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Antioxidant Activity of *Sempervivum tectorum* and Its Components

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The antioxidant properties of components of leaf extracts of the evergreen plant, *Sempervivum tectorum* (ST), have been evaluated using UV irradiated liposomal systems containing the spin trap 5-(diethoxyphosphoryl)-5-methyl-pyrroline-*N*-oxide. Decreases in free radical activity in the liposomal systems as measured by electron paramagnetic resonance (EPR) spectroscopy demonstrate that the lipophilic ST juice components, kaempferol (KA) and kaempferol-3-glucoside (KG) contribute significantly to the antioxidant properties of the juice. EPR spectral simulation established the presence of oxygen and carbon centered free radical adducts. The mixtures with low pH, citric and malic acid, and ST juice reveal increased EPR signals from oxygen centered radicals in comparison to the control, pointing to the important role of pH in oxygen radical formation. Parallel assays that measured thiobarbituric acid related substances confirm the antioxidant effects of KA and KG and explain the results of spin trapping experiments complicated by low pH's.

KEYWORDS: *Sempervivum tectorum* extract; EPR; kaempferol; kaempferol-3-glucoside; liposomes; antioxidants

INTRODUCTION

Sempervivum tectorum is an evergreen plant with crassulacean acid metabolism (*1*). It belongs to a large family of the Crassulaceae (*2*, *3*). Juice squeezed from leaves of the plant has been used as folk medicine in many countries (*4*).

S. tectorum extract lowered lipid levels in rats (*5*−*7*) and exhibited antimicrobial (*4*) and in vitro antioxidant properties (*8*, *9*). Previously, we extracted, in ethyl acetate soluble, oligomeric (0.07%) and, in ethyl acetate insoluble compounds, polymeric polyphenols (0.13%) from fresh leaves of *S. tectorum* and identified kaempferol as the unique aglycon of the oligomeric polyphenolic fraction (*4*). Kaempferol and its glycosides belong to the flavonoids, a large class of compounds which are ubiquitous in plants occurring mainly in fruit, vegetables, tea, and skin of tubers and roots. Flavonoids may protect plants from photooxygenation damage caused by singlet oxygen, because especially high concentrations of them are found in peel, leaves and skin. Flavonoids usually contain a number of phenolic hydroxyl groups, which are considered to have antioxidant activity. These compounds can act as reducing agents, hydrogen-donating antioxidants, metal chelators, and singlet oxygen quenchers (*10*−*12*). The hydroxyl groups in the flavonoids participate in glycoside formation, and most of the naturally occurring flavonoids are in that form.

Juice squeezed from fresh leaves of *S. tectorum* contains approximately 1.4 mg of total phenols/mL (*4*), as well as citric (up to 20 mg/mL), malic (up to 14 mg/mL), and ascorbic (205 mg/kg of fresh leaves) acids, as determined with RP-HPLC (unpublished results).

The aim of the present work was to determine the contributions of kaempferol, kaempferol-3-glucoside, and citric, malic, and ascorbic acids to the antioxidant activity of *S. tectorum* (ST) juice in a biologically relevant environment. To mimic a cellular membrane, liposomes composed of egg lecithin and phosphatidylserine were used. Juice components, individually and in mixtures, were entrapped in liposomes, and their antioxidant activity was compared to the antioxidant activity of ST juice and the oligomeric polyphenols extracted from *S. tectorum*. The concentrations of the components used in the model systems were comparable to those found in juice from the leaves of *S. tectorum*.

