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Antioxidative Effect of Kaempferol and Its Equimolar Mixture with Phenyltin Compounds on UV-Irradiated Liposome Membranes

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The work investigates the possibility of the protective action of kaempferol on phosphatidylcholine liposome membranes exposed to the pro-oxidative action of diphenyltin dichloride (DPhT) and triphenyltin chloride (TPhT) induced by UV radiation ($\lambda = 253.7$ nm). The concentrations of kaempferol and its equimolar mixtures with DPhT and TPhT were determined so that they induce 50% inhibition in oxidation of liposomes irradiated with UV. They are 11.6, 10.0, and 4.5 μM/L, which constitute the following sequence of antioxidative activity: kaempferol/triphenyltin > kaempferol/diphenyltin > kaempferol. This relationship is confirmed by the results on the antiradical ability of kaempferol and its mixtures with DPhT and TPhT toward the free radical 2,2-diphenyl-1-picrylhydrazyl. Similar sequences obtained in both studies suggest a possible mechanism of the antiradical action of the mixtures as free radical scavengers. Kaempferol's ability, then documented, to form complexes with phenyltins indicates (a) a possible way to liquidate the peroxidation caused by the free radical forms of phenyltins and (b) the stabilizing role of chelating in the antioxidative action of the kaempferol/phenyltins. The differentiation in the action of the compounds studied may, among others, result from different localizations in the liposome membrane, which is indicated by the results of the fluorometric studies.

KEYWORDS: Kaempferol; phenyltin; antioxidants; complexes; fluidity; liposomes

INTRODUCTION

Ultraviolet radiation is in general harmful to biological membranes (*1*−*8*). It is thought that the main cause of structural and functional changes induced in membranes by the radiation is membrane lipid peroxidation (*9*−*11*). This is why many studies on the interaction between UV radiation and membranes have been conducted on model lipid membranes (*11*−*14*). In turn, heavy metals also disorganize biological membranes, affecting membrane lipids among others (*15*). In particular, they may cooperate with UV radiation, the effects of the cooperation being diverse depending on the kind of metal and membrane (*16*−*18*). 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 (*19*−*21*). Our previous studies on the effect of organic tin and lead compounds on biological and model membranes (*22*−*28*) 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 on phosphatidylcholine (PC) liposomes and how to counteract that effect by the protective action of a selected flavonoid such as kaempferol.

Studies on the antioxidative action of flavonoids—a group of compounds not yet well-known in this respect—have been conducted very intensely in recent years (*21*, *29*−*34*). However, there are, as yet, no studies on the mutual interaction between flavonoid substances, which are present in plant cells and have antioxidative properties, and compounds of the organometallic group present in the environment due to their use as, for example, fungicides (*35*), biocides (*36*), antifouling agents (*37*, *38*), and wood preservatives and a plastics stabilizer (*39*) that may affect membrane lipid peroxidation. Kaempferol is a representative of flavonoids that occur in the human diet in, among others, endives, leeks, broccoli, teas, grapefruit, strawberries, and gooseberries (*30*, *40*).

In the present work the pro-oxidative behavior has been shown of diphenyltin dichloride (DPhT) and triphenyltin chloride (TPhT), induced by UV radiation at a wavelength of ∼254 nm with respect to PC liposome membranes. The

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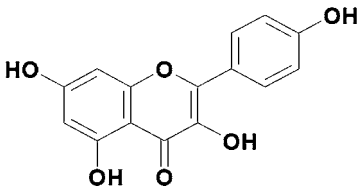

Figure 1. Chemical structure of the kaempferol molecule.

possibility of protective action by kaempferol toward a membrane exposed to both UV radiation and, additionally, the DPhT and TPhT compounds was investigated. The antioxidative action of equimolar mixtures of kaempferol with diphenyl- and triphenyltins has been compared with the activity shown by kaempferol alone. The parameter of that activity were assumed as the concentration of a compound (or mixture) that caused a 50% reduction in membrane oxidation. A mechanism for the antioxidative action of the mixture was proposed, which was determined by its ability to reduce the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. The DPPH• radical, owing to its stability and long lifetime, is often used in studies of the flavonoid red−ox processes (*41*−*44*). Then, owing to the known associative properties of flavonoids toward ions of some metals, for example, Cu, Al, Mn, and Fe (*20*, *45*, *46*), the possibilities of complex formations of kaempferol with *n*-phenyltin compounds have been investigated. The fluorometric studies performed using the 1,6-diphenyl-1,3,5-hexatriene (DPH) probe allowed for the detection of differences in the behavior of the associations of kaempferol 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. Phosphatidylcholine was extracted from egg, as described elsewhere (*47*). Kaempferol (see the molecular structure in **Figure 1**) and 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma Chemical Co. (St. Louis, MO). Organotin compounds, 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 (1,6-diphenyl-1,3,5-hexatriene) was purchased from Molecular Probes Inc. (Eugene, OR). The remaining chemicals were of an analytical grade.

