 °C using a modular spectrofluorometer PTI QuantaMaster model QM-6/2003 SE (Photon Technology International, Inc., NJ). IF (%) and Q (%) results were representative of four independent experiments, and additions of methanol were used as blanks in both assays because stock solutions of rosemary compounds were prepared in this organic solvent.

Generalized Polarization of Laurdan. The steady-state generalized polarization of the fluorescent probe Laurdan shows specific values for the gel and liquid-crystalline phases of phospholipids, and it is independent of the phospholipid polar head, acyl chain composition, or pH ranging from 4 to 10 (*27*). This parameter relies on the spectral shifts that this probe undergoes depending on the chromophore's surrounding polarity and solvent dynamics (*27*). Excitation generalized polarization (GP_{ex}) values are given by

$$GP_{ex} = (I_{440} - I_{480})/(I_{440} + I_{480}) \quad (4)$$

where I_{440} and I_{480} are the fluorescence intensities at 440 and 480 nm, respectively, obtained from the emission scan acquisition (420−550 nm) after excitation at 380 nm.

For this purpose, DMPC multilamellar vesicles (MLVs) model membranes (0.2 mM) containing rosemary compounds at different molar ratios were prepared as described for steady-state anisotropy measurements. Aliquots of Laurdan were directly added into the lipid dispersion to obtain a probe/lipid molar ratio of 1/500. Samples were incubated for 15 min above the gel to liquid-crystalline phase transition temperature (30 °C), and measurements were taken immediately thereafter. GP_{ex} measurements were recorded at temperatures below and above the phospholipid temperature transition, that is, at 5 and 37 °C, respectively.

Statistical Analysis. Curve fitting and plots were performed by using Origin 7.5 software (OriginLab Corp., Northampton, MA). Statistical analysis (average and standard deviation) was performed using the Statistical Package for Social Sciences (SPSS Inc., v. 14.0 for Windows, Chicago, IL). A minimum of four independent replicates were used in every experiment unless otherwise stated. Statistical significance was set at $p < 0.05$, unless explicitly indicated otherwise. All of the data were tested for normality. Student's t test for unpaired data was used to identify significant differences.

RESULTS

Characterization of the Composition of Rosemary Extracts by HPLC-DAD-ESI-MS/MS Analysis. The identification of the major components of both water-soluble and non-water-soluble extracts was carried out by HPLC analysis coupled to a photodiode array detector and mass spectrometer (**Figure 2**; **Tables 1** and **2**). The analysis of the water-soluble extract revealed that its main component was rosmarinic acid because its UV, MS, and MS/MS spectra were in agreement with previous results (*28*, *29*) (**Table 1**). Fragmentation of rosmarinic acid negative parental ion $[M - H]^-$ with m/z at 359 produced the ion fragments at m/z 197 $[M - H -$ caffeic acid$]^-$, 161 $[M - H - 197]^-$, 179 [caffeic acid $- H]^-$, and 135

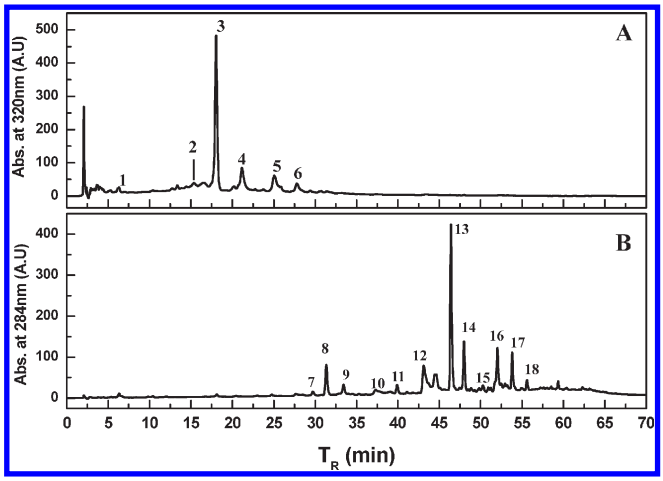

Figure 2. HPLC-DAD chromatogram of water-soluble (**A**) and non-water-soluble (**B**) extracts from rosemary leaves recorded at 320 and 284 nm, respectively.

$[179 - COO]^-$, which is typical of phenolic acids containing caffeoyl moieties in their structures (**Figure 1**). Other minor compounds were also found in the extract, mainly hydroxycinnamic acids and glycosylated flavonoids (**Table 1**). Unknown compounds (UNK) 3, 4, 5, and 8 showed a UV spectrum typical of hydroxycinnamic acids (*29*). With the exception of UNK 3, it was not possible to determine MS and MS/MS spectra of UNK 4, 5, and 8 due to their low concentration in the extract. An m/z difference of 162 from the negative parental ion 521 of UNK 3 showed the presence of hexose in this compound, and the MS/MS spectra of the remaining fragment (m/z 359) showed an identical fragmentation as rosmarinic acid. This allowed identification of UNK 3 as a glycosylated hydroxycinnamic acid with a caffeoyl residue like rosmarinic acid (**Table 1**).