In the liposome systems, ultraviolet radiation initiated free radical reactions, which resulted in short-lived, lipid-derived free radicals that were stabilized by use of the spin trapping method. Amounts and types of free radicals were determined using electron paramagnetic resonance (EPR) spectroscopy (*13*). The nitrone spin traps used react with short-lived free radicals,

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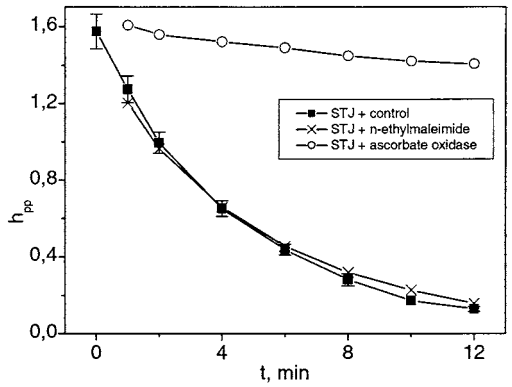

Figure 1. EPR spectral intensity decrease of Tempone in pure *S. tectorum* juice (12.5% v/v) and in the presence of inhibitors *N*-ethylmaleimide (10 mmol/L) or ascorbate oxidase (1000 U/mL).

transforming them into more stable nitroxide free radicals (free radical adducts) which can be detected by EPR. Because the intensity of the EPR spectra is proportional to the number of free radical adducts, an intensity decrease was expected in the presence of antioxidant compound(s) that could scavenge the initial free radicals or inhibit the formation of secondary forms (*14*). To confirm lipid peroxidation as the source of free radicals, the thiobarbituric active reactive substances (TBARS) assay was performed on selected systems.

MATERIALS AND METHODS

Plant Material. *S. tectorum* plants were collected from the Botanical Garden, Ljubljana, Slovenia. Juice squeezed from clean, fresh leaves of *S. tectorum* was centrifuged and diluted with water to 25% v/v. Oligomeric polyphenols (OP) were extracted with ethyl acetate from fresh leaves of *S. tectorum* according to the procedure described by reference 4.

Preparation of PC/PS Liposomes. The antioxidant activity of each compound or mixture was examined after it was entrapped in liposomes. Liposomes were prepared from egg lecithin (PC) (Sigma Chemical Co. St. Louis, MO), and phosphatidylserine (PS) (Sigma Chemical Co.) in the weight ratio of 9/1 by the thin film method (*15*). Phospholipid solutions containing kaempferol (KA) (Extrasynthese, Genay, France), kaempferol-3-glucoside (KG) (Extrasynthese, Genay, France), or oligomeric polyphenols were dissolved in a chloroform/ethanol solution. After the removal of the solvent under reduced pressure, the thin film formed on the wall of the flask was hydrated by the addition of 2 mL of water followed by shaking of the flask until the lipids were removed from the wall. The multilamellar vesicles (MLV) formed had a total concentration of 50 mg/mL of phospholipids. The final concentration of KA or KG in liposome dispersion was 18 mmol/L, (i.e., 5 and 8 mg/mL, respectively) and the concentration of OP was 8.5 mg/mL.

In some experiments, during hydration, aqueous solutions of 100 mmol/L citric acid or malic acid (both Kemika, Zagreb, Croatia) or their mixture (50 mmol/L of each), or juice from the leaves of *S. tectorum* (25% v/v) were added, instead of pure water, to the flask with the thin film of lipids. For EPR experiments, ST juice was mixed with ascorbate oxidase (1000 U/ml, final concentration) in order to stop the reduction of spin adduct by ascorbate (see **Figure 1**). Because addition of acids or ST juice decreased the pH value of the solution to 2.5 for malic acid and juice and to 2.3 for citric acid, the influence of pH on the EPR spectra was also investigated. Instead of water, phosphate buffered saline (PBS) with pH varying between 2.5 and 7.4 was used for hydration.

Initiation of Oxygen Radical Reactions. Radical reactions were initiated by irradiation of liposome suspensions with UV light (λ = 365 nm). In experiments involving concentration variation, the liposome suspension was diluted with "empty liposomes" in such a way that the final concentration of KA or KG varied from 0 to 18 mmol/L, and the OP concentration varied from 0 to 8.5 mg/mL.