Liposome Preparation and Induction of Peroxidation. A chloroform solution of egg yolk phosphatidylcholine was dried under vacuum in a nitrogen atmosphere (*48*). A 50 mM Tris-HCl [(hydroxymethyl)-aminomethane] buffer of pH 7.4 was added, and the sample was vortexed to obtain a milky suspension of multilamellar vesicles. The final concentration of lipid in the vesicle suspension was 1.5 mg mL^{-1}. Such a suspension was then sonicated for 10 min with a 20 kHz sonicator (equipped with a titanium probe) to obtain small vesicles. The vesicle suspension was then centrifuged to remove titanium particles. The DPhT, TPhT, and kaempferol (alone) or equimolar mixtures of kaempferol with an organotin compound (DPhT or TPhT) were then added from a concentrated methanol solution (2×10^3 M/L) to the stirred sample of suspended vesicles. The content of methanol never exceeded 2% of the final 8 mL volume of the sample. The concentrations of the compounds studied changed in the range of 2.5−20 μM/L. Lipid peroxidation in the egg phospholipid liposomes was induced by ultraviolet radiation—the bactericidal lamp (253.7 nm) intensity was 3.0 mW/cm^2. The accumulation of phospholipid peroxidation products was estimated by determination of 2-thiobarbituric acid reactive products (TBARS) in the incubation medium (*49*). The amount of reaction products was determined by measuring the increase in absorbance at 535 nm (Specol 11, Zeiss Jena). The percentage of PC liposome oxidation induction or oxidation inhibition was calculated on the basis of the relationship % induction/inhibition $= (1 - \Delta A_A/\Delta A_O) \times 100$, where ΔA_A is the absorption increase (for $\lambda = 535$ nm) after 30 min of light exposure with antioxidant (kaempferol or its eqimolar mixture with DPhT or TPhT) added and ΔA_O is the absorption increase (for $\lambda = 535$ 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 2,2-diphenyl-1-picrylhydrazyl (DPPH•) of absorption ~0.9 was mixed with a proper amount of methanol solution or liposome dispersion at 0.25 mM PC/L of an antioxidant (kaempferol or equimolar mixture of kaempferol with DPhT or TPhT). The mixture was then incubated for an hour at room temperature in darkness. During incubation a reduction occurred of a part of the free radical form DPPH•, depending on concentration of the antioxidant added. Then absorption was measured at the characteristic for DPPH• wavelength equal to 517 nm (*42*). The measurements were done for concentrations of compounds studied in the range of 2.5−20 μM/L. The amount of DPPH• reduction (expressed in percentage), in the presence of a set antioxidant concentration, was calculated using the formula % reduction $= (1 - \Delta A_A/\Delta A_O) \times 100$, where ΔA_O is the DPPH• absorption increase at $\lambda = 517$ nm and $t = 0$, before antioxidant addition, and ΔA_A is the DPPH absorption increase at $\lambda = 517$ nm after 1 h of DPPH• incubation with an antioxidant.

Complexation of Organic Forms of Tin with Kaempferol. The studies of complexation of diphenyl- and triphenyltin with kaempferol were made using the spectrophotometric method. They were done in methanol and in the presence of PC liposomes suspended in a phosphate buffer of pH 7.4. A constant amount of kaempferol, to obtain 48 μM/L concentration, was added to 2 mL of methanol (or liposome dispersion containing 0.3 mg of PC), and then an organometallic compound was titrated until its concentration equaled that of kaempferol. Absorption of the stirred samples was measured at 250−600 nm and room temperature, in a 1 cm cuvette with a UV−vis spectrophotometer model 2401 (Shimadzu). The parameter of complexation was assumed as the value of absorbance increase $\Delta A\uparrow$ ($\lambda = \max$) at the peak coming from the complex and the value of absorbance decrease $\Delta A\downarrow$ ($\lambda = \max$) at the peak coming from the free kaempferol.

Fluidity Study. The fluidity experiments were done on liposome membranes, which were subjected to the action of kaempferol and its equimolar mixture with DPhT or TPhT. The fluorescent probe DPH was used at 1 μM/L concentration. The measurements were performed with an SFM 25 spectrofluorometer equipped with a thermostatic attachment (Kontron, Zurich, Switzerland) at 25 °C. The excitation and emission wavelengths were 354 and 429 nm, respectively. The polarizatin (*P*) and anisotropy (*A*) coefficients were calculated according to the formulas (*50*−*52*)

$$P = (I_{||} - GI_{\perp})/(I_{||} + GI_{\perp}); \qquad A = (I_{||} - GI_{\perp})/(I_{||} + 2GI_{\perp})$$

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 the perpendicular direction, and *G* is the diffraction constant.

