Antioxidant Activity of Flavones from *Scutellaria baicalensis* in Lecithin Liposomes

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Trihydroxyflavones of *Scutellaria baicalensis*, Antioxidant Activity, Liposome, Peroxidation, MDA

The antioxidant effect of a trihydroxyflavone extract from *Scutellaria baicalensis* on oxidation induced by ultraviolet light, was studied with phosphatidylcholine liposome membrane. Also, as standards, the antioxidative activity of baicalin, wogonin, baicalein and butylated hydroxytoluene (BHT) was investigated. Comparison of the protective effects of the compounds studied against photoinduced lipid peroxidation in lecithin liposome membranes showed that: (1) the inhibitory effect of those compounds (at 1.2 mol% antioxidant content in liposomes) on TBA reactive materials from lipid peroxidation decreased in the order of baicalin > BHT ≅ *Scutellaria baicalensis*. These were found much greater than wogonin and baicalein; (2) the depressed effect of those compounds (at 1.1 mol% compounds content in liposomes) on the production of conjugated dienes (proportional to oxidation index) could be classified as follows: *Scutellaria baicalensis* ≅ baicalin > BHT, these three were found more active much greather than baicalein and wogonin. Results obtained by ESR measurement confirm that *Scutellaria baicalensis* extract and the BHT compound significantly depressed the effect of liposome oxidation.

It was found that the new trihydroxyflavones of *Scutellaria baicalensis*, ensured a very satisfactory concentration-dependent protection of the liposome membrane against UV-induced oxidation. These findings suggest that some of the beneficial effects of the extract of the *Scutellaria baicalensis* can be mediated in certain diseases (for example in skin diseases) by their ability to scavenge free radicals and by their protective effect on lipid peroxidation caused by sunlight irradiation.

Introduction

Flavonoids are a group of phenolic compounds widely occuring in the plant kingdom and are present in common foods for example: tea; fruits (citrus, grapes; apples, cherries,berries); vegetables (onions, kale, broccoli, parsley); nuts; beans and red wine. They are dietary compound (≈1 g/day) of pharmacological interest because of their antioxidant activity (Bonino *et al.,* 1996; Chen *et al.,* 1996; Guo *et al.,* 1996; Gabrielska *et al.,* 1997; Rasetti *et al.,* 1997). Free radicals, the products of oxidation processes, play the main role in the human organism in certain diseases. They can cause cardiovascular disease, chronic inflammation, atherosclerosis, different cancers and accelerate the alteration processes (Holman *et al.,* 1996). Natural phenolic compounds could protect people against those diseases (Kamei *et al.,* 1996; Varma, 1996; Holman *et al.,* 1996), probably owing to their antioxidant properties.

Dried roots of *Scutellaria baicalensis* are a very old, well-known drug in traditional Chinese medicine for the treatment of bronchitis, hepatitis, diarrhoea and tumors (Tang and Eisenbrand, 1992; Zhou *et al.,* 1997). We extract from hairy root cultures of *Scutellaria baicalensis* to prepare a trihydroxyflavone with a very high efficiency. The extract contains baicalin (in 75%), and other nonidentified flavones as derivatives of wogonin and baicalein (Sokół-Łętowska, 1977).

The objective of the work was to study the protective effect of this new extract of *S. baicalensis*

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against UV-induced peroxidation on phosphatidylcholine vesicles as a model membrane. For comparison the antioxidant activity of pure baicalin, wogonin, baicalein and the known synthetic antioxidant BHT were measured. Also by using the ESR method the alleviating effect of *S. baicalensis* and BHT was evaluated.

Materials and Methods

Chemicals

Egg yolk lecithin was prepared in our laboratory by the method described by Singleton *et al.* (1965). All the compounds studied are presented in Fig. 1. Trihydroxyflavone of *S. baicalensis* extract was prepared in the Department of Fruit and Vegetable Technology Agricultural University of Wrocław, by methanol extraction from hairy root of scullcap. The composition of the extract was determined by HPLC (Zhou *et al.*, 1997). Baicalin, wogonin and baicalein were from Nacalai Tesque (Kyoto), butylated hydroxytoluene (BHT) from Sigma (Deisenhofen). Stearic acid spin label with 4,4-dimethyloxazolidine-N-oxyl group at the 5-th and 16 carbon positions were from Sigma Chem. Co. (St. Louis). These will be referred to as SA-5 and SA-16, respectively. All other chemicals were of analytical grade from commercial sources.