Measurement of the Redox Activity of Tested Samples. Because the EPR spectra intensity depends not only on the antioxidant activity of tested substances but also on their capability to reduce or oxidize the nitroxide group of the adduct to the EPR invisible hydroxylamine, it was necessary to investigate the redox activity of these substances. For this purpose, a 5×10^{-5} mol/L solution of the spin probe Tempone was added to the aqueous solution of ST juice (12.5% v/v), and its water soluble components (100 mmol/L citric acid or malic acid). For the tested substances, which had limited aqueous solubility (OP 8.5 mg/mL and KA or KG 18 mmol/L), Tempone was added to the liposome dispersion with the incorporated substances. In each case, the EPR spectral intensity decrease due to the reduction of the nitroxide to the EPR-invisible hydroxylamine was measured with time. To identify the sources of reduction, the samples exhibiting such reduction were mixed either with *N*-ethylmaleimide (Aldrich Chemical Co., Inc. Milwaukee, WI) (final concentration 10 mmol/L), which inhibits SH groups, or with ascorbate oxidase (Sigma Chemical Co.) (final concentration 1000 U/mL), which catalyzes the aerobic oxidation of ascorbic acid.

Spin Trapping. The spin trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) (OXIS Research, Portland, OR) (0.1 mol/L) was mixed in equal volume with 30 μL of liposome suspension prior to irradiation. In preliminary experiments, the dependence of the DEPMPO-adducts' EPR spectral intensity on the time of irradiation was measured. On the basis of these experiments, 2 h irradiation was chosen as a convenient time to get an acceptable signal-to-noise ratio.

EPR Measurements. After the irradiation period of the liposome system, a portion of the sample was transferred to a glass capillary for EPR measurements. Measurements were performed on an ESP 300 X-band spectrometer (Bruker, Rheinstetten, Germany) at room temperature. In the intensity decay studies, the amplitude of the low-field line of the EPR spectrum of the DEPMPO/$OH^{\bullet}$ free radical adduct was measured as a function of time.

In the studies of antioxidant effectiveness, the EPR spectrum was measured immediately after the UV irradiation period, and the contribution of different free radical adducts to the overall spectrum was determined by the computer simulation of the EPR spectra using the program PEST WinSIM (*16*).

TBARS Assay. Determination of the percent inhibition of peroxidation in liposomes by added substances involved utilization of the method described by Pelle et al. (*17*). By this method, the concentration of malondialdehyde, as a final product of lipid peroxidation, was measured spectrophotometrically.

RESULTS

Measurement of Redox Activity of the Tested Samples. From the Tempone EPR spectral intensity decay with time in the presence of tested samples, it is evident that the addition of ST juice to the Tempone solution resulted in a marked decrease of the EPR signal intensity **(Figure 1)**. This decrease can be attributed to either reduction of the nitroxide to the EPR-silent hydroxylamine or oxidation of the nitroxide to the equally EPR-silent nitrone. We tried to identify a source of the EPR spectral intensity decrease by adding to the TEMPONE a solution of *N*-ethylmaleimide, which inhibits SH groups, or ascorbate oxidase, which catalyzes the aerobic oxidation of ascorbic acid to dehydroascorbic acid. The addition of *N*-ethylmaleimide did not inhibit the reduction, but the presence of ascorbate oxidase in ST juice did inhibit it **(Figure 1)**. Because ascorbate oxidase almost completely stopped the reduction, we conclude that ascorbic acid is the main reducing agent found in ST juice. Therefore, in further experiments with ST juice, ascorbate oxidase was added to the sample to stop the reduction of the free radical adducts. Otherwise, the EPR spectra intensity decrease due to the antioxidant activity of ST juice cold not be distinguished from the intensity decrease due to the reduction of the adducts by ascorbate.