RESULTS

The results of studies on the pro-oxidative behavior of phenyltins toward phosphatidylcholine liposome membranes irradiated with UV light are given in **Figure 2A** (DPhT) and **Figure 2B** (TPhT). They present the relative (percent of) liposome oxidation induction caused by the compounds DPhT and TPhT at concentrations of 2.5, 5, 10, 15, and 20 μM/L, subjected 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 DPhT and TPhT present. From the relationships shown in **Figure 2** it follows that concentration and exposure time are dependent on the level of liposome photo-oxidation. It decreases after 20 min of UV exposure, in principle, most significantly for higher phenyltin concentrations (e.g., 20 μM/L). The relative liposome oxidation level, induced for instance by TPhT, is basically higher (for the high concentrations in samples of 10, 15, and 20 μM/

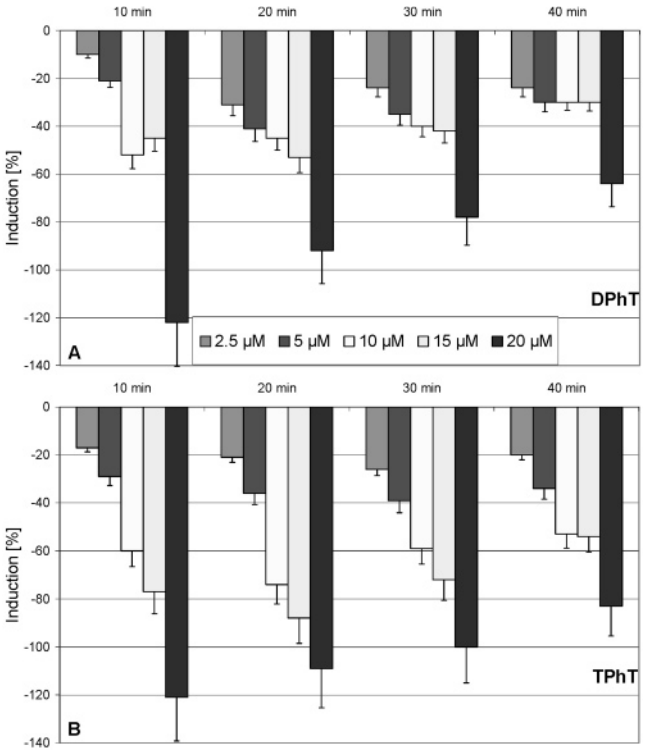

Figure 2. Dependence of photooxidation induction on the time of UV (λ = 254 nm) radiation of PC liposomes in the presence of DPhT (**A**) and TPhT (**B**). The concentration of compounds added changed from 2.5 to 20 μM/L, as indicated. The data, from three repeated experiments, represent the relative peroxidation compared to its control (without the compounds added). The mean standard deviation did not exceed 15%.

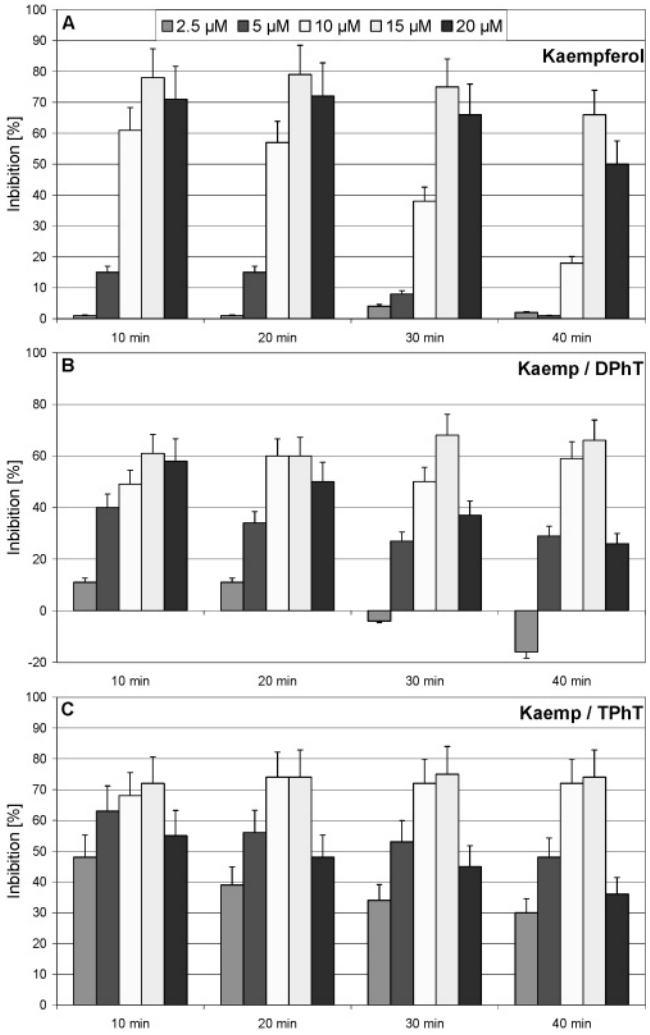

Figure 3. Dependence of oxidation inhibition on the time of UV radiation of PC liposomes in the presence of (**A**) kaempferol, (**B**) kaempferol with DPhT (1:1), and (**C**) kaempferol with TPhT (1:1). The concentration of compounds added changed from 2.5 to 20 μM/L, as indicated. The data, from three repeated experiments, represent the relative peroxidation compared to its control (without the compounds added). The mean standard deviation did not exceed 15%.