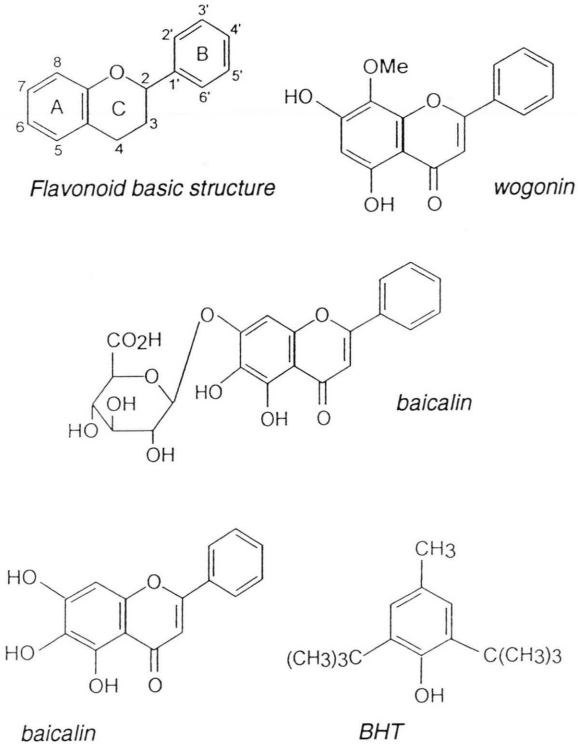

Fig. 1. The chemical structure of the compounds studied. Trihydroxyflavone of *Scutellaria baicalensis* extract contains 75% baicalin and 25% other derivatives of baicalein and wogonin.

Preparation of extract of Scutellaria baicalensis

The extract of *S. baicalensis* from 10 g of dry powdered rootstalks was extracted three times with 100 ml methanol. The solution was then filtered through Schott funnels. After methanol evaporation at 40 °C under vacuum, the residue was dried with a vacuum drier. 2 g of yellow powder was thus obtained containing 75% of baicalin.

Multilamellar liposome preparation and induction of the peroxidation

Chloroform solution of egg lecithin was evaporated under vacuum in nitrogen atmosphere. The thin film of lecithin obtained was shaken with 0.05 M Tris:HCl (hydroxymethyl) aminomethane buffer at pH 7.4 during a quarter of an hour (control liposome) or with the extract of *S. baicalensis* or with BHT during the next fifteen minutes. The compounds were added as a stock solution with ethanol. The amount of solvent never exceeded 1.1% of the final volume in the reaction, and control incubations contained the same volume of the ethanol used. The concentration of the antioxidant studied was varied in the range 1–25 μM. The liposome suspension contained 1.5 mg phosphatidylcholine (PC) per ml. In the case of the studies with baicalin, wogonin and baicalein, and for comparison to the same experiments with the extract of *S. baicalensis*, a stock solution of those compounds in ethanol was added to chloroform solution of egg yolk lecithin (at 1.2 mol% concentration) and was evaporated under vacuum in nitrogen atmosphere. The following procedure was identical as described above.

Lipid peroxidation in the phospholipid liposomes was induced by ultraviolet radiation of 3.5 mW/cm^2. The accumulation of phospholipid peroxidation products was estimated by the determination of 2-thiobarbituric acid (TBA)-reactive products in the incubation medium (Buege and Aust, 1972; Porębska-Budny *et al.*, 1992) and ex-

pressed as the increase of absorbance at 535 nm (ΔA 535). The spectra were recorded by a Specol 11 (Zeiss Jena).

Conjugated diene formation

Of the incubated multilamellar liposomes (at concentration 9 mg lecithin/ml) prepared as described above with compounds added to chloroform solution before evaporation (at 1.1 mol%), 250 μl were taken, dissolved in 3 ml of ethanol and analyzed spectroscopically for conjugated diene formation (Klein, 1970; Fiorentini *et al.*, 1989). The absorption spectra of conjugated diene were recorded in 232 nm wavelength by a UV-VIS spectrophotometer (Zeiss Jena). The increase of absorption at 232 nm was used as indicator for conjugated dienes (proportional to oxidation index), the absorption at 300 nm being taken as zero.