The compound eluting at 21.15 min showed an absorbance spectrum typical of flavonoids with an apigenin-like scaffold that is a flavone with a monohydroxylated B ring (*30*). The mass difference of 176 indicated the presence of a glucuronic acid residue in the molecule. The m/z value of the aglycone parent ion (285) suggested a molecule containing an extra hydroxyl group in the A ring, because an extra OH group in the B ring would change the UV spectrum to a luteolin-like flavone (*30*). Previous studies in rosemary have shown the occurrence of a similar compound, and this was identified as isoscutellarein-7-*O*-glucuronide (OH at position 8) (*31*, *32*). Nonetheless, the compound eluting at 21.15 min could be also identified as scutellarein-7-*O*-glucuronide (OH at position 6), according to its UV and mass spectra. The occurrence of luteolin 3′-(3″-*O*-acetyl)-*O*-glucuronide and its isomer luteolin 3′-(4″-*O*-acetyl)-*O*-glucuronide has been reported in rosemary leaves (*7*). UV spectra and mass of the negative parental ion (m/z 503) of compounds eluting at 25.5 and 27.8 min were in agreement with the spectral properties of these acetyl-glycosylated luteolins. The fragment ions found in the MS/MS spectrum and deriving from the above-mentioned compounds corresponded to the loss of the acetoxyl group $[M - H - 60]^-$, successive fragmentation of acetoxyl and glucuronic acid $[M - H - 218]^-$, and the split of the acetoxyl and carboxylic groups from the sugar moiety $[M - H - 60 - 44]^-$.

Table 2 summarizes the phytochemical composition of the non-water-soluble extract enriched in abietane diterpenes. The MS/MS spectra of rosmanol and epirosmanol showed the fragment ions at m/z 301 and 283, which are consistent with the successive loss of the lactone group $[M - H - COO]^-$ and water $[M - H - COO - H_2O]^-$. Epiisorosmanol also shared a

Table 1. Phenolic Compounds Identified by HPLC-DAD-ESI-MS/MS in the Water-Soluble Extract of Rosemary Leaves[a]

t_R (min)	λ (nm)	$[M - H]^-$	$[MS/MS]^-$	ID compound	ref
3.84	225, 265	431	371, 209	UNK 1	
6.6	240, 295, 325	179	135	caffeic acid (**1**)	*29*
12.73	255, 350			UNK 2	
13.35	289, 320	359	179, 197, 161, 135	UNK 3, tentative identification: glycosylated rosmarinic acid	
		521	(521 = 359 + 162)	$\Delta m/z$ 162 (hexose)	
15.4	221, (256), 274, 346			6-OH-luteolin-7-*O*-glucoside (**2**)	*8*
16.5	290, 329			UNK 4	
18.1	220, 290, 330	359	179, 197,161, 135	**rosmarinic acid** (**3**)	*28*
20.42	292, 326			UNK 5	
21.15	221, (239), 268, 340	461	285	isoscutellarein or scutellarein 7-*O*-glucuronide (**4**)	*31*, *32*
23.04	234, 304			UNK 6	
23.9		347	329, 301, 283	UNK 7	
25.5	220, (240), 269, 339	503	285, 399, 443	luteolin 3′-(3′′-*O*-acetyl)-*O*-glucuronide or luteolin 3′-(4′′-*O*-acetyl)-*O*-glucuronide (**5**)	*7*
25.87	293, 325			UNK 8	
27.8	220, (240), 269, 339	503	285, 443, 399	luteolin 3′-(3′′-*O*-acetyl)-*O*-glucuronide or luteolin 3′-(4′′-*O*-acetyl)-*O*-glucuronide (**6**)	*7*

[a] UNK represents unknown compounds.

Table 2. Phenolic Compounds Identified by HPLC-DAD-ESI-MS/MS in the Non-Water-Soluble Extract of Rosemary Leaves[a]

t_R (min)	λ (nm)	$[M - H]^-$	$[MS/MS]^-$	ID compound	ref
29.73	228, 287, (330)	301		hesperetin (**7**)	*22*
31.4	(206), 221, 285	345	301, 283	rosmanol (**8**)	*8*, *22*
33.5	(207), 224, 289	345	283, 301	epirosmanol (**9**)	*8*, *22*
37.3	218, (231), 275, 336	313	298	cirsimaritin (**10**)	*22*
39.9	209, (230), 289	345	283	epiisorosmanol (**11**)	*8*, *22*
41.08	245, 320	329	285	UNK 1	
43.1	210, (220), 268, 337	283	268	**genkwanin** (**12**)	*22*, *31*, *32*
44.4	245, 295	343	299	UNK 2	
44.6	245, 290	345	301	UNK 3	
	(coelution of UNK 2, 3, and 4)	363	319, 275	UNK 4	
46.4	227, 285	329	285	**carnosol** (**13**)	*8*, *22*
47.99	(203), 234, 289	343	315, 299, 287	**rosmadial** (**14**)	*22*
48.8	206, (269), 426	329	285	6,7-dehydrocarnosic acid (15)	*34*
52.03	203, 279, 410	345	301	7-keto-carnosic acid (**16**)	*34*
53.8	206, (230), 284	331	287	**carnosic acid** (**17**)	*6*
55.6	207, (232), 287	345	301, 286	12-*O*-methylcarnosic acid (**18**)	*6*

[a] UNK represents unknown compounds.

fragment ion at m/z 283. Carnosol and UNK 2 and 3 showed similar fragmentation patterns, which exhibited the loss of the lactone group $[M - H - COO]^-$. The mass difference of 44 detected in the carnosic acid compared to its parental ion was due to the split of the free carboxylic group. The methylated form of carnosic acid, 12-*O*-methylcarnosic acid, showed the successive loss of the carboxylic $[M - H - 44]^-$ and methyl $[M - H - 44 - 15]^-$ groups. All of the abietane diterpenes, rosmanol, epirosmanol, epiisoromanol, carnosol, carnosic acid, and 12-*O*-methylcarnosic acid, showed similar UV spectra with a main peak ranging from 200 and 210 nm, a shoulder at 230 nm, and a second maximum at 285 nm, typical of this class of compounds. These abietane diterpenoids are generated from the oxidation pathway of carnosic acid, which takes place in the plant leaves during the summer season or under water-stress situations (*33*). From the precursor carnosic acid, several abietane diterpenes with γ-lactone function, such as rosmanol and epirosmanol, and δ-lactone function, such as carnosol and epiisorosmanol, are formed (**Figure 1**). Rosmadial was the exception, showing a UV spectrum shifted to higher wavelength (234, 289 nm), and the maximum absorbance wavelengths of UNKs 2, 3, and 4 were also slightly shifted to higher wavelengths. Moreover, the shape of rosmadial's spectrum was broader compared to the rest of the abietane diterpenoids. Two intermediates of the carnosic acid oxidation pathway were also detected in the extract, the 6,7-dehydrocarnosic and 7-keto-carnosic acid (**Table 2**) (*34*). Other hydrophobic compounds such as the methylated flavones genkwanin and cirsimaritin and the flavanone hesperetin were also identified in the non-water-soluble extract according to their spectral features.

