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Antioxidative Effect of Quercetin and Its Equimolar Mixtures with Phenyltin Compounds on Liposome Membranes

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Our earlier studies have shown that the compounds diphenyltin dichloride (DPhT) and triphenyltin chloride (TPhT) in the presence of UVC radiation enhanced the degree of phosphatidylcholine liposome membrane oxidation (*J. Agric. Food Chem.* **2005**, *53*, 76−83). The prooxidative behavior of the compounds has now been confirmed with the electron paramagnetic resonance method, which proved the possibility that the studied compounds can exist in free radical forms. The present work investigates the possibility of the protective action of quercetin on phosphatidylcholine liposome membranes exposed to the prooxidative action of DPhT and TPhT induced by UV radiation ($\lambda = 253.7$ nm). The concentrations of quercetin and its equimolar mixtures with DPhT and TPhT were determined (and compared with well-known antioxidants as standards—trolox and butylated hydroxytoluene, also in the presence of phenyltins) as those that induce 50% inhibition in oxidation of liposomes radiated with UV. They are 5.1 ± 0.10, 2.9 ± 0.12, and 1.9 ± 0.08 μM (differences between the values are statistically significant), constituting the following sequence of antioxidative activity: quercetin:TPhT > quercetin:DPhT > quercetin. This relation is confirmed by the results on the antiradical ability of quercetin and its mixtures with DPhT and TPhT toward the free radical 1,1-diphenyl-2-picrylhydrazil. Similar sequences obtained in both studies suggest a possible mechanism of the antiradical action of the mixtures as free radical scavengers. We suggested that (i) quercetin's ability, documented by spectrophotometric, infrared attenuated total reflectance spectroscopy, ^{1}H NMR, and molecular modeling methods, to form complexes with phenyltins indicates a possible way of protection against the peroxidation caused by the free radical forms of phenyltins and (ii) the differentiation in the action of the quercetin/TPhT and quercetin/DPhT associates (statisticaly significant) may result from a different localization in the liposome membrane, which is indicated by the results of the fluorimetric studies.

KEYWORDS: Quercetin; phenyltin; antioxidants; complexes; fluidity; liposomes; molecular modeling

INTRODUCTION

Ultraviolet radiation is in general harmful to biological membranes (*1*−*5*). It is thought that the main cause of structural and functional changes induced in membranes by radiation is membrane lipid peroxidation (*6*, *7*). This is the reason why many studies on the interaction between UV radiation and membranes are conducted on model lipid membranes (*8*−*11*). In turn, heavy metals also disorganize biological membranes, affecting, among others, membrane lipids (*12*). In particular, they may cooperate with UV radiation, the effects of the cooperation being diverse depending on the kind of metal and membrane (*13*, *14*). In order to counteract the harmful effects of the agents mentioned, various practices are employed. Among others, flavonoids are used that can inhibit lipid peroxidation induced by UV radiation and the toxic effect of heavy metals on membranes (*11*, *15*−*18*). Our previous studies on the effect of organic tin and lead compounds on biological and model membranes (*18*−*25*) have directed attention to the effects caused by the concurrent action of radiation and organic tin and lead compounds on membranes. Our purpose has been to investigate the collective effect of UV radiation and organic tin compounds (OTC) on phosphatidylcholine (PC) liposomes and how to counteract that effect by the protective action of a selected flavonoid such as quercetin (Que). Que is a representative of flavonoids that occurs in the human diet, in, among other things, endives, leeks, broccoli, teas, grapefruits, strawberries, and gooseberries (*26*, *27*) and that shows a high antioxidant efficiency in various systems (*28*−*30*). Besides, the phenyltin compounds studied in different industrial applications are used as fungicides in agriculture (*31*−*34*).

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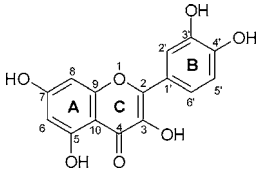

Figure 1. Chemical structure of the quercetin molecule.

In the work presented, the antioxidative action of equimolar mixtures of Que with diphenyltins and triphenyltins has been compared with the activity shown by Que alone and with the well-known synthetic antioxidants 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and butylated hydroxytoluene (BHT). A measure of this activity is the concentration of a compound (or mixture) that causes 50% reduction in membrane oxidation. A mechanism for the antioxidative action of a mixture was proposed, determined by its ability to reduce the 1,1-diphenyl-2-picrylhydrazil (DPPH•) free radical. The DPPH• radical, because of its stability and long lifetime, is often used in studies of flavonoid redox processes (*35*, *36*). Then, because of the known associative properties of flavonoids toward ions of some metals, for example, Cu, Al, Mn, and Fe (*37*−*41*), the possibility of complex formation of Que with *n*-phenyltin compounds has been investigated by ^{1}H NMR and spectrophotometric (IR and UV−vis) measurements. Using the molecular modeling method, the possibility of probable complex formation between Que and DPhT and TPhT was calculated. The fluorimetric studies, performed using the 1,6-diphenyl-1,3,5-hexatriene (DPH) probe, allowed detection of differences in the behavior of the associates of Que with DPhT and TPhT in the liposome membrane bilayer. This was helpful in discussing the possibilities of an effective protection of liposome membranes against the toxic forms of phenyltins.

MATERIALS AND METHODS

Materials. PC from eggs was purchased from Lipids Product (South Nutfield, United Kingdom). Que (see the molecular structure in **Figure 1**), DPPH, dimethyl sulfoxide (DMSO), trolox, and BHT were purchased from Sigma Aldrich Chemie GmBH (Steinheim, Germany). OTCs, namely, $(C_6H_5)_2SnCl_2$ (diphenyltin dichloride , DPhT) and $(C_6H_5)_3SnCl$ (triphenyltin chloride, TPhT), were purchased from Alfa Products (Karlsruhe, Germany). The fluorescent probe DPH was purchased from Molecular Probes Inc. (Eugene, OR). The remaining chemicals were of analytical grade.

Electron Paramagetic Resonance (EPR) Measurements of Free Radical Forms of OTC. Powders of DPhT and TPhT compounds were exposed to UVA radiation (mercury lamp Osram dulux 78, $\lambda = 320-400$ nm) for 30 min at room temperature. The sample was placed at a distance of ca. 5 cm from three lamps placed symmetrically on two opposite walls and ceiling of the box. The exposition intensity of the sample was 800 mW/m^2. The control samples were not exposed to the radiation. The spectra were recorded at a temperature of 25 °C on a standard SE/X28 electron spin resonance spectrometer operating in the X-band (made at Technical University of Wrocław, Poland) with a microwave power of 2.0 mW, a sweep time of 10 s, a time constant of 100 ms, and a modulation amplitude of 0.8 mT at 0.8×10^5 amplification. For a scientific description of spectra, the LABCARD program was used.

Liposome Preparation and Induction of Peroxidation. A chloroform solution of egg yolk PC was dried in vacuum under nitrogen atmosphere (*42*). A 50 mM concentration of Tris:HCl [(hydroxymethyl) aminomethane] buffer, pH 7.4, was added, and the sample was vortexed to obtain a milky suspension of multilamellar vesicles. The final concentration of lipids in the vesicle suspension was 1.5 g L^{-1}. Such a suspension was then sonicated for 10 min with a 20 kHz sonicator. The DPhT, TPhT, and Que (alone) or equimolar mixtures of Que with an OTC (DPhT or TPhT) were then added from a concentrated methanol solution (2×10^{-3} M) to the stirred sample of suspended vesicles. The concentration of methanol never exceeded 2% of the final 8 mL volume of the sample. The concentration of the compounds studied changed in the range from 0.625 to 20 μM (as indicated in **Figures 4** and **5**). Lipid peroxidation in the egg phospholipid liposomes was induced by ultraviolet radiation using a bactericidal lamp (253.7 nm) of 3.0 mW/cm^2 intensity. The accumulation of phospholipid peroxidation products was estimated by determination of 2-thiobarbituric acid reactive substances (TBARS) in the incubation medium (*43*). The amount of the reaction products was determined by measuring the increase in absorbance at 535 nm. The percentage of PC liposome oxidation induction or inhibition was calculated based on the relation: % induction/inhibition $= (1 - \Delta A_A/\Delta A_O)$ 100%, where ΔA_A is the absorption increase (at $\lambda = 535$ nm) after 30 min of light exposure with an antioxidant (Que or its equimolar mixture with DPhT or TPhT) added and ΔA_O is the absorption increase (at $\lambda = 535$ nm) after 30 min of light exposure of liposomes without phenyltins or antioxidant added.