L) than the oxidation level caused by DPhT of the same concentrations.

The results of measurements on the antioxidative ability of kaempferol and its equimolar mixtures with diphenyl- and triphenyltin are presented in **Figure 3**. The dependence of PC liposome oxidation inhibition is shown, caused by the compounds studied at concentrations of 2.5, 5, 10, 15, and 20 μM/L, as dependent on time of exposure to UV radiation. From the course of the dependence in **Figure 3A** (for kaempferol) it is seen that the most effective oxidation inhibition is at the 15 μM/L concentration of antioxidant. The level of oxidation inhibition by kaempferol reached even 79%. With respect to 15 μM/L at the higher 20 μM/L concentration, kaempferol shows a decreased effectiveness of photo-oxidation inhibition (from ca. 23 to 9%). It is also observed that for practically all concentrations of that flavonoid, the antioxidative efficiency decreases with the time of UV exposure. The degree of that reduction is especially high at lower concentrations of kaempferol, in other words, at 5 and 10 μM/L (e.g., for 10 μM/L and 40 min of exposure it is 71%), whereas kaempferol at the lowest concentration of 2.5 μM/L has practically no protective properties.

The antioxidative action of equimolar mixtures of kaempferol with DPhT and TPhT toward liposome membranes oxidated with UV radiation is shown in **Figure 3B** (kaempferol/DPhT) and **Figure 3C** (kaempferol/TPhT). From comparison of the relationships in panels **A** and **B** of **Figure 3** it follows that for lower concentrations of the kaempferol/DPhT mixture (5−0 μM/L) a synergic action occurs with respect to kaempferol alone at the same concentrations. Exceptions here are the action of the 10 μM/L mixture in the initial phase of photo-oxidation, and at a concentration of 2.5 μM/L, where starting from 30 min of exposure a rather small pro-oxidative effect is observed (i.e., a percent of increase in the oxidation level relative to control, which amounts to 4 and 16% after 30 and 40 min exposures, respectively). The concentration of 15 μM/L of kaempferol with DPhT is, as for kaempferol alone, the most effective in PC liposome photo-oxidation, although in fact less effective than that of kaempferol alone. Also, the inhibitory action toward liposome peroxidation by the mixture kaempferol/DPhT at 20 μM/L proved to be less effective than the inhibitory effect of kaempferol alone. The decisively greatest antioxidative properties toward photo-oxidizing PC liposomes were shown by mixtures of kaempferol with TPhT at concentrations of 2.5, 5, 10, and 15 μM/L. Additionally, at concentrations of 10 and 15 μM/L (of the mixture) the percentage of oxidation reduction did not decrease in time, and the reduction in antioxidative ability at concentrations of 2.5 and 5 μM/L was lowest compared with the reduction induced by kaempferol and its mixture with DPhT.

For a quantitative picture of the differences in the antioxidative ability of both mixture and kaempferol, **Figure 4** shows a comparison of PC liposome oxidation inhibition percent as dependent on concentration after a 30-min exposure to UV

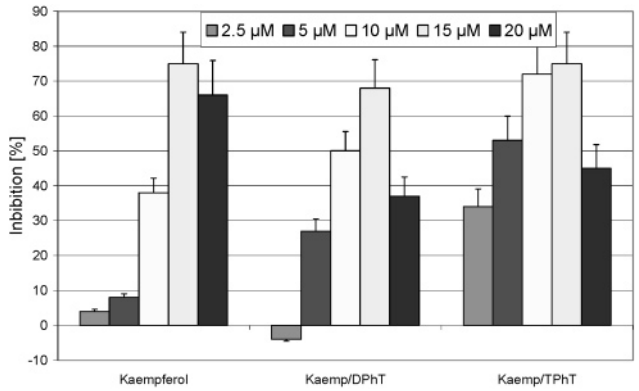

Figure 4. Dependence of percent of inhibition of PC liposome membrane oxidation after 30 min of exposure to UV radiation (calculated on the basis of results presented in **Figure 1**) on the concentration of kaempferol and its equimolar mixtures with DPhT and TPhT. The concentration of compounds added changed from 2.5 to 20 μM/L, as indicated. The mean standard deviation did not exceed 15%.

radiation. From the relationships shown in **Figure 4** it follows that mixtures of phenyltin and kaempferol at lower concentrations (2.5, 5, and 10 μM/L) exhibit an ability lower than that of kaempferol to oxidize liposome lipid membranes (with one exception: kaempferol/DPhT at 2.5 μM/L), and at 15 μM/L the mixture kaempferol/TPhT has the inhibition ability at the level of kaempferol. Only at 20 μM/L does kaempferol have a higher ability than both of the mixtures to protect the membranes against oxidation. The values of IC_{50} (i.e., concentrations that cause 50% reduction in peroxidation after 30 min of exposure to UV) determined on the basis of **Figure 4** are given in **Table 1**. The sequence of the antioxidative activities of the compounds studied is kaempferol/TPhT > kaempferol/DPhT > kaempferol.