Antioxidant Capacity: TEAC and TBARS Assays. The results obtained with a liposome-free-based antioxidant assay (TEAC) indicated that the hydrophilic rosemary extract containing rosmarinic acid showed higher antioxidant activity than diterpene-enriched lipophilic extract (**Table 3**). However, when the antioxidant activities of their respective major components was compared, no significant differences were observed between the water-soluble rosmarinic acid and hydrophobic diterpenes such as carnosic acid and carnosol. The oxidized diterpene rosmadial showed slightly lower antioxidant capacity, whereas the lowest activity was observed for the flavone genkwanin (**Table 3**). No significant differences were observed between the TEAC values determined either in water or in ethanol, although in most cases slightly higher values were obtained in ethanol.

In contrast, the results obtained in a membrane-based antioxidant assay (TBARS) showed that the non-water-soluble extract was more powerful as an antioxidant than the water-soluble extract (**Figure 3A**). Moreover, the hydrophobic diterpene carnosol exhibited the highest antioxidative capacity (**Figure 3B**) as revealed by the IC_{50} values (**Table 3**). Carnosol showed an IC_{50} value almost 4.4 times lower than those of rosmarinic acid and rosmadial and 5.7 times lower than that of carnosic acid. In both

antioxidant assays, TEAC and TBARS, the flavone genkwanin showed the lowest antioxidant activity. With regard to the rosemary extracts, the non-water-soluble extract showed an antioxidant activity almost 4-fold higher than that of the water-soluble extract as measured by TBARS assay, which is in agreement with the behavior observed for their respective major compounds carnosol and rosmarinic acid. These differences in antioxidant behavior between lipophilic and hydrophilic compounds have been also described for other antioxidant polyphenols and in comparative studies between ascorbic acid, β-carotene, and α-tocopherol (*24*, *35*). Moreover, similar differential antioxidant behavior of rosemary water-soluble and non-water-soluble compounds has been previously pointed out by other authors (*9*, *10*). In the later study, rosmarinic acid showed antioxidant activity similar to that of the diterpenes when $^{\bullet}OH$ or $^{\bullet-}O_2$ radicals were produced in the aqueous phase. In contrast, carnosic acid and carnosol behaved as better antioxidants than the hydrosoluble rosmarinic acid when lipoperoxyl radicals were generated.

Steady-State Fluorescence Anisotropy Measurements. The effect of rosemary bioactive compounds on the structural properties of phospholipid membranes was investigated by measuring the steady-state fluorescence anisotropy, $\langle r \rangle$, of the fluorescent probes PA-DPH and TMA-DPH, which are widely used to monitor the structural order of acyl chains in different regions of the bilayer. TMA-DPH and DPH locate their fluorophore groups at 12 and 7.8 Å from the center of the bilayer, respectively (*36*, *37*). Nevertheless, PA-DPH location is unclear due to its dependence on the pH value. Anyhow, DPH does not distribute homogeneously inside the bilayer (*38*), but the presence of charged groups in TMA-DPH or PA-DPH probes fixes their position in the membrane in a more shallow location than that of the parental DPH probe (*36*). These probes were incorporated into DMPC vesicles containing increasing molar percentages of the isolated rosemary compounds, and the anisotropy values were monitored at temperatures below and above the gel to liquid-crystalline phase transition.

Table 3. Antioxidant Capacity of Rosemary Extracts and Their Respective Major Compounds

	TEAC[a] (H_2O)	TEAC[a] (EtOH)	TBARS[b] IC_{50} (mg/L)
compounds			
rosmarinic acid	3.655 ± 0.073	3.823 ± 0.139	1.09 ± 0.09
carnosic acid	3.565 ± 0.050	3.808 ± 0.132	1.37 ± 0.05
carnosol	3.566 ± 0.214	3.257 ± 0.210	0.24 ± 0.02
rosmadial	1.963 ± 0.083	2.078 ± 0.027	1.10 ± 0.08
genkwanin	1.045 ± 0.064	1.022 ± 0.054	14.68 ± 0.96
extracts			
water-soluble	1.640 ± 0.021	1.820 ± 0.050	3.87 ± 0.28
non-water-soluble	0.762 ± 0.011	0.900 ± 0.023	1.15 ± 0.15

[a] TEAC values were expressed as Trolox equivalents needed for neutralizing the $ABTS^{\bullet+}$ radical (mmol of Trolox/g of compound or extract). Results are expressed as average ± SD (n = 4). [b] TBARS values were determined in a membrane system (EYPC SUVs) and expressed as the concentration of compound/extract able to inhibit TBARS formation at 50% (IC_{50}) in mg/L. Results are expressed as average ± SD (n = 4).