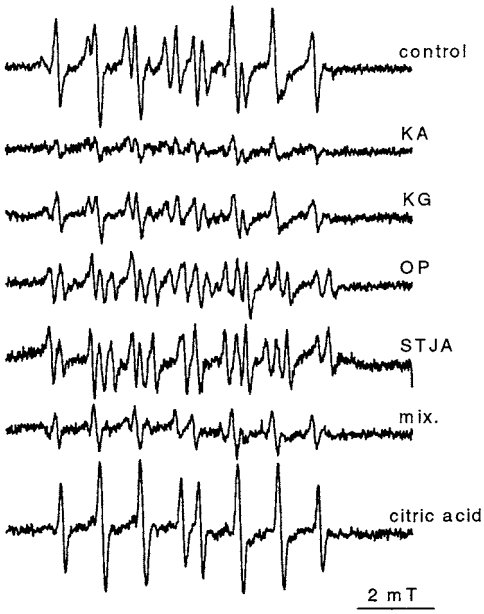

Figure 2. EPR spectra of DEPMPO radical adducts obtained in PC/PS liposomes after 2 h of irradiation with UV light ($\lambda = 365$ nm) in control and in the presence of different antioxidants: KA = kaempferol (1.2 mmol/L), KG = kaempferol-3-glucoside (1.2 mmol/L), OP = oligomeric polyphenols (4.3 mg/mL) extracted from leaves of *S. tectorum*, STJA = juice squeezed from the leaves of *S. tectorum* (12.5% v/v) in the presence of ascorbate oxidase (1000 U/mL), mix = mixture of KA (4.5 mmol/L), KG (4.5 mmol/L), malic acid (25 mmol/L) and citric acid (25 mmol/L), and citric acid (50 mmol/L). (Final concentrations are given.)

The oligomeric polyphenols from *S. tectorum*, pure KA, KG, citric, or malic acids did not show any redox activity during the 1 h measurement.

UV Irradiation and Free Radical Formation. The EPR spectra of DEPMPO radical adducts in PC/PS liposomes after 2 h irradiation with UV light ($\lambda = 365$ nm) in empty liposomes (control) and in the liposomes with different compounds or mixtures are presented in **Figure 2**. A system containing malic acid gave results almost identical to those of citric acid.

EPR spectral simulations revealed that the EPR spectra in **Figure 2** were composed of three components, the hydroxyl radical adduct DEPMPO−OH•, the superoxide or lipid hydroperoxide radical adduct DEPMPO−OOH•(−OOL•) and a carbon-centered radical adduct, with hyperfine coupling constants that are comparable to those already published (*18*, *19*) **(Figure 3)**.

The ratios between the components of the EPR spectra obtained with different antioxidants is different, indicating that the different substances inhibit DEPMPO adduct formation in different ways. The relative ratio between the radical adducts within the spectrum as well as the total intensities of the EPR spectra, with respect to the control (empty liposomes), are given in **Table 1**.

The concentration dependencies of the antioxidant activity of KA and oligomeric polyphenols are presented in **Figure 4**. The decrease of the EPR spectral intensity is presented for each free radical adduct separately as well as for the sum of portions of all three radical adducts.

KA and KG were found to be the most effective antioxidant compounds among the tested substances in *S. tectorum* leaves **(Figure 2** and **Table 2)**. KA strongly inhibited the formation of all three free radical adducts, and at 4.5 mmol/L concentration, the signal became negligible. This means that KA inhibited the formation of free radicals or their interaction with the spin trap. The ratio between the radical adducts did not change with the concentration of KA **(Figure 4)**.