ESR measurements

The multilamellar liposomes (at concentration 40 mg lecithin/ml of veronal-acetate buffer at pH 7.4) were UV-irradiated during 6 hours with a bactericidal lamp at intensity of 3.5 mW/cm^2. During irradiation the suspension was stirred. Every hour 20 μl of irradiated liposomes were added to a spin label probe and vigorously vortexed for 3 min and placed in a glas capillary (1 mm i.d.). An appropriate amount of spin probe was used to maintain the label to lipid molecular ratio 1:1000. Two stearic acid spin probes were used; SA-5 and SA-16. The ratio of the antioxidant studied to lecithin was 1:20. All spectra were recorded at temperature 300K on the standard SE/X28 electron spin resonance spectrometer operating in the X-band (made in Technical University of Wrocław), at microwave power 2.0 mW, sweep time 10 s and time constant 100 ms. For scientific description of ESR spectra the LABCAD programme was used.

The ESR spectral parameters, rotational correlation time τ_c and order parameter S, were evaluated from following equations:

$$\tau_c = 6.5 \cdot 10^{-10}\, w_o\, [(h_o/h_{-1})^{0.5} - 1],$$

where w_o, h_o, and h_{-1} are parameters taken from ESR spectrum; w_0 is the midfield line width, h_0 and h_{-1} are mid- and high-field line amplitudes;

$$S = (A_{||} - A_{\perp}/A_{zz} - A_{xx}) \cdot (a/a')$$

where $A_{||}$ and $A_{\perp}$ are the measured maximum and minimum hyperfine splitting constances, respectively, A_{zz} and A_{xx} are the hyperfine splitting tensors measured for the probes in the crystal matrix, a and a' are the isotropic hyperfine splitting factors for the nitroxides in a crystal and in the membrane, respectively (Kocherginsky and Swartz, 1995).

Results

The effect of ultraviolet-induced phosphatidylcholine peroxidation on the time of radiation of liposome suspension in the presence of different concentrations of the *S. baicalensis* extract is shown in Fig. 2. For comparison, the concentration-dependent effect of BHT is also shown antioxidant. The increase of absorbance at 535 nm (ΔA_{535}) is caused by changes in the concentration of TBA-reactive products in the medium. It is evident that *S. baicalensis* and BHT inhibit the phospholipid peroxidation in liposomal membranes and that the extent of this inhibition depends directly on the compound concentration. It can also be seen that the inhibition potency of *S. baicalensis* on the liposome peroxidation is only slightly smaller than the inhibition potency of BHT compounds for the smallest concentration of antioxidant equal to 1.5 μM and is the same for the highter concentration equal to 3.0 μM. A similar effect of antioxidant activity of *S. baicalensis* extract and BHT compounds was confirmed in the second experiment. Fig. 3 demonstrates the influence of the extract studied and BHT compound at chosen concentration (1.1 mol%) on the increase of absorbance at 232 nm, i.e. on the decrease of conjugated diene production (or oxidation index) of the liposome membrane. It is shown in Fig. 3 that production of conjugated diene, which take place during UV radiation, is efficiently inhibited by *S. baicalensis* and a little less inhibited by BHT. Comparative results of measurement of the antioxidant activity of *S. baicalensis* and the standard compounds of baicalin, baicalein and wogonin, which are contained in the extract of *S. baicalensis*, are presented in Figs 4 and 5. It is seen in the figures that baicalin exhibits the greatest effect on concentration of TBA-reactive products and on concentration of conjugated dienes formed during UV radiation of the liposome suspension. Smaller