Reduction of DPPH• Free Radical. A methanol solution of the free radical DPPH• of absorption approximately 0.9 was mixed with a proper amount of methanol solution of an antioxidant (Que or equimolar mixture of Que with OTC). The mixture was then incubated for an hour at room temperature and in darkness. During incubation, a reduction occurred of a part of the free radical form DPPH•, dependent on the concentration of the antioxidant added. Then, absorption was measured at a characteristic DPPH• wavelength equal to 517 nm (*35*). The measurements were made for concentrations of the compounds studied in the range 2.5−20 μM. The amount of DPPH• reduction (expressed in percentage), in the presence of a set antioxidant concentration, was calculated using the formula: % reduction $= (1 - \Delta A_A/\Delta A_O)$ 100%, where ΔA_O is the DPPH• absorption increased at $\lambda = 517$ nm and $t = 0$, before the antioxidant addition, and ΔA_A is the DPPH• absorption increased at $\lambda = 535$ nm after 1 h of DPPH• incubation with an antioxidant. Absorption of the stirred samples was measured at room temperature, in a 1 cm cuvette with the UV−vis spectrophotometer model 2401 (Shimadzu).

Complexation Studies and Determination of Chemical Equilibrium Constants. The studies on complexation of OTC with Que were performed using the spectrophotometric method, with methanol as the solvent and in the presence of PC liposomes suspended in a phosphate buffer, pH 7.4. A constant amount of Que, to obtain a 49 μM concentration, was added to 2 mL of methanol (or liposome dispersion containing 0.1 mg PC/mL), and then, an OTC was titrated. Concentrations of DPhT and TPhT are given in the caption to **Figure 7**. Absorption of the stirred samples was measured at 250−600 nm at room temperature, in a 1 cm cuvette with UV−vis spectrophotometer model 2401 (Shimadzu). Equilibrium constants for OTC binding to Que were calculated using the Bensi−Hildebrand law (*44*). Stoichiometry of the complexes Que/DPhT (1:1) and Que/TPhT (1:1) were determined by the method of the modified Job's plot. Fitting of the curves to experimental data, as shown by the equation

$$A_c = \frac{K\epsilon_c[\mathrm{OTC}][\mathrm{Que}]_0}{1 + K[\mathrm{OTC}]}$$

was done using the nonlinear least-squares (NLLS) Marquardt−Levenberg algorithm implemented in the gnuplot program (version 4.0), where A_c is the absorption at $\lambda = 430$ nm (a wavelength characteristic for a complex), K is the binding constant, ϵ_c is the molar extinction coefficient for a complex, [OTC] is the concentration of OTC as a titrate, and $[\mathrm{Que}]_0$ is the initial concentration of Que.

^{1}H NMR and Infrared Attenuated Total Reflectance Spectroscopy (IR-ATR) Studies. ^{1}H NMR spectra were collected for samples of a 0.5 mL mixture of Que with DPhT (1:1) or Que with TPhT (1:8) and for pure Que or OCT compounds in DMSO in 5 mm NMR tubes. ^{1}H NMR spectra were recorded on the Bruker Avance DRX spectrometer. ^{1}H NMR parameters of 300 MHz were as follows: spectral window, 6173 Hz; digital resolution, 0.188 Hz; pulse width, 4.5 μs (30° flip angle); acquisition and delay times, 2.65 and 1 s, respectively; and acquisition temperature, 25 °C.

The IR spectra of an equimolar mixture of Que with DPhT (1:1) or Que with TPhT (1:1) and for pure Que in solid form (after the evaporation of methanol from the mixture) were obtained on an FTIR Mattson IR 300 apparatus using the ZnSn ATR method. The spectra represent the average of 128 scans at 2 cm^{-1} resolution in the spectral range of 4000−800 cm^{-1}.

Fluidity Study. The fluidity experiments were done on liposome membranes, which were subjected to the action of Que and their equimolar mixture with OTC. The fluorescent probe DPH was used at a 0.1 μM/L concentration. The measurements were performed with an SFM 25 spectrofluorimeter equipped with a thermostatic attachment (Kontron, Zurich, Switzerland), at 25 °C. The excitation and emission wavelengths were 354 and 429 nm, respectively. The anisotropy coefficients (r) were calculated according to the formulas (*45*):

$$r = \frac{I_{||} - GI_{\perp}}{I_{||} + 2GI_{\perp}}$$

where $I_{||}$ is the intensity of fluorescence emitted in a direction parallel to the polarization plane of the light, $I_{\perp}$ is the intensity of fluorescence emitted in a perpendicular direction, and G is an instrumental correction factor.

Quantum Mechanical Computations. Closed-shell structures of Ph_2Sn^{++}, Ph_3Sn^{+}, and Que were built with the help of the molecular modeling package Sybyl version 6.4 (Sybyl version 6.4, 1991−1997). Their geometries were optimized using Gaussian 03 (*46*) with the methods of ab initio Hartree−Fock and then using the B3LYP potential (*47*) of the density functional theory (DFT). A generalized basis set and relativistic effective core potentials were used for tin (*48*), oxygen, and carbon (*49*). Also, for hydrogen atoms, the generalized basis set (*50*) was applied but without any core potential. Such optimized structures of DPhT and TPhT were catenated with the optimized Que structure to form the initial supermolecular structures of possible complexes of DPhT/Que and TPhT/Que. The geometries of the supermolecular structures were optimized in the same manner and using the same methods, basis sets, and effective core potentials as before. Having performed the optimizations of the supermolecules in vacuo, we decided to take into account the self-consistent reaction field (SCRF) forces exerted on the supermolecules by water medium and to check if the supermolecules were stable in such conditions. However, the most interesting results expected from these computations were the optimized geometries of Que/DPhT and Que/TPhT complexes in water. We used Gaussian 03, modeled the reaction field of water by the conducting polarizable continuum model (CPCM), and used the B3LYP potential (which includes electron correlation and exchange effects) and the same functional basis set and pseudopotentials as used before in in vacuo computations. The cavity was formed according to the CPCM technique implemented in Gaussian 03 with additional spheres on hydrogens. The water accessible surface of the cavity was considered the contact surface with the water medium. The frequency computations in the same model chemistry have shown the stability of the structures of Que/DPhT and Que/TPhT complexes.

Statistical Analysis and Calculation of IC_{50}. The fitting by least-squares method was applied to an initial linear dependence of the % of oxidation inhibition after 30 min of induction of oxidation (or % of DPPH free radical quenching) on concentration of the compounds studied. In the case of trolox and its OTC mixtures, the nonlinear function ($y = ax^b + c$) was fitted. On this basis, for $y = 50\%$, the value of $x = IC_{50}$ was calculated. The error of IC_{50} was calculated by a complete differential method. The maximal errors of the fitted parameters were taken as their mean square errors. The intervals of $IC_{50} \pm$ error for Que and BHT (and also for their mixtures with OTC) do not overlap and are as follows: oxidation experiments, $IC_{50}^{QUE} \in$ (5.02, 5.22) μM, $IC_{50}^{QUE/DPhT} \in$ (2.82, 2.94) μM, $IC_{50}^{QUE/TPhT} \in$ (1.82, 1.98) μM, $IC_{50}^{BHT} \in$ (2.65, 2.87) μM, $IC_{50}^{BHT/DPhT} \in$ (3.06, 3.30) μM, and $IC_{50}^{QUE/TPhT} \in$ (3.48, 3.68) μM; quenching of DPPH free radical, $IC_{50}^{QUE} \in$ (8.07, 8.33) μM, $IC_{50}^{QUE/DPhT} \in$ (7.00, 7.80) μM, and $IC_{50}^{QUE/TPhT} \in$ (5.90, 6.30) μM. Therefore, we may judge that for low concentrations of the compounds (to ca. 5.2 μM in the case of Que and the mixture with OTC, to ca. 3.7 μM in the case of BHT and the mixture with OTC), the differences are significant statistically. The differences for trolox and their mixtures with OTC are statistically insignificant.

The fitting of straight lines to results of fluorescence anisotropy vs concentration of compounds studied was performed by the linear regression method. The slope coefficients are in ranges as follows: Que $\in$ (2.48, 2.61) $\times 10^4$ M^{-1}, Que/DPhT $\in$ (3.95, 4.18) $\times 10^4$ M^{-1}, and Que/TPhT $\in$ (2.08, 2.83) $\times 10^4$ M^{-1}. The differences between slope coefficients are statistically significant.