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 a spontaneous red−ox reaction. The results of these experiments are presented in **Figure 5**. Panels **A** and **B** show the dependence of percent reduction of the free radical DPPH$^{\bullet}$ after 1 h of incubation with an antioxidant in methanol (**A**) and in the presence of PC liposomes (**B**) as a function of the reducer concentration (kaempferol and the mixtures kaempferol/DPhT and kaempferol/TPhT). On the basis of the relationship of **Figure 5**, the concentrations were determined (IC_{50} or IC_{20} in the case of DPPH$^{\bullet}$ in the presence of liposomes) that cause 50% (or 20%) reduction of DPPH$^{\bullet}$ by the antioxidant. The values of the parameters IC_{50} and IC_{20}, given in **Table 1**, constitute the following sequence of antiradical activity: kaempferol/TPhT > kaempferol/DPhT > kaempferol. It is valid for experiments with DPPH$^{\bullet}$ radical in methanol (**Figure 5A**) and in the presence of methanol (**Figure 5B**).

To explain the action of diphenyl- and triphenyltin mixtures with kaempferol, the possibility of a complex formation between several molecules was investigated. A representative set of electron absorption spectra of kaempferol titrated with DPhT compound (**A**) and TPhT (**B**) in methanol can be seen in **Figure 6**. Dependences similar to those in **Figure 6** were also obtained for the process of complex formation between kaempferol and DPhT or TPhT in a liposome suspension. In panels **A** and **B** of **Figure 6** one of the peaks from kaempferol is marked $A_{Kaempferol}$, the absorption of which decreased during titration with DPhT and TPhT, and another new peak from molecules of the complexes is marked $A_{Complex}$. These are associations of kaempferol molecules with DPhT (**A**) and TPhT (**B**). The peak absorption from a complex increased with increasing concentration of DPhT chloride (**Figure 6A**, the final concentration ratio reaching 1:1) and TPhT chloride (**Figure 6B**, the final concentration ratio reaching 1:4). The assumed parameter of the phenyltin complex formation is the increase in absorption peak from a complex (ΔA_{CO}) and the decrease in peak absorption from kaempferol (ΔA_{KA}) for equimolar mixtures of kaempferol/phenyltin. Values of ΔA are given in **Table 1**.

To explain the diversity in the antioxidative action of the compounds studied with respect to PC liposome membranes, the percent of relative changes was determined in the fluorescence of the DPH probe present in the liposome membrane due to absorbed antioxidants (the studied compounds). The results of the relative changes in polarization and anisotropy are presented in **Figure 7**. The largest changes in both of the parameters are caused by the mixture kaempferol/DPhT. They depend on the mixture concentration and reach values of 120 and 142% in the case of polarization and anisotropy increase caused by the highest 5 μM/L concentration of kaempferol/DPhT. Decisively smaller changes in polarization and anisotropy of DPH probe were induced by the kaempferol/TPhT mixture. In that case the relative increases in the probe polarization and anisotropy reached the values 74% (39% ↓) and 85% (40% ↓), respectively, for the 5 μM/L concentration of kaempferol/TPhT. Changes in DPH probe parameters induced by kaempferol alone are a little smaller than those induced by the kaempferol/TPhT mixture; thus, for instance, the polarization decreased by 12% and anisotropy by 14% for 5 μM/L kaempferol.

DISCUSSION

The pro-oxidative behavior of the chlorides DPhT and TPhT induced by UV radiation with respect to PC liposomes, shown in **Figure 2**, follows from the effect on membranes of the free radical products of photoreduction of both phenyltins and water molecules. The problem of the photodestruction of triphenyltin molecules was treated by, among others, Navio et al. (*53*). It is suggested that the final products of irradiating TPhT chloride with light that imitates sunlight are diphenyl- and monophenyltins (most probably also inorganic tin). An intermediate product of that process can be the phenyl radical (Ph*). It undergoes transformations as a result of further interaction with, for example, oxygen molecules (O_2) or the hydroxyl radicals (*OH) that results from water hydrolysis. This may generate the formation of other phenyl derivatives, such as PhO_2* and PhOH. Most probably the free phenyl radicals, formed during exposure of the liposome dispersion containing phenyltin compounds to UV radiation, are responsible for the relative increases in the liposome oxidation shown in **Figure 2**. The possibility of the creation of some free radical forms of the chlorides DPhT and TPhT in the crystalline phase of the compounds after 2 h of exposure to UV radiation (λ = 330 nm) was confirmed with the EPR method (our unpublished data).

The development of a free radical during photoinduced degradation (at λ > 300 nm) of tributyltin (TBT) in the presence of Fe(III) ions was suggested by Mailhot and others (*54*). They have found that the main product of the interaction is a gradual dealkylation of TBT molecules resulting in the formation of di- and monobutyltin and the final formation of inorganic tin. The authors suggest that the intermediate products of TBT photodegradation can be, among other products, butyl radicals (TBT* and TBT-O_2*). According to the authors, they develop with the participation of hydroxyl radicals formed in parallel red−ox reactions of Fe(III) ions, which then initiate the oxidation of TBT molecules.

