# Effects of Flavonoids of Ginseng Leaves on Erythrocyte Membranes against Singlet Oxygen Caused Damage

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**Abstract**□ It has been well known that extended exposure to reactive oxygens causes severe damage to susceptible biomolecules. In this study, the effects of flavonoids including trifolin and kaempferol from Ginseng leaves on singlet oxygen induced photohemolysis of erythrocytes and free radical scavenging activities were investigated.

Each flavonoid aglycone (5-50 $\mu$M) such as kaempferol, quercetin or baicalein exhibited a high protective effect against the photohemolysis. They protected the cells by scavenging $^1O_2$ and free radicals. Although the free radical scavenging activities of the flavonoid glycosides were not much lower than those of their corresponding aglycones, their insolubility into lipid bilayers of membrane made them less effective in preventing the photohemolysis induced by $^1O_2$.

The $^1O_2$ and free radical scavenging activities of flavonoids were estimated by the decomposition of the flavonoid by $^1O_2$ and the bleaching of free radicals by the flavonoid, respectively. The solubilization of the flavonoid into micells or erythrocytes was deduced from spectrophotometric and microscopic observations.

The cooperation of L-ascorbic acid and a flavonoid, and a possible involvement of lipoxygenase or cyclooxygenase in the photohemolysis mechanism were discussed.

**Keywords**□ *Panax ginseng* C.A. Meyer, ginseng leaves, flavonoids, singlet oxygen, photohemolysis.

## Introduction

The major forms of electronically activated and reactive states of oxygen are singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^-$), hydroxyl radical (·OH) and hydrogen peroxide ($H_2O_2$). These can be produced significantly in cells by a variety of processes including high energy irradiation, photosensitization, phagocytosis and several enzymatic reactions.[1-7]

The biological consequences of excessive production of reactive oxygen may involve mutations, inflammation, cell lethality, carcinogenesis and protein denaturation related to skin aging.

Several lines of evidence support the view that $^1O_2$, among the reactive oxygen species, has the greatest significance in the cell which have endogenous photosensitizable molecules, and frequent opportunities of being exposed to a variety of xenobiotics. Recently Tyrrell *et al.* proposed that $^1O_2$, but not $H_2O_2$ or ·OH, plays an important role in the inactivation of cultured human cells by UV-A.[9] And Foote *et al.* reviewed that protein damage in light-exposed skin is the result of $^1O_2$-mediated polymerization and the photocarcinogenesis has been attributed to the action of $^1O_2$.[5]

Kanofsky *et al.* identified the human eosinophils as the first living cells able to generate $^1O_2$ and reported eosinophils are also able to damage normal tissues in a variety of inflammatory disease states.[10]

Besides, the flavonoids widely used as therapeutic agents are known to act as strong scavengers of the active oxygen species, and react with peroxy radicals involving termination of radical chain reactions during the autoxidation of polyunsaturated

fatty acids.[11-15]

Ginseng leaves which have been used as a remedy for skin disorder or a deodorant since thousands of years,[16] were reported to have antioxidative, anti-gout and growth stimulation activities.[17-19]

This paper deals with the effects of several flavonoids, especially those from Ginseng leaves, on $^1O_2$-induced hemolysis of rabbit erythrocytes in order to evaluate the flavonoids as practical protectors for cellular damage caused by $^1O_2$. The obtained results indicated that not only scavenging of reactive oxygens or free radicals but also the solubilization into lipid bilayer of cellular membrane should be the important factors for a flavonoid to protect cellular membranes against $^1O_2$.

## Materials and Methods

### Apparatuses and instruments

Electronic absorption spectra were measured with a spectrophotometer (CE-5500) of Cecil Co. and fluorescent spectra with a spectrofluorometer (SPF-500C™) of SLM-AMINCO Co.. Nikon Optiphot Episcopic Fluorescence Microscope was used in the observation of erythrocytes, and Spectronic 20 D of Bausch & Lomb Co. equipped with CETRON CE-A 30 phototube sensitive to red light was utilized for the determination of % transmittance in photohemolysis experiment.