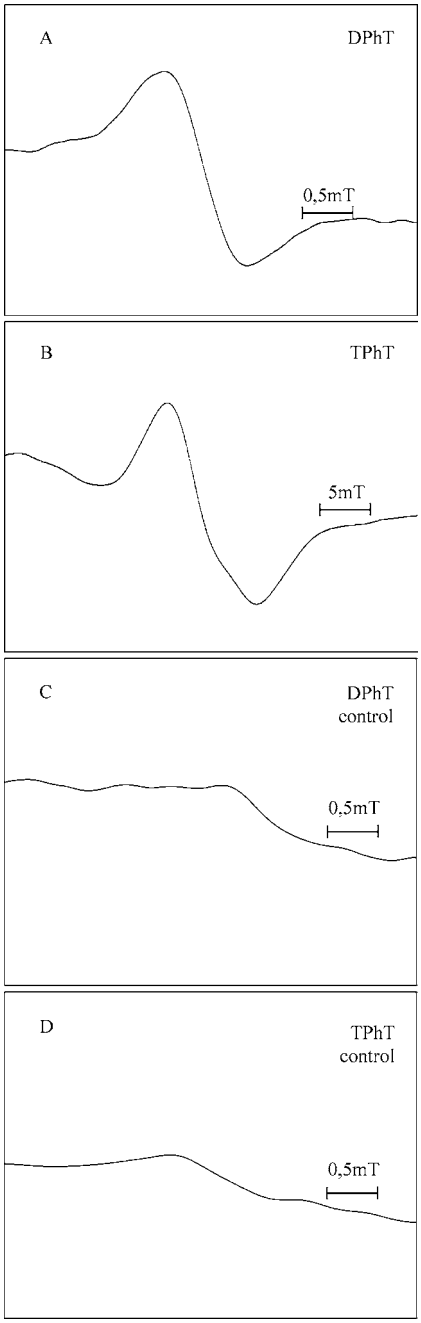

Figure 2. EPR spectra of DPhT (**A**) and TPhT (**B**) compounds (in solid state) after 30 min of exposure to UV radiation. Spectra **C** and **D** are of unradiated DPhT and TPhT (control), respectively.

RESULTS

The results of the EPR studies on free radical forms of the compounds DPhT and TPhT, which are induced by UV radiation, are presented in **Figure 2A** (DPhT) and **B** (TPhT), while **Figure 2C**,**D** shows the control spectra in the absence of UV radiation. The spectra (**Figure 2A**,**B**) are characteristic for

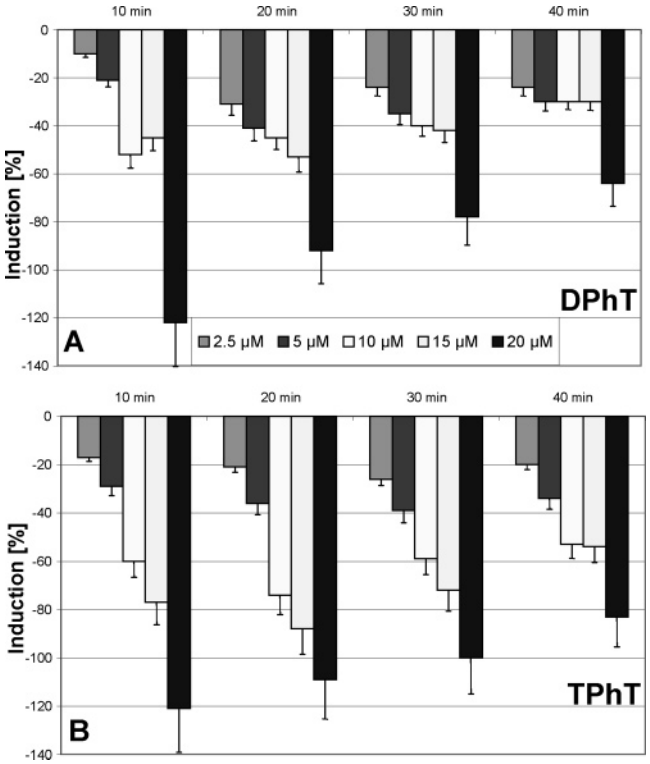

Figure 3. Dependence of photooxidation induction on the time of UV (λ = 253.7 nm) radiation of PC liposomes in the presence of DPhT (**A**) and TPhT (**B**). The concentration of compounds added changed in the range from 2.5 to 20 μM, as indicated. The data, from three repeated experiments, represent the relative peroxidation as compared to its control (without the compounds added). The mean standard deviation did not exceed 15% (*18*).

the existence of the free radicals, which appear as a result of photodestruction (dephenylation) of DPhT and TPhT molecules; the calculated coefficients of spectroscopic dispersion *g* are 2.0113 and 2.0042 for TPhT and DPhT, respectively. The EPR spectrum for TPhT (**Figure 2B**) shows a larger breadth between the maxima (expressed in mT) than it is for DPhT, which means that a greater number of free radicals are created during TPhT exposition to UV as compared to that during DPhT radiation. The results of the studies on prooxidative behavior of phenyltins on PC membrane radiated with UVC are given in **Figure 3A** (for DPhT) and **Figure 3B** (for TPhT) (the results were previously published in *J. Agric. Food Chem.* **2005**, *53*, 76−83). They present the relative (in %) oxidation induction of liposomes caused by OTC, corresponding to 40 min of UV exposure. The percentage of oxidation induction was determined with respect to control, in other words, by the level of liposome oxidation without OTC. As it follows from **Figure 3**, the level of membrane oxidation is dependent on both the concentration of OTC and the exposition time to UV. The liposome oxidation level, induced for instance by TPhT, is basically higher (for its concentration in samples higher than 10 μM) than the oxidation level caused by the same concentration of DPhT.

The results on the antioxidative ability of Que and its equimolar mixtures with DPhT and TPhT are presented in **Figure 4**, whereas **Figure 5** shows (for comparison) the results on the antioxidative ability of two standard substances, trolox (**Figure 5A**), BHT (**Figure 5B**), and their equimolar mixtures with OTC (after 30 min of oxidation induction time). Trolox is a hydrophilic antioxidant, whereas BHT is more hydrophobic than hydrophilic and not very soluble in water. The dependence of PC liposomes oxidation inhibition on exposure time to UV

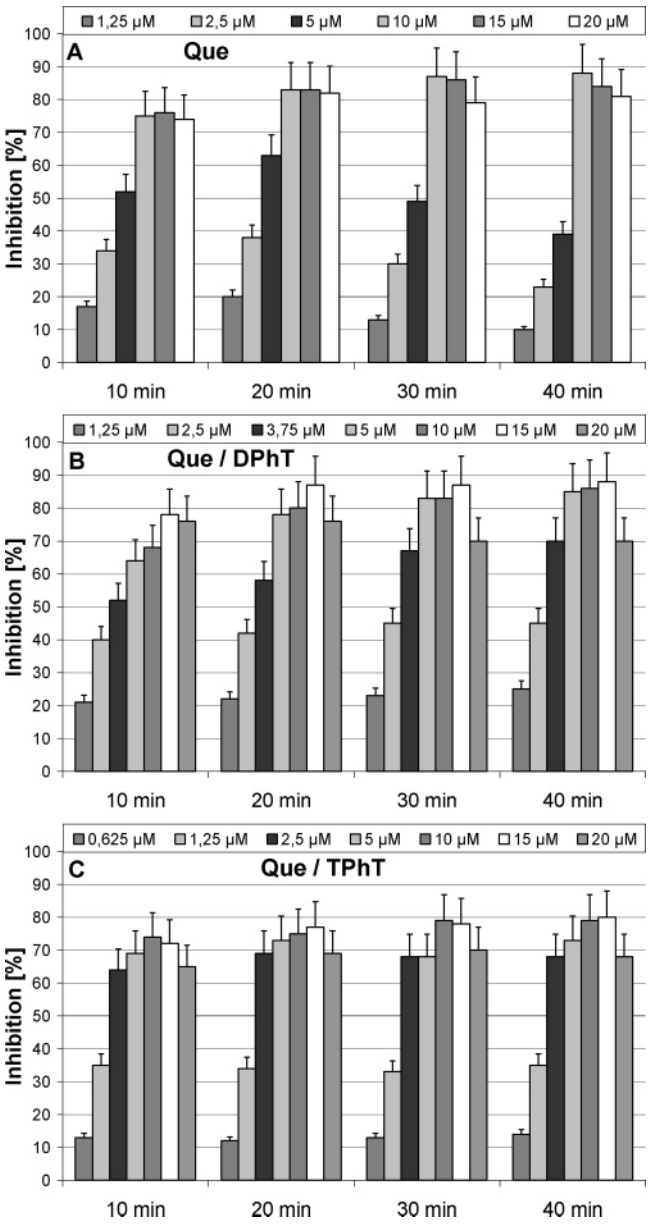

Figure 4. Dependence of oxidation inhibition on the time of UV radiation of PC liposomes in the presence of (**A**) quercetin, (**B**) quercetin with DPhT (1:1), and (**C**) quercetin with TPhT (1:1). The concentration of the compounds added changed in the range from 0.625 to 20 μM, as indicated. The data, from three repeated experiments (n = 6), represent the relative peroxidation as compared to its control (without the compounds added). The mean standard deviation did not exceed 15%.