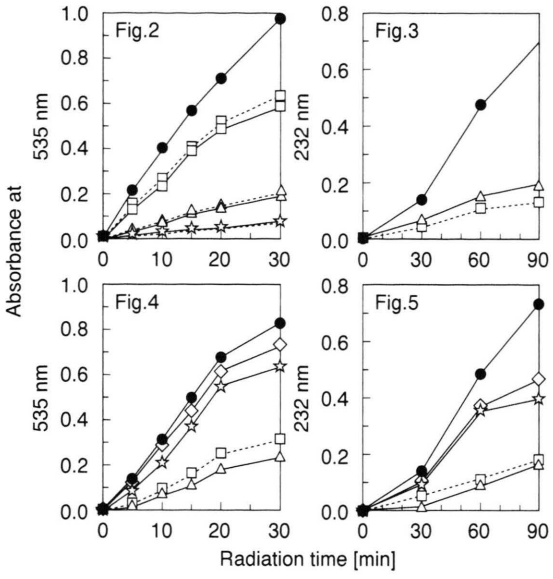

Fig. 2. Inhibition of UV-induced oxidation of egg yolk lecithin liposomes (estimated by the determination of 2-thiobarbituric acid (TBA)-reactive products in the medium of liposome and expressed as the increase of absorbance at 535 nm) by extract of *Scutellaria baicalensis* at different concentration and to comparison by BHT. Intensity of UV radiation was 3.5 mW/cm^2, concentration of lecithin was 1.5 mg/ml. Data are the means of six samples of three experiments with different liposome preparation. Filled circles and continuous line – control; dotted line – flavonoids of *Sc. baicalensis*; continuous line – BHT; The concentration of antioxidant present in the liposome suspension was 1.5 μM (open squares); 3.0 μM. (open triangles) and 6.0 μM (open asterisks).

Fig. 3. Effect of studied extract of *Scutellaria baicalensis* (dotted line and open squares) and to comparison of BHT (continuous line and open triangles) on the production of conjugated dienes in liposomes, for 1.1 mol% of antioxidant content in liposomes, after different time of UV irradiation. Data are the means of four probes of two experiments on different lipid preparation. Intensity of UV radiation was 3.5 mW/cm^{-2}. Continuous line and filled circles was for unmodified liposomes.

Fig. 4. Inhibition of UV-induced oxidation of lecithin liposomes (estimated by the determination of 2-thiobarbituric acid (TBA)-reactive products in the medium of liposome and expressed as the increase of absorbance at 535 nm) by the compounds presence in the extract of *Sc. baicalensis*: baicalin (open triangles); baicalein (open asterisks); wogonin (open rhombus) and to comparison for extract of *Sc. baicalensis* (dotted line and open squares). Continuous line and filled circles – control. The concentration of antioxidant present in liposomes was 1.2 mol%. Experimental conditions are the same as those in Fig. 2.

Fig. 5. Effect of studied flavones, at 1.2 mol% content in liposomes , on the production of conjugated dienes in liposomes membranes after different time of irradiation: open triangles – baicalin; open squers and dotted line – extract of *Sc. baicalensis;* open asterisks – baicalein; open rombus – wogonin; filled circles – control. Experimental conditions are the same as those in Fig. 3.

antioxidant activity has trihydroxyflavone of *S.* baicalensis, and the smallest – baicalein and wogonin. These differences in the antioxidant activity are well illustrated in Fig. 6. It shows percent inhibition of the four compounds studied: baicalin (1), *S. baicalensis* extract (2), baicalein (3) and wogonin (4), calculated on the basis of the results presented in Fig. 4. Percent of inhibition was calculated as follows:% INHIBITION = $(\Delta A_{535} - \Delta A'_{535})/\Delta A_{535} \cdot 100\%$; where ΔA_{535} – increase in absorbance at 535 nm after 30 min of UV radiation of liposomes in the absence of flavones; $\Delta A'_{535}$ – increase of absorbance at 535 nm after 30 min UV radiation of liposomes in the presence of flavones.

The inhibitory effect of *S. baicalensis* extract and BHT on lipid peroxidation of lecithin liposomes incubated with and without the compounds studied under the UV radiation were investigated with ESR measurements of spin labels (Ondrias *et al.* 1989; Misic *et al.* 1991). Two stearic acid spin probes were used: SA-5 and SA-16. From the ESR spectra of spin probe SA-5 and SA-16 incorporated into liposome membranes the order parameter (S) and apparent rotational correlation time (τ_c) were calculated, respectively. The rotational correlation time did not change during the time of UV radiation of control liposomes and pretreated with an antioxidant. The order parameter of spin probe SA-5 in the lecithin liposomes versus time of UV radiation are presented in Fig. 7. The order parameter of the spin probe in the contol liposomes decreased with time of lipid peroxidation. *S. baicalensis* extract and BHT considerably reduced the decrease of order parameter with the oxidation time.