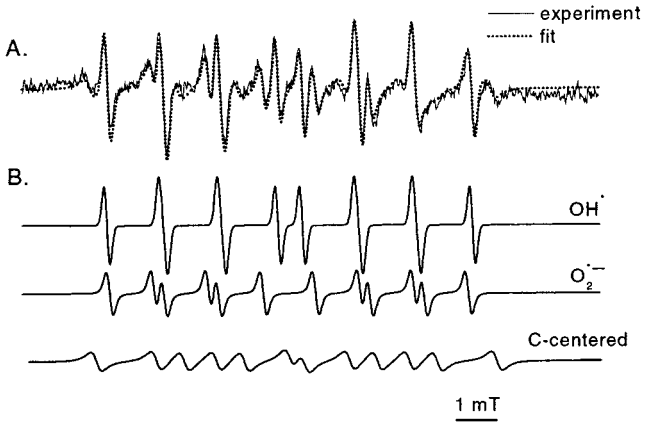

Figure 3. EPR spectrum of DEPMPO-adducts in PC/PS liposomes after 2 h irradiation with UV light ($\lambda = 365$ nm). A. (—) experiment, (···) the best fit, obtained as a superposition of the spectral components. B. of DEPMPO–OH• ($a_N = 14.14$ G, $a_{H1} = 13.0.09$ G, $a_{H2} = 0.37$ G, $a_P = 47.41$ G), DEPMPO–OOH•(–OOL•) ($a_N = 13.2$ G, $a_{H1} = 10.7$ G, $a_{H2} = 0.62$ G, $a_{H2} = 0.39$ G, $a_P = 49.8$ G) and a carbon-centered radical adduct ($a_N = 14.5$ G, $a_{H1} = 21.3$ G, $a_P = 46.74$ G).

While KG also decreased the EPR signal intensity, it was less efficient than KA **(Table 1)**. At the same 1.2 mmol/L concentration, the EPR spectral intensity (the sum of all three adducts) decreased by 49% in the presence of KG, while in the presence of KA, it decreased by 76% **(Table 1)**. In the samples with 9 mmol/L of KA, no EPR spectrum was observed, indicating that at this concentration, KA completely stopped the formation of radical adducts, while at the same concentration of KG, the intensity of the EPR spectrum decreased by 71%.

There is a distinct difference between the samples protected by KA in comparison to those protected with OP, the natural extracts of *S. tectorum*. In the samples protected by KA, the intensity of all free radical adducts decreased equally with increasing concentration of KA. However, in the samples containing OP, the formation of carbon-centered free radical adducts increases with increasing OP concentration, while oxygen-centered free radical adduct levels are similar to the KA systems.

Inclusion of citric or malic acid in the liposome systems increased the EPR spectral intensity as compared to the control **(Figure 2** and **Table 2)**.

Effect of pH on the EPR Spectra. The EPR spectral intensity of all free radical adducts increased in liposomal dispersions buffered at lower pH's **(Table 2)**. These data highlighted the need to include pH effects in evaluations of spectra from systems containing ST juice, the mixture similar to ST juice, citric acid, and malic acid, whose pH values are about 2.5.

TBARS Assay. TBARS assay results included in **Table 2** indicate that some lipid peroxidation occurred during the preparation of liposomes, because A_0 in the control is higher than in the liposome dispersions with KA, KG, and mixture of KA, KG, malic acid, and citric acid, indicating that KA and KG inhibit the formation of free radicals during preparation of liposomes. It is interesting to note that the natural extract from *S. tectorum*, OP's and juice squeezed from leaves (ST juice) does not prevent formation of free radicals during the preparation of liposomes, but inhibits further formation of free radicals during irradiation. Citric and malic acids show no protective

Table 1. EPR Spectral Intensities and Intensity Ratios Relative to Control Levels of DEPMPO-Adducts Formed during UV Irradiation of PC/PS Liposomes with Entrapped Antioxidants[a]

sample	concn	OH•[b]	OOH• (OOL•)[b]	C-centered radical[b]	intensity[c] (%)
control (H_2O)		21.4 ± 0.2	36.9 ± 0.8	42 ± 1	100
PBS pH 2.5		32.4 ± 0.6	26.2 ± 0.2	41 ± 5	205
KA	9.0 mmol/L	0	0	0	0
	1.2 mmol/L	20.5 ± 0.4	32.2 ± 1.1	47 ± 1	24
KG	9.0 mmol/L	19.0 ± 1.0	30 ± 2	51 ± 2	29
	1.2 mmol/L	18.4 ± 0.6	31.7 ± 1.8	50 ± 2	51
OP	4.3 mg/mL	8.2 ± 0.1	9.7 ± 0.1	82 ± 1	63
	1.1 mg/mL	12.0 ± 0.1	23.1 ± 0.2	64 ± 3	75
citric acid	50 mmol/L	35.7 ± 2	34.1 ± 1	30 ± 1	286
malic acid	50 mmol/L	28.7 ± 0.1	25.2 ± 2	46 ± 2	312
STJA (H_2O)	12.5% v/v	12.2 ± 0.2	4.8 ± 0.1	82 ± 0.2	164
mix (with KG)	*d*	43.4 ± 0.6	14.25 ± 0.9	73 ± 1	43