Figure 4 shows the variation of the fluorescence anisotropy of PA-DPH with the temperature in DMPC vesicles containing different amounts of the bioactive compounds under study. None of the compounds had a significant effect on the anisotropy values of PA-DPH in the gel phase compared to pure phospholipid. In contrast, the anisotropy plots corresponding to DMPC vesicles containing carnosic acid, carnosol, rosmadial, or genkwanin (panel **A**, **B**, **C**, or **D**, respectively, of **Figure 4**) showed a dose-dependent increase of the probe anisotropy values ($p < 0.001$) at temperatures above the gel to liquid-crystalline phase transition, thus indicating a decrease of the mobility of the phospholipid acyl chains compared to pure DMPC. Experiments carried out with the TMA-DPH probe showed similar results (data not shown for brevity), and these results were in agreement with those previously obtained using a deeper localized probe such as DPH (*16*). In all three cases, the hydrophilic antioxidant rosmarinic acid (**Figure 4E**) hardly affected the anisotropy values of any probe at the gel or liquid-crystalline phase.

The weak antioxidant flavone genkwanin and carnosol increased $\langle r \rangle$ the most, followed by rosmadial and to a lesser extent carnosic acid. These results did not correlate with the antioxidant activities obtained in both TEAC and TBARS assays in which the flavone genkwanin did not show any protective action against oxidation reactions. Therefore, control experiments were carried out to check whether the increased values of anisotropy observed in the presence of these compounds were due to additional quenching effects rather than to an increase of lipid order. It has been reported that the anisotropy parameter is dependent on the microfluidity of the probe's surrounding environment as well as on quenching events that affect the probe's fluorescence lifetime (*39*). In these control assays, it was observed that genkwanin progressively quenched the fluorescence of the probe as it was incorporated into the PA-DPH- or TMA-DPH-labeled vesicles, almost extinguishing the probe's fluorescence at 20 mol % of genkwanin. In addition, this quenching effect was even observed when the fluorescence was measured in solution (data not shown). Therefore, other alternative fluorescent probes, that is, octadecylrhodamine and Laurdan, were used as their

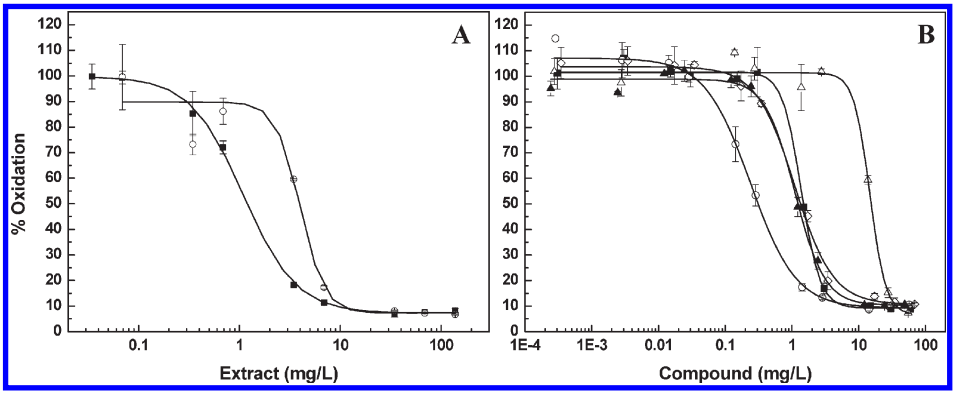

Figure 3. Antioxidant activity measured in a membrane-based system (TBARS assay) of (**A**) rosemary extracts [water-soluble extract (○) and non-water-soluble extract (■)] and of (**B**) their respective major compounds [rosmarinic acid (◇), carnosic acid (■), carnosol (○), rosmadial (▲), and genkwanin (△)]. The percentage of oxidation was expressed as nanomoles of TBARS per milligram of lipid. Results are expressed as average ± SD (n = 4).