Discussion

Trihydoxyflavones from the root of *S. baicalensis* contein ca. 75% baicalin and 25% of other derivatives including baicalein and wogonin which was found by the HPLC method. A relative simple extraction allows to obtain flavones preparation from dry mass of scullcap with ca. 20% efficiency

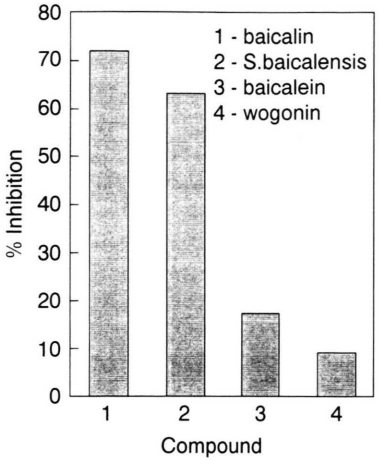

Fig. 6. Percent inhibition of lecithin liposome oxidation (estimated on the basis of results present in Fig. 4, see in the text) for different flavonoids studied. Concentration of flavone content in liposomes was 1.2 mol%. 1 – baicalin; 2 – extract of *Sc. baicalensis*; 3 – baicalein; 4 – wogonin.

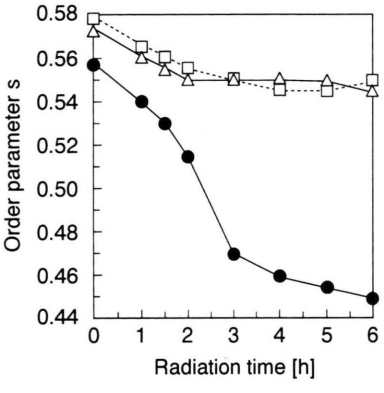

Fig. 7. Dependence of the order parameter S in liposomes on time of UV irradiation of multilamellar lecithin suspension: unmodified (continuous line filled circles) and modifed with extract of *Sc.baicalesis* (dotted line and open squares) or with BHT (continuous line and open triangles). Concentration of antioxidant present in multilamellar liposomes was 5 mol%. Deviation of order parameter was $\Delta S = 0.02$.

(see Materials and Methods). Potential healing properties of the extract, for instance treating skin diseases caused by sunlight (dry root of *S. baicalensis* has been used since long ago in the traditional Chinese medicine for treating many diseases), may be connected with its antioxidant properties (Hollman, 1996). The results of investigation shown in Figs. 2 and 3 allow to conclude that the extract *S. baicalensis* exhibits high, concentration dependent, antioxidant activity in lecithin liposome membranes irradiated with UV light. This activity can be expressed by concentrations causing 50% inhibition of lecithin liposome oxidation (IC_{50}) for extract of *S. baicalensis* and for BHT, which are 1.3 and 1.1 μM, respectively. BHT is known to be a very effective antioxidant (Ondrias et al., 1989). The antioxidant activity of flavone extract towards BHT is well illustrated by the summary given in Table I of percent inhibition of both compounds as dependent on concentration. The results given in Figs 4, 5 and 6, where the antioxidant activity of *S. baicalensis* extract is compared with the standards of the basic extract components, i.e. baicalin, baicalein and wogonin, show that baicalin exhibits the highest 72% inhibition of oxidation. 75% of this compound is present in the extract. The degree of oxidation inhibition of baicalein and wogonin (in an experiment presented in Fig. 4) was 19% and 9% respectively. It can thus be concluded that mostly baicalin is responsible for the antioxidant properties of *S. baicalensis* extract. The chemical structure of baicalin and baicalein (see Fig. 1) differ in a glucuronic acid substitute in the ring A at C7 position. This moiety may thus be thought to play an essential role in the antioxidant action of baicalin and *S. baicalensis* extract in liposome membranes exposed to UV light. It is probable that substituent of glucuronic acid makes the baicalin molecule to assume optimum orientation in the liposome membrane, enabling it to capture most effectively the free radicals that attack the membrane. An increased biological activity of flavonoids just with glucoside substituent was noticed by Kamei *et al.* (1996a, 1996b) (Koide *et al.,* 1996), who found a

Table I. Percent inhibition (defined as $[1 - \Delta A_{535}'/\Delta A_{535}]$ 100%, $\Delta A_{535}'$ – absorption after 30 min of UV radiation at 535 nm in the presence of antioxidant, ΔA_{535} – in the absence of antioxidant) of liposome oxidation induced by UV radiation at intensity 3.5 mW/cm^2. Concentration of lecithin was 1.5 mg/ml.