radiation is shown for the compounds studied at different concentrations (in the range from 0.625 to 20 μM). The level of oxidation inhibition by Que reached 86%. In comparison to 15 μM (and also to 10 μM) above 20 μM concentration, Que showed a decreased effectiveness of photooxidation inhibition. It is also observed that after 20 min of UV exposition and for 1.25, 2.5, and 5 μM concentrations of Que, the antioxidative efficiency decreases with the time of UV exposure. The degree of the decrease reaches the value of ca. 40%. The antioxidative action of equimolar mixtures of Que with OTC on liposome membranes oxidated with UV radiation is shown in **Figure 4B** (Que/DPhT) and **Figure 4C** (Que/TPhT). From comparison of the relationships in panels **A** and **B** of **Figure 4**, it follows that for lower concentrations of the Que/DPhT mixture (0−5 μM) the stronger protective action of Que takes place than the action

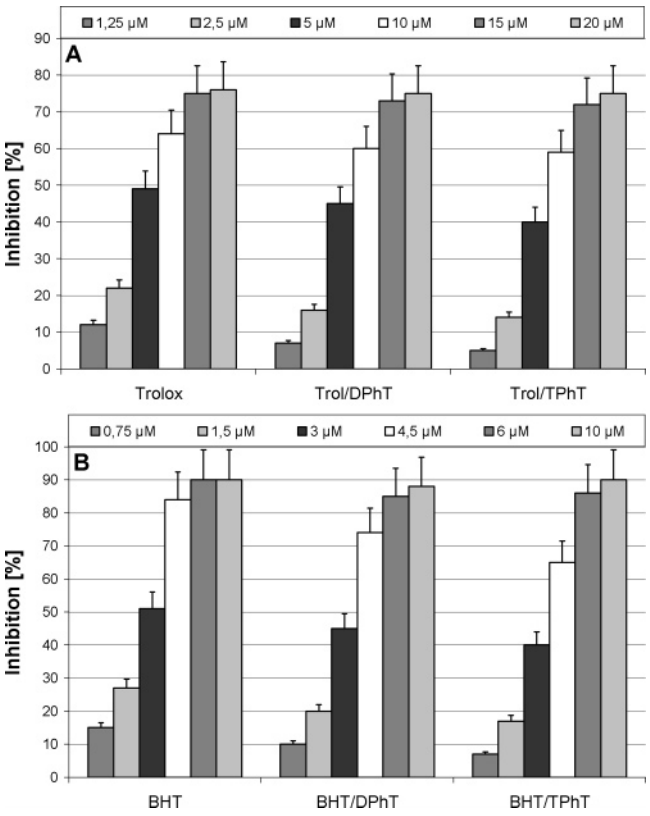

Figure 5. Dependence of oxidation inhibition on the time of UV radiation of PC liposomes in the presence of (**A**) trolox and its mixture with DPhT and with TPhT and (**B**) BHT and its mixture with DPhT and with TPhT. The concentration of the compounds added changed from 1.25 to 20 μM (trolox) and from 0.75 to 10 μM (BHT), as indicated. The data, from three repeated experiments ($n = 6$), represent the relative peroxidation as compared to its control (without the compounds added). The mean standard deviation did not exceed 15%.

of Que alone at the same concentrations. The concentration of 20 μM Que/DPhT is, as for Que alone, less effective in PC liposome protection against photooxidation. The decisively greater antioxidative properties against photooxidation of PC liposomes were shown by mixtures of Que with TPhT (**Figure 4C**) in comparison to Que alone and Que/DPhT mixture at the low concentrations (0−5 μM). Additionally, at all concentrations of the mixtures used in the study, the percentage of oxidation reduction did not decrease in time. Moreover, in **Table 1**, the parameters IC_{50} (in μM) are collected, that is, concentrations at which 50% inhibition of oxidation occurs. For these parameters (calculated on the basis of a graphic relationship between oxidation percent at the 30th min of oxidation and antioxidant concentration), the activity sequence was obtained as follows: Que:TPhT > Que:DPhT > Que (as IC_{50} values are in sequence: 1.9 ± 0.08 μM < 2.9 ± 0.12 μM < 5.1 ± 0.10 μM); the differences between the values are significant statistically.

The antioxidative properties of trolox, BHT, and its equimolar mixtures with OTC against PC liposomes oxidation induced by UV radiation are shown in **Figure 5**. There was shown in it % of inhibition of liposome oxidation dependence on the concentration of antioxidants (in the range from 0.75 to 20 μM, as indicated) for a single oxidation time equal to 30 min. Taking into account IC_{50} values (as shown in **Table 1**), we can state that inhibition of trolox and its OTC mixtures in comparison to Que and its OTC mixtures is lower. The protection of liposomes against OTC free radicals or against UV in case of Que and its mixtures is better than in the case of trolox and its OTC mixtures. IC_{50} values for trolox and its OTC mixtures do not differ significantly. Antioxidant properties of BHT and Que and also of BHT/OTC and Que/OTC can also be read from their IC_{50} values presented in **Table 1**. The comparison of the values for BHT (2.7 ± 0.11 μM) with that for Que (5.1 ± 0.10 μM) show that BHT is a better antioxidant than Que, and this fact is statistically significant. However, in the case of their OTC mixture, such a judgment is unjustified. Both Que/OTC mixtures have significantly lower IC_{50} values (Que/DPhT, 2.9 ± 0.12 μM; Que/TPhT, 1.9 ± 0.08 μM) than IC_{50} values for BHT/OTC mixtures (BHT/DPhT, 3.1 ± 0.11 μM; BHT/TPhT, 3.6 ± 0.10 μM). As it follows from above, BHT in the presence of OTC is a worse antioxidant than Que/OTC mixtures and this difference is statistically significant.

However, to suggest a probable mechanism of the antioxidant action of the compounds studied, it was necessary to investigate their ability to scavenge the free radical $DPPH^{\bullet}$ in spontaneous redox reactions. The results of such experiments are presented in **Figure 6**. **Figure 6** shows the dependence of percent reduction of the free radical $DPPH^{\bullet}$ after 1 h of incubation with an antioxidant in methanol as a function of the reducer concentration (Que and Que/OTC). On the basis of the relationships of **Figure 6**, the concentrations were determined (IC_{50}, in μM) that cause 50% quenching of $DPPH^{\bullet}$ by the antioxidant. The values of the parameters IC_{50}^{DPPH} given in **Table 1** constitute the following sequence of antiradical activity: Que/TPhT > Que/DPhT > Que.

In order to explain the action of the DPhT and TPhT mixture with Que, the possibility of complex formation between these molecules was investigated. A representative set of electron absorption spectra of Que titrated with DPhT compound (**A**) and TPhT (**B**) in methanol can be seen in **Figure 7**. Dependencies similar to those in **Figure 7** were also obtained for the process of complex formation between Que and DPhT or TPhT in a liposome suspension (spectra not presented here). In **Figure 7A**,**B**, one of the peaks coming from Que is marked ΔA_{QUE}, which absorption decreased during titration with DPhT (and TPhT), and a new peak marked ΔA_{CO} assigned the complexes. These are evidence of association of Que molecules with DPhT (**A**) and TPhT (**B**). The peak absorption from a complex increased with an increasing concentration of DPhT (in **Figure 7A** the final concentration ratio Que/DPhT reaching 1:1.8) and TPhT (in **Figure 7B** the final concentration ratio Que/TPhT reaching 1:28). The obvious parameter of the phenyltin complex formation is a binding constant K (*36*). Values of K are given in **Table 1**. It can be noticed that the binding constants for Que/DPhT in metanol ($K^{met} = 31350 \pm 4200$ M^{-1}) and in PC liposomes samples ($K^{PC} = 28900 \pm 2200$ M^{-1}) are comparable and differ insignificantly but the binding constants for Que/TPhT are significantly lower and differ by 2 orders of magnitude ($K^{met} = 300 \pm 40$ M^{-1}, $K^{PC} = 16740 \pm 2140$ M^{-1}). From quantum mechanical computations, we can judge that the reason for the stronger binding of Que/DPhT complex is a five member ring formed in this supermolecule in comparison to quasi-hydrogen bonds in the Que/TPhT complex.