### Plant materials

Ginseng leaves were collected at a farm located in Kanghwa (Kyongkee-Do, Korea) in September 1989 and air-dried at a temperature below 40 °C.

### Chemicals

Kaempferol, quercetin, rutin, rose-bengal, L-ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), soybean lipoxygenase (Type I, EC 1.13.11.12, 134,000 units/mg protein), linolenic acid and thiobarbituric acid were purchased from Sigma Chemical Co. Polyoxyethylene (12) nonylphenyl ether (Trade name; ENP-12) was supplied by Nihon emulsion Co. Baicalein and baicalin were isolated from *Scutellaria baicalensis* Georgy in our laboratory.[15] And trifolin was isolated from Ginseng leaves by following procedure for this study. TLC was silica gel 60 F-254 glass sheets (Merck), and column chromatography was achieved on silica gel-60 (Merck) and Sephadex LH-20 (Pharmacia).

### Extraction and isolation of trifolin from Ginseng leaves

Crushed leaves (300g) were extracted with methanol (4 litre, 10 days) at room temperature and concentrated to dryness in a vacuum. The methanolic extract (32g) was dissolved in water and then extracted with ethyl ether. The ethyl ether extract didn't contain flavonoids (inspection by TLC) and was discarded. Aqueous fraction was introduced on the polyamide column ($80 \times 4$ cm) and eluted with water and 50% methanol. 50% Methanolic extract was chromatographed on a silica gel column using the elution solvent of chloroform-methanol-water (60/30/5). The fraction containing flavonoid was submitted to Sephadex LH-20 column chromatography yielding 0.54 g of trifolin.

### Hydrolysis of trifolin to obtain kaempferol

2 mg of Trifolin was hydrolysed in 10 m*l* of 2 M HCl for an hour at 95 °C, then extracted with 20 m*l* of ethyl acetate, and evaporated to dryness to obtain the aglycone. The aglycone was identified as kaempferol by chromatographic (TLC and HPLC) and spectroscopic (UV) comparisons with authentic kaempferol.

### Preparation of erythrocytes

The blood collected from a rabbit was centrifuged at 3000 rpm for 5 min to separate erythrocytes and serum, then washed and diluted with 0.9% saline phosphate buffer, pH 7.4 to prepare the erythrocyte suspension (15.0 million cells/m*l*). The erythrocyte was used either immediately or within a few hours, while stored at 4 °C in a refrigerator.

### Measurement of photohemolysis

The reaction mixture for photohemolysis determination consists of a test group containing 3.5 m*l*

of erythrocyte suspension, 50 $\mu l$ of ethanolic sample solution and a control group containing only 50 $\mu l$ of ethanol. After the mixture was pre-incubated for 30 min at the dark place, 0.5 m$l$ of 12 $\mu$M rose-bengal as a photosensitizer was added per tube, and then sealed with a laboratory sealing film. Then the tubes placed in 50 × 20 × 25 cm rectangular hexahedron box of which the inside had been painted black were irradiated for 15 min by a 20 Watt fluorescent lamp at a distance of 5 cm. The degree of hemolysis of the erythrocytes was measured by % transmittance at 700 nm at intervals of 15 min. The increase in % transmittance at 700 nm was proportional to the degree of hemolysis of the erythrocytes.[7] Every step of the above experiment was carried out in the constant room temperature of 27 °C. The activity of the sample in protecting the cells against active oxygen species was defined as half-time of hemolysis ($\tau_{50}$), the time required for 50% of the erythrocytes added to be hemolysed.

### Free radical scavenging activity

Free radical scavenging activity was determined by the modified method of Fujita *et al.*[20] using a moderately stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). 1.0 m$l$ of ethanolic sample solution (up to 200 mM) was added to 0.5 m$l$ of 0.2 mM DPPH methanolic solution, and allowed to stand for 10 min at 25 °C. The amount of free radicals in the mixture was measured by absorbance at 517 nm. The free radical scavenging activity ($SC_{50}$) was defined as the concentration of the sample required for 50% of the free radicals to be scavenged.