Antioxidant	Percent inhibition [%] of liposome oxidation induced by UV radiation at concentration in μM			
	1.5	3.0	6.0	25
Sc. baicalensis	62	89	96	98
BHT	64	90	95	97

flavonoid-mediated suppresion of tumor growth of cells and that both the number and place of OH groups around B phenol and glucose attachment at A phenol play an important role in the tumor suppression. High antioxidant activity of flavonoids extracted from *Sideratis javalambrensis* with a glucose group as compared with compounds without such group was found for gossypin and 7,8-dihydroxyflavone with a glucose group in C8 position of A phenol (Rios *et al.*, 1992). It was also found that both compounds cause inhibition of inflammation, which the authors belive may be due to antioxidant properties of the flavonoids.

Oxidation of lipid membranes, according to literature data cited by Chatterjee and Agarwal (1988), is the reason of the decrease in the fluidity of those membranes. However, there are data suggesting that the oxidation causes an increase at membrane fluidity (Delmelle, 1978; Grzelińska *et al.*, 1979; Ondrias *et al.*, 1989 and Misic *et al.*, 1991). The results of ESR studies presented in Fig. 7 shows that the order parameter *S* of the nitroxide spin probe SA-5 in liposomes oxidized with UV light decreases with exposure time and thus with the degree of oxidation (from a value 0.56 for nonradiated membrane to 0.45 after 6 hours of UV radiation). Addition of *S. baicalensis* extract and BHT resulted in an increase of the parameter *S* compared to control to the value 0.55 after 6 hours of radiation. The increase in parameter *S* from 0.45 for a probe in oxidized liposome membrane to ca. 0.55 for a membrane with the extract added may indicate that the membrane fluidity after addition of antioxidant was approximately similar to that of nonoxidized liposome membrane, for which the order was 0.56 (deviation of order parameter was $\Delta S = 0.02$). This in turn speaks in favour of an effective antioxidant action of *S. baicalensis* comparable to the action of BHT. A similar dependence of the parameter *S* of spin probe SA-5 in liposome undergoing autooxidation with and without addition of flavonoids and caffeyl derivatives of propolis was obtained by Misic *et al.*, (1992). Order parameter obtained in their experiment was 0.53 for unoxidized sample and decreased to the value of about 0.47 after 96 hours incubation at 50 °C.

The results of their experiment as well as those obtained by Barratt and Laggner, (1974) permit to interpret them as a decrease in parameter *S* resulting from pH changes of the medium (between 7.3 and 6.2). A decreased value of pH induced decreased ionization of the acid group of spin probe and the resultant change in its motional anisotropy that determines the value of parameter *S*. In the case of the presented ESR studies the changes in pH of liposome dispersion were in fact lower (changing from 7.5 to 7.2), but one can't exclude local, greater pH changes close to the membrane than those recorded for the sample. Those changes may be a consequence of the effect of UV light on the surface of the liposomal membrane. Changes on the membrane surface might modified the mobility of the spin probe locatad at the 5-th carbon of the alkyl chain in the hydrophobic region of the lipid bilayer. Significant changes at the membrane surface is supported by observation that the correlation time τ_c (determined from the measurements using the probe located at the 16-th carbon atom of the hydrocarbon chain) does not change during UV irradiation.

In conclusion, it is evident that *S.baicalensis* extract possesses high potency to reduce lipid peroxidation in phosphatidylcholine liposomes. The extract probably may be considered scavenger of free radicals. Our results suggest that some of the beneficial effects of the *S. baicalensis* extract can be mediated in various diseases, especially skin diseases caused by sunlight radiation, through its ability to scavenge free radicals and thus exert a protective effect against lipid peroxidation.

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