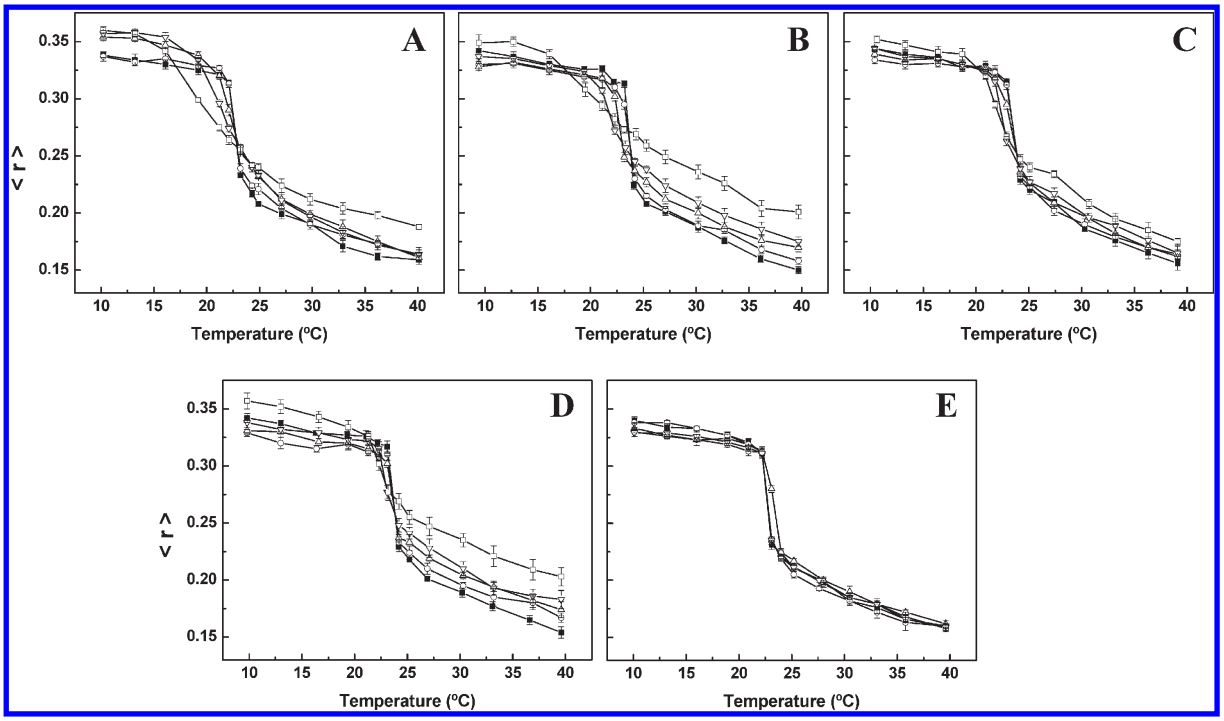

Figure 4. Steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene phenylpropionic acid (PA-DPH) incorporated in dimiristoylphosphatidylcholine (DMPC) vesicles containing 0 (■), 2 (○), 5 (△), 10 (▽), or 20% M (□) carnosic acid (**A**), carnosol (**B**), rosmadial (**C**), genkwanin (**D**), or rosmarinic acid (**E**). Results are expressed as average ± SD (n = 4).

fluorescence properties were not affected by the presence of genkwanin or other rosemary compounds, neither in membrane nor in solution.

Bilayer-to-Micelle Transition and Octadecylrhodamine B Self-Quenching Fluorescence in Membranes Containing Rosemary Compounds. The capability of the rosemary compounds to prevent bilayer-to-micelle transition upon the addition of TX-100 was determined by using the self-quenching of C18-Rh in membranes. **Figure 5** shows titration curves of detergent-induced bilayer-to-micelle transition in the absence and in the presence of increasing concentrations of rosemary compounds. Hence, the more the curve is shifted to the right, the more detergent was required to complete the bilayer-to-micelle transition. A more stable bilayer requires more TX-100 to disrupt its structure. With the exception of carnosic acid, there were no significant differences at 2 mol % of each added compound, but significant differences compared to controls were found ($p < 0.001$) at higher concentrations of the diterpenes carnosic acid, carnosol, and rosmadial. No significant changes were observed for the flavone genkwanin until 20 mol % concentration in the membrane was reached ($p < 0.001$). Thus, carnosic acid was the compound that shifted the titration curves the most (**Figure 5A**), followed by its derivatives carnosol (**Figure 5B**) and rosmadial (**Figure 5C**), in that order. However, the gradual rise of genkwanin concentration in C18-Rh-labeled membranes did not alter the bilayer-to-micelle titration curves when compared to pure phospholipid (**Figure 5D**). This effect was more evident when IC_{50} values for each compound were determined (**Figure 6A**) from their corresponding TX-100 titration curves. The IC_{50} values obtained showed how the presence of the antioxidant diterpenes (carnosic acid, carnosol, and rosmadial) in the membrane required higher amounts of detergent to carry out the micelle formation ($p < 0.001$), whereas the weak antioxidant flavone genkwanin slightly raised the IC_{50} value when its membrane concentration was as high as 20 mol % (**Figure 6A**).

The fluorescent properties of octadecylrhodamine in membrane can be also used for testing the effect of added compounds on membrane packing by measuring the extent of the probe's self-quenching (%Q). The addition of rosemary compounds to C18-Rh-labeled membranes also increased the extent of the self-quenching of this probe [Q (%)] ($p < 0.001$), the diterpene carnosic acid being the compound that promoted the strongest effect (**Figure 6B**). The hydrophilic compound rosmarinic acid affected significantly only the self-quenching of the probe ($p < 0.001$) compared to the control at concentrations > 10 mol %. Carnosic acid increased the percentage of self-quenching up to 85% when its concentration in the membrane was as low as 5 mol %. Carnosol also increased the Q (%) of the probe, but it was less potent than carnosic acid because it needed almost 20 mol % to reach a similar level of self-quenching. Rosmadial also enhanced Q (%) values, although to a lower extent compared to carnosic acid or carnosol (78% at 20 mol %), whereas the flavone genkwanin and the hydrophilic compound rosmarinic acid had a mild effect on C18-Rh self-quenching.

At a concentration of 20 mol %, the diterpene carnosic acid caused a 58% increase in the probe's self-quenching when compared to initial self-quenching values (i.e., in the absence of added compound), and carnosol and rosmadial enhanced this value by 51 and 35.5%, respectively. The non-antioxidant flavone genkwanin and the hydrophilic antioxidant rosmarinic acid slightly modified initial self-quenching values, causing increases of 28 and 24%, respectively, when added at 20 mol % to C18-Rh-labeled membrane.

Lipid/Water Interface Hydration State of Membranes Containing Rosemary Diterpenes. The effect of rosemary diterpenes on the lipid/water interface of model membranes was also studied by using Laurdan, a surface-located fluorescent probe. Rosmarinic acid and genkwanin were not employed in the Laurdan experiments because these compounds did not show strong effects on C18-Rh self-quenching and none of them exerted a significant perturbation on model membranes as previously reported (*16*).