[a] Antioxidants: KA, KG, OP, citric acid, STJA (1000 U/mL), mixture of KA, KG, citric and malic acids (mix), concentrated = final concentrations. [b] Percent contribution of free radical adduct to total EPR spectral intensity.[b] [c] The sum of all radical adduct contributions normalized to the value of empty liposomes. [d] Mixture of KA (4.5 mmol/L), KG (4.5 mmol/L), MA (25 mmol/L) and CA (25 mmol/L).

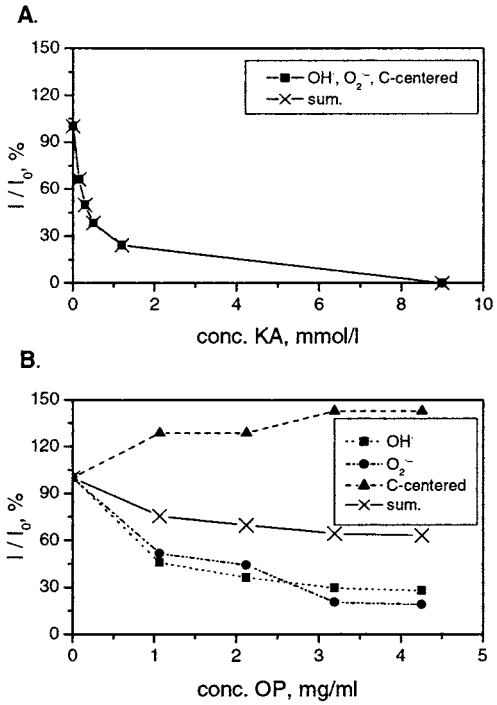

Figure 4. Concentration dependencies of antioxidant activity of (A) kaempferol and (B) OP for each free radical adduct separately as well as for the sum of portions of all three radicals (final concentrations).

effect against the formation of free radicals, neither during preparation of liposomes, nor during irradiation.

The TBARS assay confirmed the EPR results for KA, KG, OP, and mixture, showing no change in absorbance after irradiation, but does not confirm the EPR results of STJA, where an EPR spectral intensity increase is observed.

DISCUSSION

The amount of DEPMPO radical adducts detected in the liposomes with KA or KG after UV irradiation was quite low. The effect of KA was slightly greater than that of KG. This result is in accordance with the results of other studies that show that KA with 3,5,7,4′ free OH groups is a relatively good antioxidant (*10*). Its antioxidant activity reflects its flavonol ring structure with one OH group at 4′ on the B ring (*10*). However, according to the same authors, the single OH group on the B ring has no effect on the antioxidant activity. Only the 5,7-dihydroxy groups on the A ring and possibly on the C ring contribute to the antioxidant activity in kaempferol.

Table 2. The Amplitude of OH• Radical Adduct (h_{OH} = in the Presence of Antioxidants, h_0 = Control), the EPR Spectra Intensity (I and I_0, Respectively, Are the Sum of All Adducts) Measured in the Presence of Different Antioxidants after Irradiation (for 2 h at λ = 365 Nm), the Peroxide Number (A_0 = before Irradiation, and A_{tot} = after Irradiation) Measured by the TBARS Assay, and the Relative Differences between the Irradiated and the Non-Irradiated Samples ($A_{irr} = A_{tot} - A_0$)

antioxidant	EPR[a]		TBARS[a]		
	h_{OH}/h_0 (%)	I/I_0 (%)	A_0	A_{tot}	A_{irr}
control (H_2O)	100	100	0.43	0.93	0.50
PBS pH 2.5	322	205	0.50	0.67	0.17
KA (9 mmol/L)	0	0	0.17	0.17	0.00
KG (9 mmol/L)	26	29	0.17	0.20	0.03
OP (4.3 mg/mL)	28	63	0.37	0.38	0.01
CA (50 mmol/L)	337	286	0.46	1.15	0.65
MA (50 mmol/L)	389	312	0.44	0.91	0.47
mix[b]	43	43	0.20	0.15	–0.05
STJA (12.5% v/v)	99	165	0.45	0.45	0.00

[a] Standard deviations are no more than 10%. [b] Mixture of KA (4.5 mmol/L), KG (4.5 mmol/L) MA (25 mmol/L) and CA (25 mmol/L) (final concentrations).