The possible places of coordination of OTC with the Que molecule follow also from measurements conducted with the ^1H NMR method on mixtures of Que with DPhT and TPhT in DMSO. The ^1H NMR spectra of Que/DPhT (1:1) mixture and pure Que and DPhT are tabulated. Visible is (**Figure 8 A**) a broadening of respective signals coming from the C5 and C═O groups and C3′ and C4′ in the spectrum from Que/DPhT

Table 1. Antioxidative (IC_{50}), Antiradical Activities with Respect to DPPH Free Radical (IC_{50}^{DPPH}) and Binding Constants *K* of DPhT and TPhT to Quercetin in Methanol and in PC Lipid Membranes Samples[a]

	μM				K (M^{-1})	
compounds	IC_{50}^{PC}	IC_{50}^{trolox}	IC_{50}^{BHT}	IC_{50}^{DPPH}	methanol	lipid
Que	5.1 ± 0.10	7.4 ± 6.9	2.7 ± 0.11	8.2 ± 0.13		
Que/DPhT	2.9 ± 0.12	8.7 ± 7.7	3.1 ± 0.11	7.4 ± 0.40	31350 ± 4200	28900 ± 2 200
Que/TPhT	1.9 ± 0.08	9.3 ± 8.5	3.6 ± 0.10	6.1 ± 0.20	300 ± 40	16740 ± 2 140

[a] IC_{50} and IC_{50}^{DPPH} denote the antioxidant concentration causing 50% inhibition of liposome membrane oxidation after 30 min of UV radiation and 50% reduction in the free radical form of DPPH• in methanol after 1 h of incubation with an antioxidant, respectively. The details of IC_{50} parameters and the binding constants *K* determination are described in the Materials and Methods.

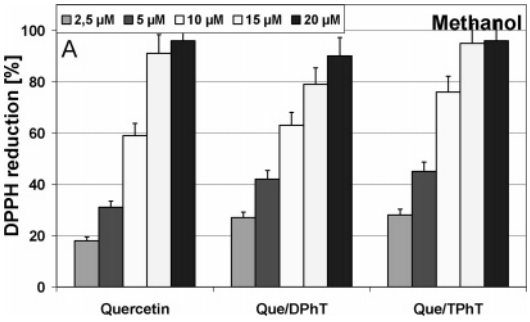

Figure 6. Dependence of the free radical DPPH reduction present in the methanol solution with quercetin or their equimolar mixture with DPhT and TPhT on the antioxidant concentration. The amount of the compounds added changed in the range from 2.5 to 20 μM, as indicated. The data are averages of six or four probes. The mean standard deviation did not exceed 8%.

mixture, whereas no such changes are visible in the case of the spectrum from the Que/TPhT mixture (**Figure 8B**).

The existence of intermolecular interactions in the Que/TPhT and Que/DPhT mixtures was also confirmed by studies conducted with the IR method. **Figure 9** presents an IR spectrum coming from the C═O group (**Figure 9A**, left panel, wave number 1660 cm^{-1}) and C−OH groups (**Figure 9A**, right panel, wave number 1230−1480 cm^{-1}) of Que molecules and equimolar mixtures of Que with TPhT (**Figure 9B**) and DPhT (**Figure 9C**). In an insert in **Figure 9** for the TPhT (**Figure 9B**, lower plot) and DPhT (**Figure 9C**, lower plot) mixtures, there are differential curves (mixture band minus Que curve). In **Table 2** are given frequencies (expressed in cm^{-1}) of oscillations for bands of the groups C═O and C−OH of the Que molecule and its equimolar mixtures with TPhT and DPhT. The frequency band of C═O of Que (**Figure 9A**, bold line) is divided into two components: one of 1666 cm^{-1} frequency (33% share) and the other 1656 cm^{-1} (67% share). The band of the carbonyl group of the equimolar mixtures Que/TPhT (**Figure 9B**) has been in fact shifted a little with a concurrent small change in the component intensities of the band. The differential spectrum in the lower part of **Figure 9B** (left panel) illustrates well the slight change in the band. Displacement of the same band in the mixture Que/DPhT is substantially greater as compared to the changes observed with the Qur/TPhT mixture. In this case, only one component remains in the band (**Table 2**) and relatively large changes are presented by the differential curve (**Figure 9C**, left panel). In the Que IR spectrum, the frequency band ascribed to the C−OH groups (**Figure 9A** and **Table 2**) are observed, among others, as frequencies ascribed to the groups C3′−OH, C4′−OH, and C7−OH (1475−1415 cm^{-1} range), C5−OH and C3−OH (1415−1340 cm^{-1} range), and C3′−OH and C5−OH (1340−1290 cm^{-1} range). In the case of the Que/TPhT interactions, there were found basically only changes referring to the band 1475−1415 cm^{-1} that are clearly visible

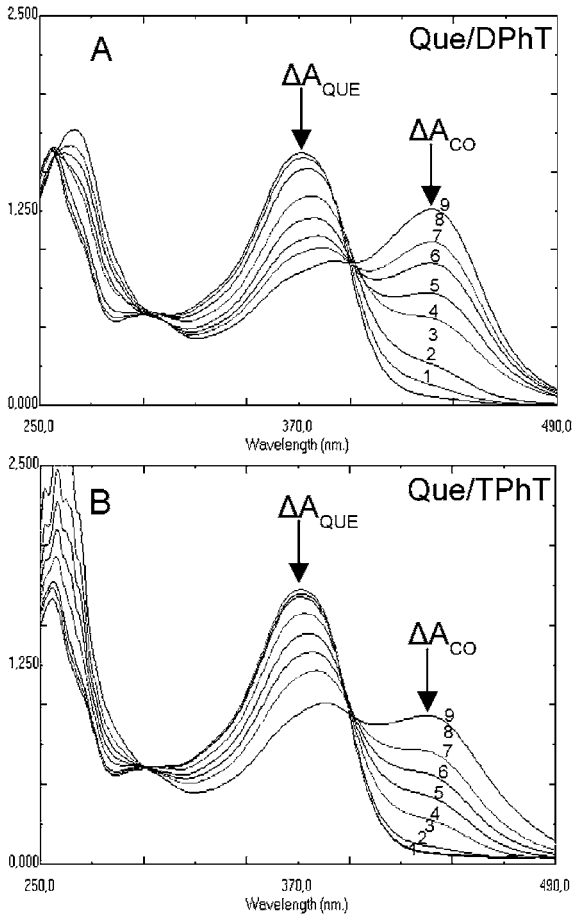

Figure 7. Compilation of the electron absorption spectra of quercetin in methanol, titrated with DPhT (**A**) or TPhT (**B**). The final concentration ratio for Que/DPhT was 1:1.8, and for Que/TPhT, it was 1:28. The initial concentration of quercetin was 49 μM. The peaks coming from quercetin (ΔA_{QUE}) and complex (ΔA_{CO}) are indicated. The concentration of the OTC was as follows. For DPhT: 1, 9.7; 2, 19.3; 3, 28.8; 4, 38.3; 5, 47.6; 6, 56.9; 7, 66.0; 8, 75.1; and 9, 84.1 μM. For TPhT: 1, 38.6; 2, 95.2; 3, 272.0; 4, 434.7; 5, 583.3; 6, 720.0; 7, 846.2; 8, 963.0; and 9, 1018.2 μM, as indicated.

on the differential plot (**Figure 9B**, right panel, lower plot) and compiled in **Table 2**. A displacement of two peaks of the band occurred toward higher frequencies, and their intensities increased. In the case of the Que/DPhT mixture, relatively large changes are found in the whole range of the C−OH group spectra. On the basis of the differential spectrum (**Figure 9C**, right panel, lower plot), an observed fact is, among others, a disappearance of the component of 1371 cm^{-1} frequency and a marked reduction in intensity of another component together with its shift from 1317 to 1321 cm^{-1}. Also, in the IR spectra of TPhT and DPhT (not presented here), larger differences were