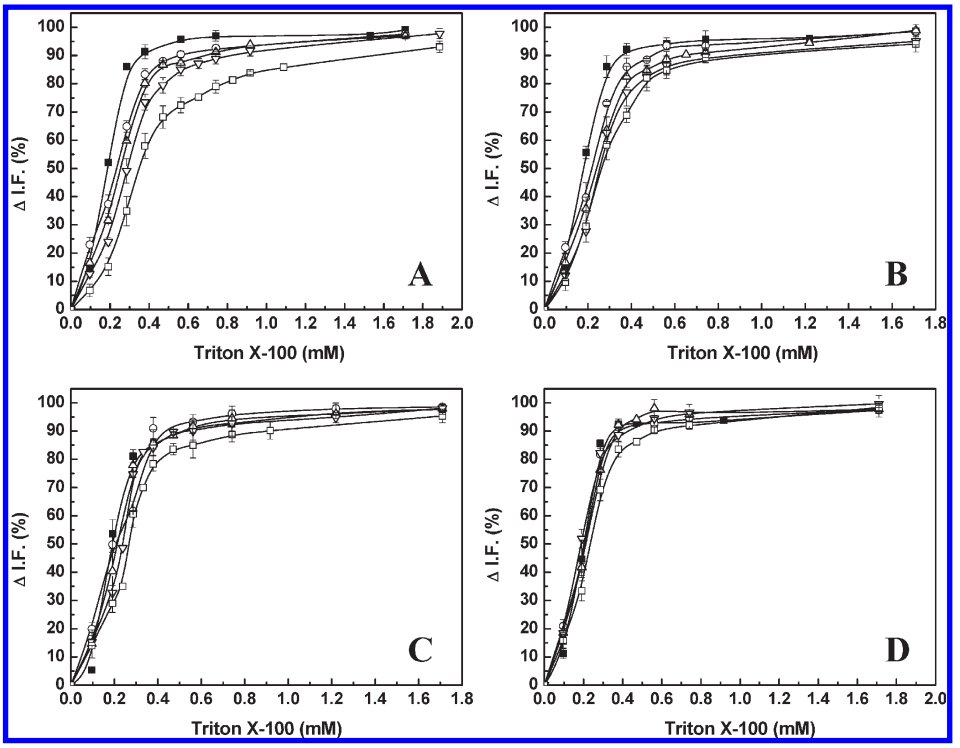

Figure 5. Bilayer-to-micelle transition upon addition of Triton X-100 (TX-100): titration curves of TX-100 added to C18-Rh-labeled membranes containing 0 (■), 2 (○), 5 (△), 10 (▽), or 20% M (□) carnosic acid (**A**), carnosol (**B**), rosmadial (**C**), and genkwanin (**D**). Small unilamellar vesicles of DMPC containing 1.8 mol % of octadecylrhodamine B were used as model membrane system, and the titration experiments were performed at 37 °C. Results are expressed as average ± SD (n = 4).

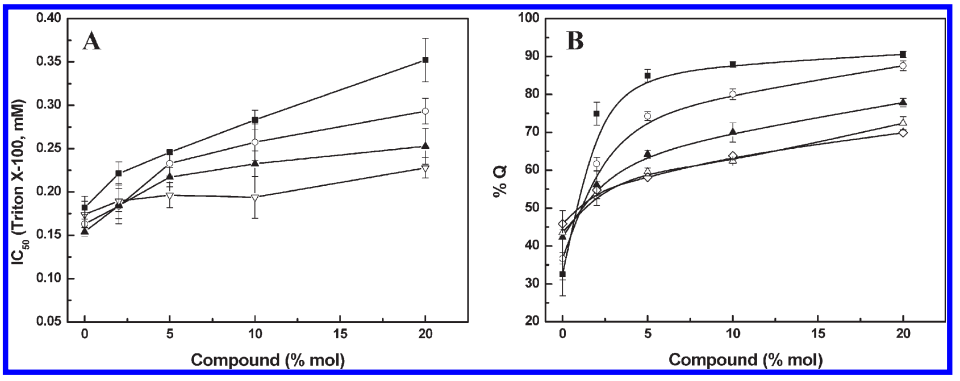

Figure 6. (**A**) Variation of the IC_{50} values (TX-100 concentration needed to achieve 50% of the bilayer-to-micelle transition) corresponding to the TX-100 titration curves of DMPC vesicles containing increasing concentrations of carnosic acid (■), carnosol (○), rosmadial (▲), and genkwanin (▽) and labeled with octadecylrhodamine B. (**B**) Effect of the addition of increasing amounts of carnosic acid (■), carnosol (○), rosmadial (▲), genkwanin (△), and rosmarinic acid (◇) on the octadecylrhodamine B self-quenching (%Q) incorporated into DMPC vesicles. Results are expressed as average ± SD (n = 4).

Laurdan fluorescent properties are sensitive to polarity or hydration state of the lipid/water interface as well as to water molecules' dynamics surrounding Laurdan's chromophore. Such effects can be detected by changes in fluorescence intensities at 440 and 490 nm, which are summarized by the GP_{ex} value (eq 4). Thus, an increase in the GP_{ex} value would reveal a decrease in water free movement or hydration and vice versa.

Figure 7 shows the changes in the GP_{ex} values of DMPC membranes in the presence of increasing concentrations of rosemary diterpenes at two temperatures, 5 and 37 °C, that is, below and above the gel to liquid-crystalline phase transition. No significant changes were observed in the GP_{ex} values when rosemary diterpenes were incorporated into DMPC membranes at 5 °C, with the exception of rosmadial, which decreased the GP_{ex} value (from 0.493 to 0.433) when added at the highest concentration tested, that is, 20 mol % ($p < 0.05$). This decrease indicates a shift of the fluorescence emission intensity to 490 nm in the presence of rosmadial. In contrast, the addition of rosemary diterpenes to membranes in the liquid-crystalline state (**Figure 7**, 37 °C), gradually increased GP_{ex} values. No significant differences in the GP_{ex} values were observed at a diterpene concentration of 2 mol % compared to those in the absence of compounds. However, a statistically significant increase in Laurdan's GP_{ex} value compared to the control at 37 °C was observed ($p < 0.001$) when the concentration of carnosic acid, carnosol, or rosmadial was ≥5 mol %. The diterpenes rosmadial and carnosol caused greater effects than carnosic acid, which was already noted at low concentrations (2−5 mol %). Rosmadial and carnosol increased GP_{ex} values from −0.140 to +0.004 and −0.004, respectively, when added at 20 mol %, whereas carnosic acid slightly increased