Table 1. Antioxidative (IC_{50}^{PC}) and Antiradical Activities with Respect to DPPH (IC_{50}^{DPPH} or IC_{20}^{DPPH}) and Complexation Parameter ΔA of Kaempferol with Diphenyltin (DPhT) and Triphenyltin (TPhT) in Methanol and in the Presence of Model PC Lipid Membrane[a]

compound	IC_{50}^{PC} (μM)	IC_{50}^{DPPH} in methanol (μM)	IC_{20}^{DPPH} in lipid (μM)	ΔA_{KA} in methanol	ΔA_{CO} in methanol	ΔA_{KA} in lipid	ΔA_{CO} in lipid
kaempferol	11.5	10.2	9.0				
kaempferol/DPhT	9.3	9.3	7.2	0.64↓	1.26↑	0.35↓	0.73↑
kaempferol/TPhT	3.0	8.1	6.2	0.14↓	0.25↑	0.26↓	0.37↑

[a] IC_{50}^{PC} and IC_{50}^{DPPH} denote the antioxidant concentration causing 50% inhibition of liposome membrane oxidation after 30 min of UV irradiation and 50% reduction of the free radical form of DPPH• in methanol (or 20% in the case of the liposome medium) after 1 h of incubation with an antioxidant, respectively. ΔA_{KA} and ΔA_{CO} denote changes of peak absorbance coming from kaempferol (↓ decrease) and a complex of kaempferol with phenyltins (↑ increase), respectively.

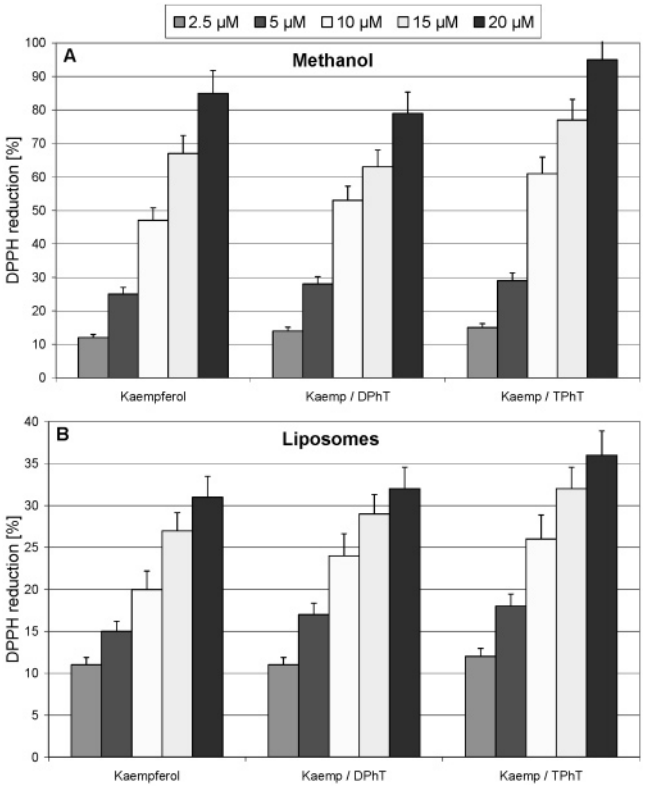

Figure 5. Dependence of the free radical DPPH• reduction present in the methanol solution (**A**) or liposome dispersion (**B**) with kaempferol or their equimolar mixture with DPhT and TPhT on the antioxidant concentration. The amount of the compounds added changed from 2.5 to 20 μM/L, as indicated. The data are the average of six or four probes. The mean standard deviation did not exceed 8%.