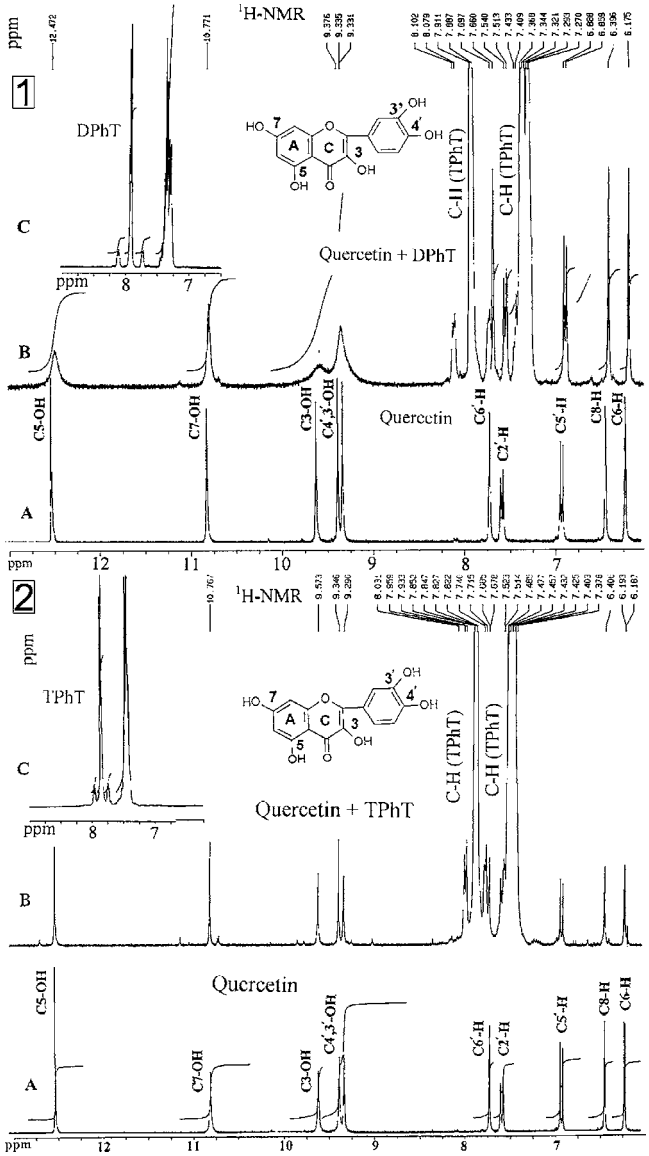

Figure 8. ^{1}H NMR spectra (300 MHz). Upper panel (1): (**A**) quercetin with DMSO, (**B**) quercetin with DPhT mixture (their molar ratio was 1:1), and (**C**) DPhT compounds. Lower panel (2): (**A**) quercetin with DMSO, (**B**) quercetin with the TPhT mixture (molar ratio was 1:8), and (**C**) TPhT compounds.

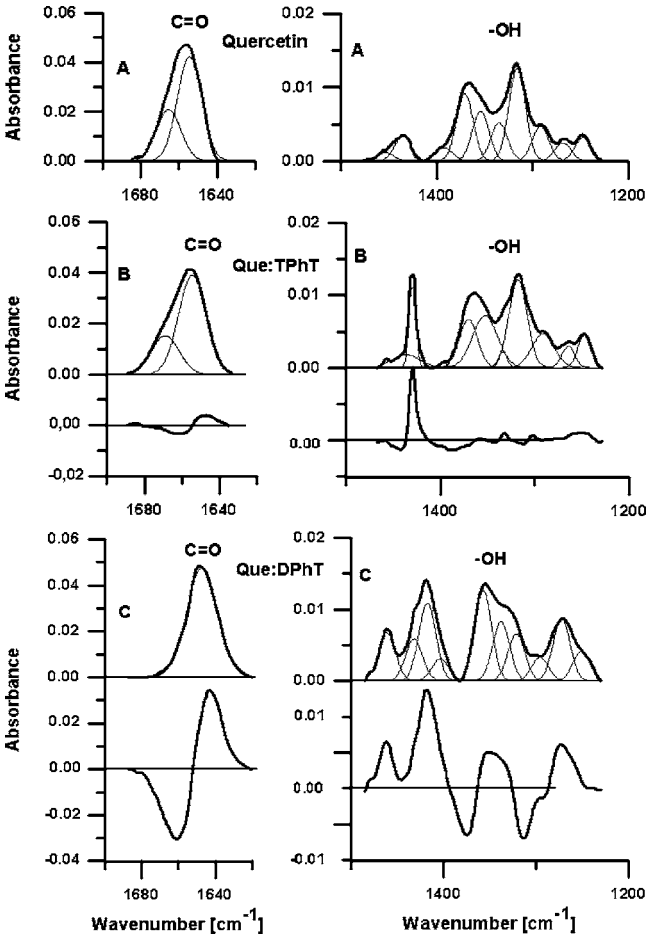

Figure 9. IR spectra of quercetin and their equimolar mixture with phenyltin compounds after evaporation of the methanol solvent at room temperature. Left panel: bands coming from –C═O group, (**A**) quercetin, (**B**) quercetin with TPhT (1:1; lower differential curve), and (**C**) quercetin with DPhT (1:1; lower differential curve). Right panel: bands coming from C–OH groups, (**A**) quercetin, (**B**) quercetin with TPhT (1:1; differential curve lower), and (**C**) quercetin with DPhT (1:1; differential curve lower).

found for the Que/DPhT (1:1) mixture than for the Que/TPhT (1:1) mixture. Thus, it was found that the frequency of Sn-phenyl oscillations (1428.2 cm^{-1}) undergoes only a small change (to 1429.9 cm^{-1}) in the case of the TPhT/Que mixture (1:1) while the signal corresponding to Sn-phenyl oscillations in the case of the DPhT/Que mixture is shifted from 1432 to 1418.8 cm^{-1}.

To explain the diversity in the antioxidative action of the studied compounds with respect to PC liposome membranes, the anisotropy changes due to the absorbed antioxidants were determined in the fluorescence of the DPH probe present in the liposome membrane. The results of the changes in anisotropy are presented in **Figure 10**. The control value (0.117) of anisotropy in PC membrane at 25 °C is in a good agreement with results obtained by other authors (*51*). The largest changes in anisotropy are caused by the mixture Que/DPhT. They depend on the mixture concentration and reach values of 0.300 (i.e., a relative increase by 156%) in the case of anisotropy increase caused by (the highest) 5 μM concentrations of Que/DPhT. Smaller changes (but statistically significant) in the fluorescence anisotropy of the DPH probe were induced by the Que/TPhT mixture (at higher concentration ranges from 3 to 5 μM). In that case, the relative increase in the anisotropy reached the value 0.274 (i.e., a relative increase by 134%) for the 5 μM concentration of Que/TPhT. Changes in DPH probe parameter induced by Que alone (anisotropy reaches the value 0.242 for 5 μM of Que) are smaller (statistically significant) than those induced by both mixtures; thus, for instance, the anisotropy decreased by 49% for 5 μM Que, in comparison to the action of Que/DPhT at the same concentration.