### Soybean lipoxygenase inhibition assay

Inhibition of soybean lipoxygenase was assayed by the method of Bawmann.[21] The activity was defined as the concentration of sample for 50% inhibition ($IC_{50}$) of soybean lipoxygenase.

## Results

### Effects of flavonoids on the rose-bengal sensitized photohemolysis

Singlet oxygen generated from rose-bengal (RB) sensitization induced the hemolysis of rabbit erythrocytes (Fig. 1). Both presence of RB and irradiation were required to hemolyse erythrocytes significantly in 120 min of post-incubation time. When irradiated in the presence of RB, erythrocytes were hemolysed by 50% in a 32 min (half-time of hemolysis, $\tau_{50}$ = 32 min). We used this condition as a control. In most cases, the hemolysis was measured for 120 min of post-incubation time.

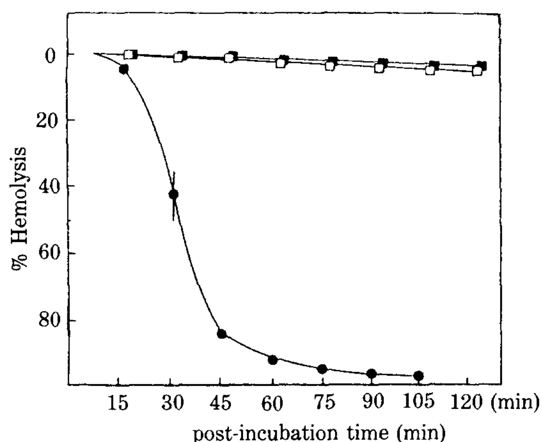


**Fig. 1.** Rose-bengal sensitized photohemolysis of rabbit erythrocytes.
●-● ; with rose-bengal, irradiation
□-□ ; without rose-bengal, irradiation
■-■ ; with rose-bengal, no irradiation

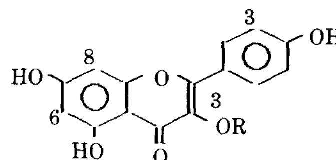


**Fig. 2.** Trifolin (R = galactose) and kaempferol (R = H)

The effects of kaempferol (Fig. 2) and several flavonoids on the cells against singlet oxygen were indicated in Fig. 3 and Table 1.

As referred in Table 1, flavonoid aglycones including quercetin, kaempferol and baicalein significantly suppressed the singlet oxygen induced hemolysis at micromolar concentrations in a dose dependent manner. And among the flavonoid glycosides tested, trifolin also showed protective effect but rutin and baicalin were not protective or rather hemolytic above 100 $\mu$M.

**Table 1.** Effects of some flavonoid aglycones and their glycosides on the rose-bengal sensitized photohemolysis of rabbit erythrocytes

| Compound | $\tau_{50}$ (Half-time of hemolysis)* | | | | | |
|---|---|---|---|---|---|---|
| | Conc. ($\mu$M) | 5 | 10 | 25 | 50 | 100 |
| Flavonoid aglycones | | | | | | |
| kaempferol (*3,5,7,4'-tetrahydroxyflavone*) | | 40 | 54 | 102 | 280 | |
| quercetin (*3,5,7,3',4'-pentahydroxyflavone*) | | 40 | 57 | 123 | 315 | |
| baicalein (*5,6,7-trihydroxyflavone*) | | | 38 | 45 | 106 | |
| Flavonoid glycosides | | | | | | |
| trifolin (*kaempferol 3-galactoside*) | | | | 75 | 83 | 100 |
| rutin (*quercetin 3-rutinoside*) | | | | 32 | 32 | 30 |
| baicalin (*baicalein 7-gulucronide*) | | | | | 32 | 26 |
| References | | | | | | |
| nordihydroguaiaretic acid | | 75 | 200 | | | |
| $\alpha$-tocopherol | | | | | 45 | |