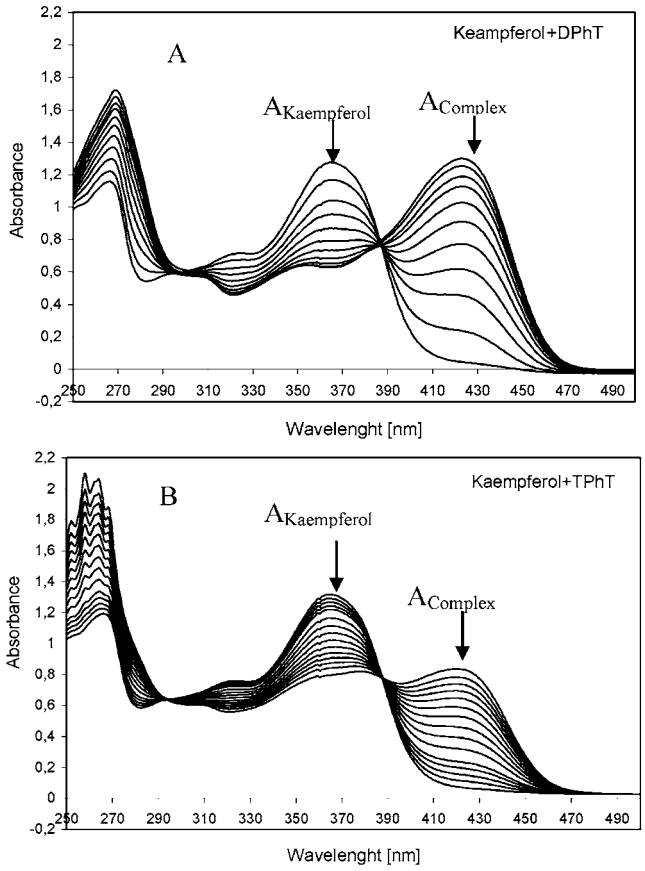

Figure 6. Compilation of electron absorption spectra of kaempferol in methanol, titrated with DPhT (**A**) or TPhT (**B**). The final concentration ratio of kaempferol to DPhT was 1:1 and that of kaempferol to TPhT 1:4. The initial concentration of kaempferol was 48.9 μM/L. Peaks from kaempferol ($A_{Kaempferol}$) and complex ($A_{Complex}$) are indicated.

The protective properties of kaempferol demonstrated with respect to liposome membranes that undergo oxidation induced by organic tin compounds and UV radiation (**Figures 3** and **4**) result most probably from several mechanisms of the flavonoid action. One of them is kaempferol's ability to scavenge free radicals. Such was the suggestion raised on the basis of the relationships obtained from studies on the antiradical activity of kaempferol and its equimolar mixtures with DPhT and TPhT with respect to the free radical DPPH. Values of the parameters IC_{50} (in the presence of methanol) and IC_{20} (in the presence of liposomes) determined in relation to that radical (**Table 1**) constitute a sequence of the compounds' activity, which is similar to that obtained in antioxidant activity studies. This high concordance of the relationships may suggest one of the mechanisms of antioxidative activity of kaempferol and its equimolar mixtures with phenyltin as free radical scavengers.

It should be added that a comparison of the parameter IC_{50}^{PC} within a series of the compounds studied allows one to formulate the sequence of their antioxidative activity that reflects well their activity in the range of lower and medium concentrations, as, for example, the concentration of 10 μM/L. The results of the antioxidative activity of the compounds studied in that concentration range also allow one to note a stabilizing role of TPhT (and also of DPhT at 5 and 10 μM/L) in the antioxidant action of kaempferol. It consists of that the degree of PC liposome photo-oxidation by equimolar mixtures of kaempferol and TPhT of 2.5, 5, and 10 μM/L concentrations is higher (45, 30, and 20%, respectively) than the degree of inhibition shown by kaempferol alone at identical concentrations. At the higher, 15 μM/L, concentration of the compounds studied the activity sequence is kaempferol/TPhT = kaempferol = kaempferol/DPhT, whereas at 20 μM/L the most effective is kaempferol, less so the mixture kaempferol/TPhT, and the lowest activity is exhibited by the kaempferol/DPhT mixture. The decrease in antioxidative activity of equimolar mixtures of kaempferol with

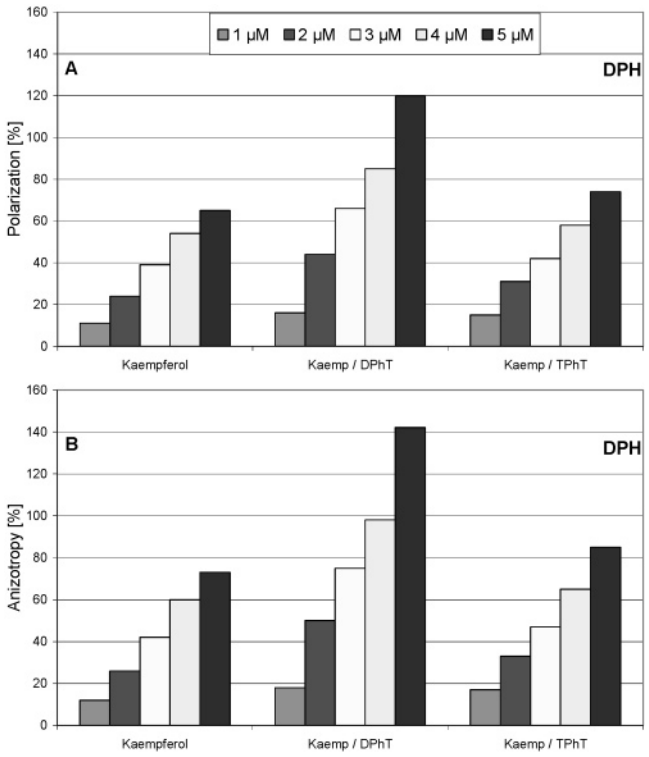

Figure 7. Polarization (**A**) and anisotropy (**B**) coefficients versus concentration of kaempferol and its equimolar mixture with DPhT or TPhT present in the dispersion of liposome membrane with fluorescent probe DPH at 25 °C. The experiment was repeated twice.

phenyltin, compared with the activity of kaempferol alone (for example, for the highest 20 μM/L concentration) may result from the increasing interaction of the associates, which develop as a result of the chelating properties of kaempferol, as shown in the present work. It cannot be excluded that at higher concentrations the mutual interaction between the associate molecules results in the formation of layered polymer structures of the sandwich type. That may involve, among others, the hydroxyl groups of a flavonoid in hydrogen bonds between molecules in the polymer structure and thus reduce the antiradical ability of those structures (*55*).