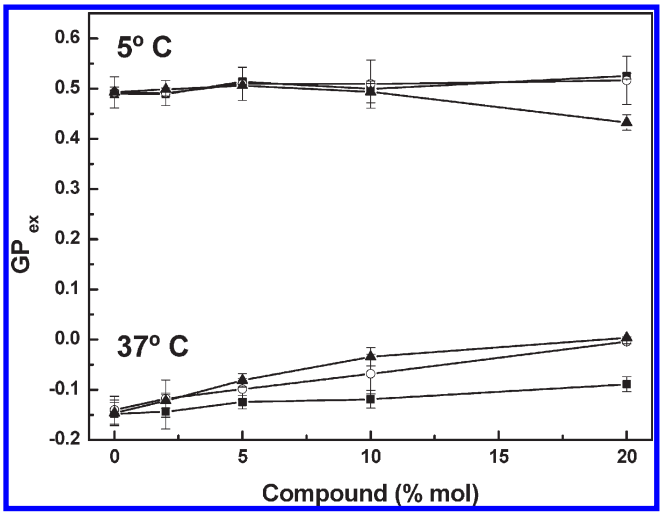

Figure 7. GP_{ex} values of Laurdan incorporated into DMPC vesicles in the gel phase (5 °C) and in the liquid-crystalline phase (37 °C) and containing increasing amounts of the rosemary diterpenes carnosic acid (■), carnosol (○), and rosmadial (▲). Results are expressed as average ± SD ($n = 4$).

the GP_{ex} value in membranes at the fluid state at the same concentration (from −0.140 to −0.089).

DISCUSSION

The HPLC-DAD-ESI-MS/MS analysis revealed that rosmarinic acid, the diterpenes carnosic acid, carnosol, and rosmadial, and the flavone genkwanin were the major compounds in the water-soluble and non-water-soluble extracts of rosemary leaves, respectively. It has been reported that rosmarinic acid, carnosic acid, carnosol, rosmadial, and genkwanin are the compounds most frequently found in rosemary extracts regardless of the extraction method employed or the manufacturer (*22*). The antioxidant capacity measurements performed in the present study using these major compounds showed that hydrophobic diterpenes and the hydrophilic rosmarinic acid were responsible for the antioxidant capacity of rosemary leaf extracts as previously reported (*5*, *6*, *40*, *41*). However, the ways in which these compounds operate seem to be quite different. In the presence of model membranes (TBARS), the non-water-soluble extract showed higher antioxidant capacity than the water-soluble extract, whereas it was vice versa in the absence of a membrane-based system (TEAC). Similar results were obtained when purified major compounds of the corresponding extracts were tested. Carnosol was demonstrated to be the strongest antioxidant compound in the TBARS assay, whereas no significant differences in the TEAC values were obtained between the hydrophobic diterpenes carnosol and carnosic acid and the hydrosoluble compound rosmarinic acid.

TEAC and TBARS methods rely on different antioxidant mechanisms. TEAC is based on single electron transfer (SET) mechanisms (*42*) in which the antioxidant compound tested donates an electron to the $ABTS^{\bullet+}$ cationic radical to neutralize it. On the other hand, the TBARS assay mimics a lipid peroxidation reaction under physiological conditions at which the antioxidant molecule studied can quench the reaction at different stages because lipid peroxidation proceeds in several steps known as initiation, propagation (chain reaction), and termination. The initiation phase starts when the free radicals generated in the aqueous phase react with membrane lipids, producing lipid peroxyl radicals that propagate across the membrane by reacting with adjacent phospholipid acyl chains (*43*, *44*). The propagation stage ends when other membrane components neutralize peroxyl and other lipidic radicals by hydrogen or electron donation. Nevertheless, these neutralizing agents can also exert a stabilizing and rigidifying effect on model membranes that could slow the dynamics of the lipid peroxidation reaction. Both the membrane stabilizer and radical scavenger effects would act synergically in cutting down the propagation phase of lipid peroxidation. This dual property has been described for well-known antioxidants such as α-tocopherol and carotenoids (*11*, *43*, *45*), although other natural phytochemicals such as flavonoids have been also found to use similar antioxidant mechanisms (*13*, *14*). Rosemary diterpenes seem to be highly effective in scavenging radicals by electron donation as indicated by TEAC results. However, the observation arising from both antioxidant capacity assays indicated that non-water-soluble extract and its main diterpenoid components exerted additional effects on model membranes other than their electron donor properties.

We have already reported that rosemary hydrophobic diterpenes caused important biophysical perturbation of model membranes by enhancing membrane phospholipid order and promoting phospholipid/diterpene-enriched domains with altered structure (*16*). In the present study, we aimed to go further into the effect of rosemary diterpenes by using several fluorescence methodologies in correlation to antioxidant capacity measurements, which allowed us to shed light on the antioxidant mechanisms of rosemary extracts and their corresponding major components. The first evidence was derived from the steady-state anisotropy results performed with the DPH derivatives PA-DPH and TMA-DPH, which are located at different depths in the membrane compared to DPH. As we have previously observed in the DPH anisotropy assay (*16*), hydrophobic compounds, that is, diterpenes carnosic acid, carnosol, and rosmadial and flavone genkwanin, increased anisotropy ($\langle r \rangle$) values of DPH- derivative probes, whereas rosmarinic acid hardly increased the anisotropy. This could mean that this class of lipophilic compounds may enhance phospholipid packing at different depths of the membrane and restrict the fluorescent probe's free movement. Unexpectedly, the non-antioxidant flavone genkwanin exhibited a significant increase of $\langle r \rangle$ with respect to the control ($p < 0.001$), as happened to carnosol, rosmadial, and carnosic acid. Nevertheless, one should be careful when relating changes in membrane fluidity with changes in $\langle r \rangle$ values measured by steady-state fluorescence techniques. These kinds of measurements do not take into account depolarizing events caused by changes in the probe's excitation lifetime (τ) as indicated by the Perrin equation (*39*). It is a proven fact that $\langle r \rangle$ changes rely not only on viscosity changes but also on the probe's excitation lifetime (τ) changes. Therefore, any event that could cause a decrease in the τ of DPH probes, such as loss of fluorescence (quenching), would result in a net increase of the $\langle r \rangle$ values. We believe this is what occurs when genkwanin is added to DPH derivative labeled vesicles. As long as the concentration of this flavone rises in the membrane, the DPH probes gradually attenuate, being almost extinguished at 20 mol % of genkwanin. Therefore, its effect on anisotropy values was rather related to quenching processes than rigidifying effects. In fact, control experiments conducted to determine the level of quenching of DPH derivatives by the rosemary compounds revealed that genkwanin promoted a much higher quenching effect than rosemary diterpenes.