According to our experiments, KG was a less effective antioxidant than KA. In some assays, the 3-glycosylation of flavonoids reduces their activity as compared with the corresponding aglycones (*12*), which agrees well with our results. According to the conclusions made by Rice-Evans and Miller (*10*) about KG, which does not have a free OH group on position 3 in the ring C anymore, only the OH's on the A ring contribute to the antioxidant activity of the compound. If only these functional groups were responsible for radical scavenging, the same effectiveness for this compound as for KA would be expected. The reduced activity of KG implies that the contribution of the OH group on the ring C is also important for antioxidant activity of flavonoids and isoflavonoids.

Another means of evaluation of antioxidant capability involves comparison of appropriate reduction potentials of participating species. Usually, an antioxidant gives an electron to a free radical, thereby becoming a one-electron deficient daughter free radical. Antioxidant capability can then be measured by the standard reduction potential for the reduction of the daughter back to the parent species. In this situation, the

lower the reduction potential for this half reaction, the better the parent substance's antioxidant capability. Our results indicate that kaempferol has the lowest reduction potential, because it decreases the free radical level best. Although the reduction potential is the major factor determining the antioxidant capacity of flavonoids, the overall ability to act as an antioxidant is also determined by other physicochemical characteristics, such as molecular polarity, which determines the substances' distribution between polar and nonpolar media (*14*).

For the oligomeric polyphenols, a strong inhibition of $OH^{\bullet}$ and $O_2^{\bullet-}$ radical adduct formation was also observed. It was found that 4.3 mg/mL OP inhibited the formation of $OH^{\bullet}$ and $O_2^{\bullet-}$ adducts 76 and 83%, respectively. These are almost the same values as were found for 1.2 mmol/L KA or 9 mmol/L KG **(Table 1)**. The most significant difference was in the formation of the carbon centered radical adduct, which increased in the presence of OP **(Figure 4)**. Because this extract could contain traces of some other compounds besides KA and KG, like epigallocatechin or epigallocatechin-3-gallate or their polymers, it is possible that these other compounds are contributing to the increased level of carbon-centered free radical adducts. According to TBARS, OP also almost completely prevents the formation of malondialdehyde during irradiation, but is not very effective during the preparation of the liposomes **(Table 2)**. This effect can be explored with the use of such extract in the prevention of lipid autoxidation with light.

Among the water soluble compounds of ST juice, citric acid and malic acids were investigated. Citric acid has been considered an antioxidant, acidity regulator, and sequesterant or chelating agent, and malic acid has been considered as an acidity regulator only (*20*). Because a chelating agent can bind heavy metal ions and traces of heavy metal ions can catalyze the oxidation of fats or oils, citric acid should decrease the free radical formation in the system (*20*). According to our experiments, both acids caused a pronounced increase in the intensity of the EPR spectra **(Figure 2)**, and they also did not inhibit the formation of malondialdehyde in the TBARS assay **(Table 2)**. Therefore, it could be concluded that they do not express any antioxidant activity in the systems at the concentration used (50 mmol/L). The observed intensity increase can be explained by low pH values of liposome dispersions with acids, where a pH of 2.5 was measured. As demonstrated in the pH variation experiments **(Tables 1** and **2)**, EPR spectra intensity increase of all radical adducts was observed with decreased pH. The most pronounced was the increase of DEPMPO−OH radical adduct.