Quantum mechanical computations indicate that Que and DPhT form a complex in water medium due to formation of a five member ring involving the tin atom of DPhT coordinating to oxygens of 3′- and 4′-hydroxyl groups in ring B of Que (see **Figure 11 A**). This result is in complete agreement with the one obtained by the ^{1}H NMR method. In **Figure 11A**, one can see that formation of the complex occurs due to relatively strong electrostatic interaction between positively charged tin and negatively charged hydroxyl oxygens and this interaction is compensated by mechanical deformation of DPhT (*23*). The bonding distances between Sn and first carbons in the phenyl rings of DPhT are 2.127 Å and are quite comparable to distances of coordination of Sn to hydroxyl oxygens of Que, which are 2.230 Å. We can conclude that formation of the Que/DPhT complex in water is more stable than formation of the Que/

Table 2. Compilation of Component Frequencies (expressed in cm^{-1}) and Their Percent Shares in the IR Spectra of Que for Bands Coming from the Groups C═O and C–OH and in the Spectra of Equimolar Mixtures of Que with DPhT and TPhT

Que, C═O		Que/TPhT		Que/DPhT	
maximum (cm^{-1})	content (%)	maximum (cm^{-1})	content (%)	maximum (cm^{-1})	content (%)
1666	33.0	1669	28.0		
1656	67.0	1654	72.0		
				1647	100.0

Que, C–OH		Que/TPhT		Que/DPhT	
maximum (cm^{-1})	content (%)	maximum (cm^{-1})	content (%)	maximum (cm^{-1})	content (%)
1454, C3′C4′	2.3	1437	6.2	1461	9.6
1436, C7	6.7	1430	8.5	1432	8.3
				1418	15.5
1392, C5	3.7	1396	0.5	1404	4.4
1371, C3	17.9	1370	14.3		
1354	13.4	1352	20.0	1357	18.1
1335	10.4	1334	2.1	1337	11.8
1316, C3′	24.8	1318	25.2	1321	9.4
1291, C5	9.4	1391	12.0	1295	4.7
1268	5.0	1264	4.4	1272	12.4
1247	6.4	1247	6.9	1251	5.9

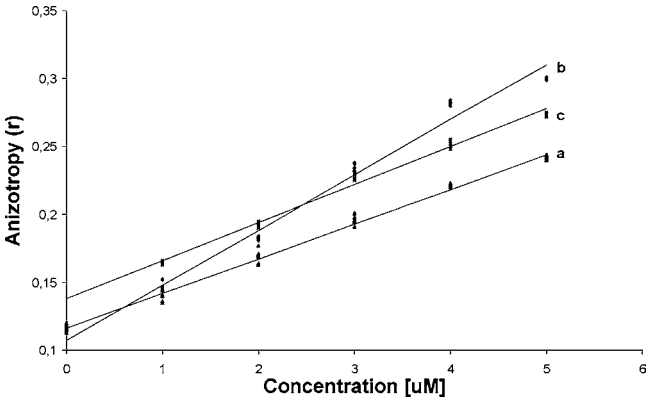

Figure 10. Anisotropy coefficients (*r*) vs the concentration of quercetin (a) and their equimolar mixtures with DPhT (b) or TPhT (c) present in the dispersion of the liposome membrane with a fluorescent probe DPH at 25 °C. Each experiment was repeated twice ($n = 6$). Linear regression lines were fitted by the least squeres method: Que ($R = 0.992$), Que/DPhT ($R = 0.997$), and Que/TPhT ($R = 0.998$).

TPhT complex (see **Figure 11B**), where weaker electrostatics and greater mechanical stiffness of TPhT causes coordination of positively charged tin of TPhT and negatively charged oxygens of Que and is not possible without mechanical work for such steric adoption of TPhT (*23*). The quantum mechanical computations show that the Que/TPhT complex in water is formed by two quasi-hydrogen bonds of phenyl group hydrogens of TPhT to hydroxyl and carbonyl oxygens of Que (H···O distance 2.463 Å). Such H···O interaction is also of electrostatic character and does not involve mechanical deformations, which are energetically expensive; therefore, the complex of Que/TPhT is weaker than the Que/DPhT complex. As one can expect, it can be formed in three possible ways due to C_3 symmetry of the TPhT cation. Because of general features of minimization algorithms, one cannot guarantee that the molecular structures obtained by modeling methods correspond to their global minima or to one of local ones. **Figure 11B** shows the one of the tree local minima geometries of the Que/TPhT complex. There are probably other local minima geometries of the Que/TPhT complex in water. The existence of more than one local minima geometries results, in general, in weaker experimental evidence of a single one, and we have not found the evidence of the Que/TPhT complex in conformation shown in **Figure 11B** in ^{1}H NMR results. In addition, as follows from our IR results, the existence of other geometries of Que/TPhT complexes is not excluded.

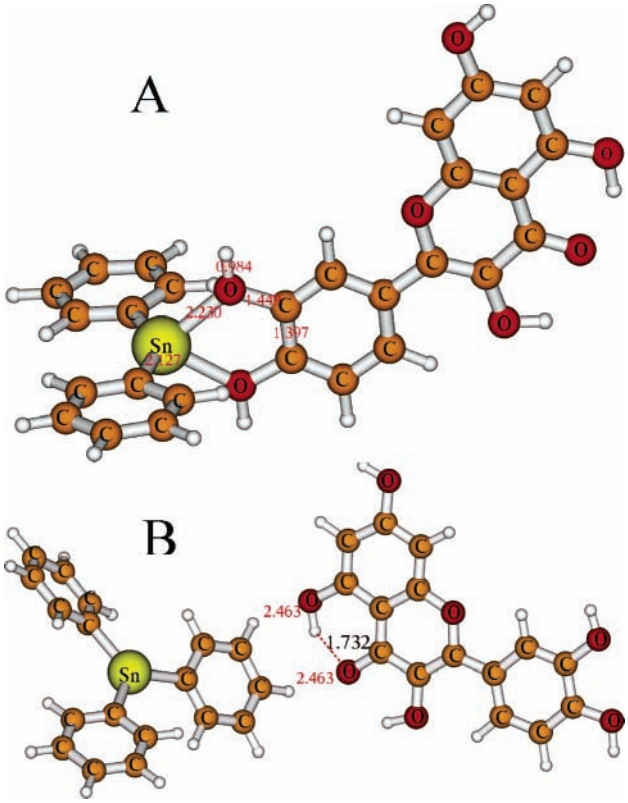

Figure 11. Complexes of quercetin with DPhT (**A**) and TPhT (**B**) were obtained with the quantum mechanical DFT method in water (see Materials and Methods). In panel **A**, there are indicated distances (in Å) between the coordinating tin atom in the DPhT molecule and the oxygen atoms of two hydroxyl groups in the ring B of quercetin (in positions C3′ and C4′). In panel **B** are indicated (in Å) distances between two hydrogen atoms of the phenyl ring in TPhT and two oxygen atoms of quercetin (of the hydroxyl group in position C5 and the carbonyl grouping in position C4 of quercetin).

DISCUSSION

In our previous paper (*18*), we presented results (which are cited in this paper as **Figure 3**) on the prooxidative effect of DPhT and TPhT compounds exposed to UV radiation on liposome membranes. We suggested that such action of the phenyltins results most probably from the compounds becoming free radical forms in the destruction process. The present results obtained using the EPR method (deemed qualitative only), shown in **Figure 2**, confirm this supposition. The EPR signals UV radiated DPhT (**Figure 2A**) and TPhT (**Figure 2B**) are characteristic for free radical forms of the compounds. However, the control spectra presented in **Figure 2C**,**D** (for unradiated DPhT and TPhT) do not show such character. The results presented in **Figure 3** also indicate that the peroxidative effect of OTC on PC liposomes (during exposition to UV) is, among others, connected to the attack of free radicals (most probably the phenyl ones, Ph) that are formed during photodestruction of OTC by UV (*52*−*54*).

The oxidation process is effectively inhibited by Que. The results on the protective action of Que as an antioxidant (**Figure**

4) clearly indicate that Que annihilates the oxidative effects of DPhT and TPhT induced by UV. Moreover, its antioxidative action at lower concentrations, that is, at 0−5 μM, is characterized by a higher level of inhibition in the presence of TPhT and DPhT than in their absence. This differentiated protective efficacy of Que in the presence of OTC relative to the antioxidative action of Que without OTC (statistically significant) is well-characterized by IC_{50} (**Table 1**). They form an activity sequence (Que/TPhT > Que/DPhT > Que), which is exactly opposite to sequences obtained for equimolar mixture of standard antioxidants with OTC (BHT > BHT/DPhT > BHT/TPhT and trolox > trolox/DPhT > trolox/TPhT). It should be noticed here (when comparing IC_{50} values) that Que exhibited a lower antioxidative activity than BHT (statistically significant) and higher than trolox, although statistically insignificant. The differentiated high protective efficacy of both the Que and the standard substances employed (BHT and trolox) depends on many factors, among others, on the molecular structure and ability of the respective compounds to scavenge free radicals, as well as on different antioxidant locations with respect to the membrane, which is attacked by free radicals from the outside. The higher antioxidative efficacy of BHT ($IC_{50} = 2.7 \pm 0.11$ μM) with respect to Que ($IC_{50} = 5.1 \pm 0.10$ μM) (both of the molecules located in the bilayer) (*55*, *56*) is to be ascribed to the protection of the hydroxyl group against destruction offered by the special steric hindrance (di-*tert*-butyl groups). Trolox, as a substance soluble in water, prevents liposomes against free radicals, if its concentration is high enough.