It has been reported that self-quenching of C18-Rh fluorescence increases due to the formation of nonfluorescent dimers in membranes containing cholesterol or when membranes go through a fluid to gel transition (*26*). A similar behavior has been observed for C18-Rh in membranes containing rosemary diterpenes in the present study. Hence, these hydrophobic diterpenes may exert a similar effect on membrane phospholipid

hydrocarbon chains, resulting in an enhanced internal packing of the bilayer. These observations were in agreement with Triton X-100 titration experiments, which indicated that incorporation of rosemary diterpenes into the membrane prevented the detergent-induced bilayer-to-micelle transition. As indicated by its IC_{50} values (**Figure 6A**) carnosic acid increased C18-Rh self-quenching the most, followed by carnosol and rosmadial, whereas the effect of genkwanin and rosmarinic acid on C18-Rh self-quenching was much lower. These results confirm that the effect of genkwanin on the DPH probe's anisotropy values did not indicate an increase of lipid order in membrane, and only the diterpenes had a significant contribution to this effect. C18-Rh self-quenching [Q (%)] and TX-100 solubilization experiments provided identical results (**Figure 6**, panels **B** and **A**) on the potency order of the diterpenes, which confirms the strong effect of carnosic acid on the phospholipid packing.

On the other hand, Laurdan's GP_{ex} studies revealed that the presence of rosemary diterpenoids in the membrane promoted a less polar and more motion-restrictive environment surrounding the chromophore, because GP_{ex} values were significantly increased when rosemary diterpenoids were added to Laurdan-labeled vesicles in the liquid-crystalline phase. Laurdan is a fluorescent probe located at the phospholipid's glycerol backbone sensitive to the polarity changes and dynamic properties of membrane's lipid/water interface. The probe's emission spectrum undergoes a blue shift when the chromophore's local environment is less polar and a red shift as the polarity increases (*27*). Laurdan's emission spectral shifts also take place when the membrane changes from gel to liquid-crystalline phase due to dipolar relaxation processes of the water molecules surrounding Laurdan's chromophore, which is closely related with dynamic and free movement of these water molecules (*27*, *46*). Spectral blue shifts due to less polar environment or restricted motion of water molecules lead to a net increase in Laurdan's GP_{ex} values. Rosmadial was the compound showing the strongest effect on the increase of GP_{ex} values followed by carnosol, whereas carnosic acid barely increased initial polarization values.

Therefore, rosmadial seemed to reduce lipid/water interface hydration, that is, fewer water molecules as well as water molecules' mobility, leading to Laurdan's spectral blue shift and consequently to increased GP_{ex} values. On the other hand, carnosic acid would decrease membrane fluidity at a deeper level by promoting hydrocarbon chain packing and increasing C18-Rh molecules' approaching and self-quenching. However, the diterpene carnosol would affect membrane fluidity at both levels (lipid/water interface and hydrophobic core), as supported by C18-Rh and Laurdan assays. This fact could account for the higher antioxidant effect observed for this compound in the membrane-based system (TBARS). This interpretation is also in agreement with the effect observed for the diterpenes on the fluorescence anisotropy experiments, in which carnosol had the strongest effect among the diterpenes on the packing order of the phospholipid bilayer when anionic or cationic DPH-derivative probes were used.

In conclusion, the fluorescence measurements carried out with C18-Rh and Laurdan demonstrate that the incorporation of the three major rosemary diterpenes in phospholipid membranes promoted a higher lipid order and packing at both the lipid/water interface and hydrophobic core of the membrane, which is in agreement with previous studies (*16*). Moreover, the diterpene carnosol would affect membrane fluidity at both the water/membrane interface and membrane hydrophobic core, as revealed by C18-Rh, fluorescence anisotropy, and Laurdan measurements, which could count for the higher antioxidant effect observed for this compound in the membrane-based antioxidant assay (TBARS). All of these effects contribute to explain the strong capability of rosemary diterpenes as antioxidant agents in lipidic environments as pointed out by other authors. The membrane-rigidifying and membrane dehydration effects described in this work for the bioactive compounds from *Rosmarinus officinalis* may account for their antioxidant capacity and cooperate with their electron donor ability in protecting lipid membranes against oxidative damage.

ABBREVIATIONS USED

$\langle r \rangle$, steady-state fluorescence anisotropy; C18-Rh, octadecylrhodamine B; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; EYPC, L-α-phosphatidylcholine tissue-derived from egg yolk; HPLC-DAD-ESI-MS/MS, liquid chromatography with diode array detection coupled to electrospray and ion-trap mass spectrometry; MLVs, multilamellar vesicles; PA-DPH, 3-(4-(6-phenyl)-1,3,5-hexatrienyl)-phenylpropionic acid; ROS, reactive oxygen species; SUVs, small unilamellar vesicles; TBARS, thiobarbituric acid reactive species; TEAC, Trolox equivalent antioxidant capacity; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; TX-100, Triton X-100.

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