The results of studies on the chelating properties of kaempferol with respect to DPhT and TPhT (a parameter of that ability being among others the increase in absorption of the peak from a comples, ΔA_{CO}) in methanol and in the presence of PC liposomes indicate the high affinity of the flavonoid to form complexes with molecules of both phenyls. It is higher with respect to DPhT than TPhT molecules, both in methanol and in the presence of liposome. For instance, the increase in absorption of the peak from the kaempferol/DPhT complex for an equimolar ratio (1:1) in the presence of liposomes is 0.35, whereas the increase in absorption of the peak from the kaempferol/TPhT complex under the same conditions was 0.26 (those values in methanol are 1.26 and 0.25, respectively). Ratios of respective increases in absorption (DPhT/TPhT) are 1.35 (in the presence of liposome) and 5.0 (in methanol). This means that the ratio of kaempferol affinities for the formation of associates with DPhT and TPhT in the presence of liposomes decreased 3.7-fold compared to the respective ratios in methanol. In other words, the association process between kaempferol and TPhT molecules in liposome membranes occurs with affinity which is only a bit lower than that for kaempferol and DPhT molecules. The fact that kaempferol forms associations with molecules of diphenyl- and triphenyltin (of different binding constant values, higher for DPhT) not only inhibits their peroxidative action but increases their antioxidative effect (synergistic effect, e.g., for the kaempferol/DPhT mixture at 5 and 10 μM/L and for the kaempferol/TPhT mixture at 2.5, 5, and 10 μM/L). It is probable that in the so-called "charge transfer" complexes that are formed in this case (*46*, *56*), the coupling of the kaempferol molecule is so changed that hydrogen can be more easily detached from the kaempferol molecule, which then reduces a met radical. Thus, the property of a flavonoid to form associations with organotins seems to constitute, as shown in the present work, a second mechanism of protective action of kaempferol with respect to liposome membranes exposed to the peroxidative action of the free radical products of photodegradation of the compounds. The chelating abilities of flavonoids toward such transition metals as iron or copper are known from the literature (*33*, *45*). They suggest one of the mechanisms of the antioxidative action of flavonoids. This mechanism consists of blocking the Fenton reaction induced by transition metals, which are the source of very reactive hydroxyl radicals.

The diversified effectiveness of the antioxidants (kaempferol and its associations with DPhT and TPhT) in their action on liposome membranes undergoing peroxidation may be connected with different degrees of their incorporation into the membrane. The large relative changes in polarization and anisotropy of the probe DPH anchored in the hydrophobic region of the liposome membrane bilayer induced by the associates of kaempferol/DPhT and the considerably smaller changes (up to ca. 39 and 40% for polarization and anisotropy, respectively) caused by kaempferol/TPhT molecules suggest a differentiated degree of liposome membrane stiffness in its interaction with both of the associates. That may indicate at deep localization of the associates kaempferol/DPhT in the membrane (in the initial part of the hydrophobic region) and a much closer to the surface localization of kaempferol/TPhT associates. This favors the hypothesis that the kaempferol/TPhT molecules assume a strategic position near the membrane surface that enables them to scavenge free radicals approaching the membrane. The kaempferol/DPhT molecules, most probably situated near the phase boundary, sweep the free radicals with a certain delay with respect to the action of the kaempferol/TPhT molecules. The different localizations of the chlorides DPhT and TPhT in the PC liposome membrane bilayer was postulated in our previous investigations. Using the methods of ^{1}H NMR, fluorometric, and molecular modeling, we have proved that among others the steric constraints favor a deeper localization of the less hydrophobic DPhT compound in the membrane bilayer than does the more hydrophobic and umbrella-like TPhT fixed to the membrane surface (*26*).

The relative changes in the polarization and anisotropy of the DPH probe induced by kaempferol, similar to those caused by its complexes with TPhT, suggest that localizations of the two antioxidants in the membrane are similar. The much lower antioxidative effectiveness of kaempferol in comparison with the complexes kaempferol/TPhT may result (in the range of low kaempferol concentrations) from the susceptibility of "free" kaempferol to the destructive action of UV radiation. With increasing kaempferol concentration the pool of undamaged molecules of that flavonoid must be on the increase and is quite sufficient for effective antioxidant action.

Summing up, one can state that the protective action of kaempferol toward phosphatidylcholine liposome membranes exposed to the pro-oxidative action of the organotins (DPhT and TPhT) under ultraviolet radiation (of 254 nm wavelength)

is connected with chelating properties of kaempferol with respect to DPhT and TPhT. These in turn inhibit the photodestruction process of both phenyltin and kaempferol molecules. The effectiveness of the protective action toward liposome membranes of the associations formed by kaempferol (kaempferol/DPhT and kaempferol/TPhT) is connected, most probably, with the ability of the complexes to scavenge free radicals and with their differentiated affinity to incorporate into the membranes.

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