The inhibitive action of Que with respect to liposome membranes subjected to oxidation induced by DPhT and TPhT under UV results most probably, as indicated earlier, from different mechanisms of the action of flavonoids. One of them is Que's ability to scavenge free radicals. Such was the suggestion raised on the basis of the relationship obtained from studies on the antiradical activity of Que and its equimolar mixtures with DPhT and TPhT with respect to the free radical DPPH$^{\bullet}$ (**Figure 6**). Values of the parameters IC_{50}^{DPPH} (Que = 8.2 ± 0.13, Que/DPhT = 7.4 ± 0.40, and Que/TPhT = 6.1 ± 0.20) determined in relation to that radical (**Table 1**) constitute a sequence (statistically significant) of the compound's activity (Que/TPhT > Que/DPhT > Que), which is in good correlation with that obtained in the antioxidant activity studies. This concordance of the relationships confirms our earlier supposition that the mechanism of the antioxidative activity of Que and its mixture with phenyltins is due to free radical scavenging. The IC_{50}^{DPPH} values for trolox and BHT are 23.5 ± 0.40 and 50 ± 0.44 μM (and do not change when there is OTC in the medium), which is not entirely in accord with the antioxidative activity series for BHT. Que and trolox set on the basis of diminishing IC_{50} values for those antioxidants (**Table 1**).

A second possible mechanism of the antioxidative action of Que with DPhT and TPhT is most probably connected with Que's chelating properties. From the literature, it follows that because of the complexation by flavonoids of metals such as Cu(II) and Fe(II) (*17*, *28*, *38*, *39*), these metals are devoid of the possibility to participate in free radical formation (e.g., $^{\bullet}$OH). By creating complexes with DPhT and TPhT (bands resulting from such complexes can be seen in **Figure 7**, and binding constants of Que in the presence of PC are higher for DPhT and lower for TPhT), Que exerts a protective action on membranes, annihilating the prooxidative effect of DPhT and TPhT in the presence of UV. Such chelating properties do not possess the applied here antioxidants trolox and BHT (our unpublished experiments indicate this). Hence, at lower concentrations of the antioxidants in the presence of OTC (**Figure 5A**,**B**), they exhibit weaker antioxidant properties than in the absence of OTC (however, Que shows exactly opposite properties; that is, in the presence of OTC, the antioxidant action of Que is more effective than without OTC).

In order to estimate the possibility of the interaction between Que and DPhT and TPhT in the complexes formed, we took the ^{1}H NMR spectra (**Figure 8A**,**B**), which showed that Que/DPhT complexes (in DMSO) are stronger than those with TPhT. They also indicated Que/DPhT coordination at probable places and did not show such places for Que/TPhT coordination, possibly due to a much weaker coordination of the molecules with each other as compared with the Que/DPhT complex. Thus, it is probable that Que forms complexes with DPhT, via hydrogen bonds, with the carbonyl and hydroxyl groups in the C3 position of the molecule and its dihydroxy groupings (C3′ and C4′) with the B ring. Also visible is a marked broadening of the signal in position C3 of the molecule and a smaller but visible broadening of the signal in positions C3′ and C4′ of Que present in the equimolar mixture with DPhT.

Interactions between Que and TPhT and Que and DPhT also follow from the IR method studies (**Figure 9** and **Table 2**). In the case of the band (ca. 1660 cm^{-1}) from the C═O group in the IR spectrum of Que, the two component parts obtained from deconvolution (of 33 and 67% intensity) most probably correspond to "pure vibrations" of the carbonyl groups and those which form hydrogen bonds, for example, as a result of partial hydration of Que or interaction with neighboring hydroxyl groups in the molecule (*57*−*59*). The intensity of the two component parts undergoes small change and a shift toward lower frequencies, indicating a relatively weak interaction between the Que and the TPhT molecule. Instead, stronger Que/DPhT interactions result in markedly greater changes in the frequency range corresponding to C═O groups. The disappearance of two components of that band with the concurrent formation of only one and its marked displacement toward lower frequencies indicate that DPhT induces interactions with all Que molecules both via the positive tin ion and via the benzene rings that participate in hydrogen bonds of the π type (*60*, *61*). The study of the changes in the IR spectrum of Que induced by interaction with TPhT molecules within the band corresponding to the C−OH group frequencies (divided into three intervals) is concerned, in principle, with a shift of only one of the bands (in the range 1475−1415 cm^{-1}) toward lower frequencies and increased intensity. This range is reported (*58*) to come from hydroxyl groups in positions C3′ and C4′ and C7. It is, thus, probable that the coordination of TPhT to Que is connected (aside from interactions with the carbonyl group and neighboring −OH groupings in C3 and C5 positions) with dihydroxy groups in the B ring of a molecule. The changes in the IR spectrum of Que induced by DPhT in the −OH group frequency band are much greater than those induced by TPhT (**Figure 9C**, lower panel) and refer basically to the whole range. Both the disappearance of some frequencies (1371 cm^{-1}) and the marked reduction of other frequencies (1317 cm^{-1}) and also displacement and an increase in intensity of other components (1460 and 1432 cm^{-1}, 1357 and 1272 cm^{-1}) may be seen as proof of the existence of strong coordination of a DPhT molecule (probably via the tin cation) both to the catecholic grouping in the B ring of the Que molecule (**Figure 11B**) and to other groupings, including the carbonyl group.

The diversified effectiveness of Que (in the presence of DPhT and TPhT) in its action on liposome membranes undergoing peroxidation may be connected with different degrees of

incorporation of Que complexes with OTC into the membranes (**Figure 10**). The large relative changes in anisotropy (**Figure 10**) of the probe DPH anchored in the hydrophobic region of the liposome membrane bilayer induced by the associates of Que/DPhT and smaller (but statistically significant) changes caused by Que/TPhT molecules suggest a different degree of liposome stiffness in its interaction with both of the associates. This may indicate a deep localization of the associates Que/DPhT in the membrane (in the border of the hydrophobic and hydrophilic region) and a localization of Que/TPhT associates much closer to the surface. Such localization is in line with the results of our earlier studies (*23*). We have shown that the more hydrophobic, umbrella-like TPhT molecule locates, for steric reasons, closer to the surface of the polar membrane region, while the less hydrophobic, but more elastic, DPhT molecule permeates deeper into the polar region of the bilayer. This favors the hypothesis that the Que/TPhT associates assume a strategic position near the surface that enables them to scavenge free radicals approaching the membrane. Also, Que molecules (like those of Que/TPhT associates) are located in the polar part, occupying that area according to a certain molecular distribution that requires the molecules to locate both at the membrane surface and within its hydrophilic region.

At lower concentrations (0−5 μM), an amount of Que is exposed to the destructive action of UV radiation (our unpublished results indicate at ca. 15%, whereas in the presence of OTC it exceeds 7%) and the rest is not sufficient to protect the membranes very well against oxidation. Hence, Que at low concentrations is least effective—the percent of inhibition changes in the range from 22 to 62%. It is higher with low (0−5 μM) Que/DPhT concentrations (in the range from ca. 40 to 83%) and even more so in the case of Que/TPhT (in the range from 64 to 72%). It is most probably due to complexes of Que with OTC that molecules within a complex stabilize each other, not allowing high photodestruction on the one hand while enforcing proper localization within the membrane on the other hand. It seems that the possible adsorption in the liposome membrane bilayer of Que and its complexes with the compounds DPhT and TPhT justifies the name "interface antioxidants", i.e., those locating in the polar region of the bilayer near the hydrophobic−hydrophilic border (*62*). In fact, they exhibit a higher antioxidant activity than does trolox (very soluble in water antioxidant—**Figures 4** and **5A**) but of higher or comparable (at low concentrations) or smaller activity (at higher concentrations) than BHT (poorly water soluble antioxidant—**Figures 4** and **5B**).

In summary, one can say that the results presented indicate a protective role of Que toward PC liposome membranes against the consequences of the free radical action of DPhTs and TPhTs on membranes exposed to UV radiation. On the basis of the results obtained, we suggest that high protection of liposome membranes by Que results from its chelating properties toward OTC and localization of the complexes Que/OTC in the bilayer that make it more stiff.

ABBREVIATIONS USED

PC, egg yolk phosphatidylcholine; Que, quercetin; TPhT, triphenyltin chloride; DPhT, diphenyltin dichloride; TBARS, thiobarbituric reactive substances; DPPH, 1,1-diphenyl-2-picrylhydrazil; DMSO, dimethyl sulfoxide; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; BHT, butylated hydroxytoluene; IR-ATR, infrared attenuated total reflectance spectroscopy; DFT, density functional theory.

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