A mixture of KA, KG, malic acid, and citric acid, comparable to ST juice, changed the ratio between hydroxyl and superoxide radical adducts in the EPR spectra **(Figure 2** and **Table 1)**. Whereas, in the presence of 9 mmol/L KA alone, the formation of oxygen radical adducts stopped completely; addition of malic and citric acids appeared to diminish the antioxidant activity of KA. Similar to the results for other samples containing acids, these results can be explained by the low pH value of mixture.

The preparation of liposomal systems with one of the acids, water, and DEPMPO exhibited spectra **(Figure 2)** similar to the mixture. Thus, we conclude that the low pH caused the extra amount of DEPMPO−OH free radical adduct in the aqueous phase, where there is limited antioxidant capacity. This interpretation of data is also supported by the negligible lipid peroxidation in this system indicated by the TBARS assay **(Table 2)**.

Juice squeezed from *S. tectorum*, to which ascorbate oxidase was added in order to stop the reduction by ascorbate, behaved differently from the mixture but, as might be expected based on compositional similarity, in the same way as OP **(Figure 4** and **Table 1)**. It diminished the amount of $OH^{\bullet}$ and $O_2^{\bullet-}$ radical adducts but significantly increased the formation of carbon centered radical adducts. Possible explanations for these differences can be based on the overall composition of ST juice and the methods of preparation of the liposome dispersion with the mixture. First, the ST juice, which could contain some other compounds with antioxidant activity, and/or substances that solubililize the hydrophobic KA and KG, thereby cause the aqueous phase of the liposomal system to have some antioxidant capacity. Thus the juice, even though it has a pH of 2.5, does not exhibit as much DEPMPO as the mixture in the EPR spectra. Also, the preparation of the mixture involved mixing the KA and KG with the lipid materials before the thin film was prepared. Thus, the KA and KG were more abundant in the lipid than in the ST juice and, hence, provided more antioxidant protection against oxygen-centered free radical formation. Finally, as indicated earlier, some other compound in ST juice and OP could be forming carbon-centered free radicals, which are captured by the DEPMPO. In contrast, the mixture, which contained only KA, KG, citric acid, and malic acid exhibited no carbon-centered free radical adduct EPR spectrum.

In this work, we have confirmed the antioxidant activity of ST juice, and we have evaluated the contribution of the individual compounds in the juice to this antioxidant activity. The results of this work support our earlier findings of antimicrobial activity of *Sempervivum* L. (*4*), and a suggestion recently stated by Shinella et al. (*21*) that antiinflammatory activities, for which *S. tectorum* is used as a folk medicine, could be explained, at least in part, by antioxidant properties of plant extracts. This study also demonstrates that careful utilization of UV irradiated liposomal systems in conjunction with EPR studies and TBARS measurements can provide insight into the antioxidant properties of both natural mixtures and their components. In contrast to the broad spectrum information provided by TBARS assays, EPR spin trapping can provide specific details about the types of chemical processes occurring in the system. In this particular study, the two data forms supported one another and revealed some interesting insights into free radical activities in both the aqueous and lipid regions of the liposomal system. In the literature, many results on antioxidant activity of plant extracts could be found. Unfortunately, the results cannot be compared with ours, because the methods used for antioxidant capability determination are different. Application of the same methods to other well characterized natural antioxidant mixtures will facilitate direct comparisons of the antioxidant capability of different plant extracts that could enable incorporation of new entries into the current antioxidant capability ranking systems.

ABBREVIATIONS USED

ST, *S. tectorum*; STJA, *S. tectorum* juice with ascorbate oxidase added; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-pyrroline-*N*-oxide; EPR, electron paramagnetic resonance; PBS, phosphate buffered saline solution; KA, kaempferol; KG, kaempferol-3-glucoside; TBARS, thiobarbituric acid related substances; RP-HPLC, reversed phase high performance liquid chromatography; OP, oligomeric polyphenols; MLV, multilamellar vesicles; PC/PS, liposomes prepared from egg lecithin (PC) and phosphatidylserine (PS); MA, malic acid; CA, citric acid.

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