*; control = 32 min

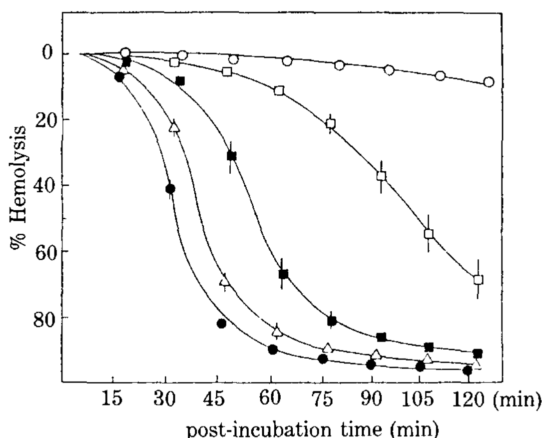


**Fig. 3.** Effect of kaempferol on the rose-bengal sensitized photohemolysis of rabbit erythrocytes. ●-● ; control △-△ ; 5$\mu$M ■-■ ; 10$\mu$M□-□; 25$\mu$M ○-○; 50$\mu$M

$\alpha$-Tocopherol, a typical antioxidant which scavenges free radicals,[22] was not so effective as expected in the suppression of photohemolysys. Nordihydroguaiaretic acid known as a potent lipoxygenase inhibitor showed a highly protective effect at very low concentrations. The contribution of free radical scavenging and lipoxygenase inhibition at the suppression of singlet oxygen induced photohemolysis was discussed in the following part.

**Table 2.** Free radical scavenging activities of several flavonoid aglycones and their glycosides

| Aglycones | $SC_{50}$ ($\mu$M) | Glycosides | $SC_{50}$ ($\mu$M) |
|---|---|---|---|
| kaempferol | 15.9 | trifolin | 25.4 |
| quercetin | 6.8 | rutin | 7.5 |
| baicalein | 15.8 | baicalin | 17.5 |

**Free radical scavening activities of flavonoids**

The free radical scavenging activities of flavonoids are shown in Table 2. The flavonoids which have the significant protective effects against singlet oxygen induced hemolysis appeared to be very reactive to the free radicals.

We thought that the flavonoids prevent the hemolysis by scavenging active oxygen species or reacting with peroxy radicals involved in termination of radical chain reactions during the hemolysis. And the activity was one of the most important factors required for their protection against the damage induced by singlet oxygen.

Since there were no significant differences in free radical scavenging activity between flavonoid aglycones and their corresponding glycosides, a possible involvement of the other factors in the photohemolysis suppression is suggested.

### Inhibition of soybean lipoxygenase by flavonoids

Flavonoids were also tested against soybean lipoxygenase (Table 3) in order to acertain whether the flavonoids affect the enzymatic peroxidation of lipid or not. All of the six flavonoids tested inhibited soybean lipoxygenase over the dose range of 40-250 $\mu$M, although the effects of trifolin, rutin and baicalin were slight. There were the marked differences between the glycosides and their corresponding aglycones, and the flavonols with 3-hydroxyl substituent (kaempferol, quercetin) appeared to be more active than a flavone, baicalein. Thus it was thought that the glycosidic substituents suppress the inhibitory activity of flavonoids on soybean lipoxygenase but 3-hydroxyl substituent is important for the lipoxygenase inhibition.

**Table 3.** Inhibition of soybean lipoxygenase by some flavonoid aglycones and their glycosides

| Aglycones | $SC_{50}$ ($\mu$M) | Glycosides | $SC_{50}$ ($\mu$M) |
|---|---|---|---|
| kaempferol | 48 | trifolin | 240 |
| quercetin | 40 | rutin | 226 |
| baicalein | 82 | baicalin | 110 |

### Effects of some phenolic compounds on the photohemolysis

Table 4 shows the effects of some phenolic compound found in *Panax ginseng* C.A. Meyer on the rose-bengal sensitized photohemolysis. All of the four phenolic compounds appeared to be less protective than trifolin but salicylic acid indicated significant protection. Besides, various cyclooxygenase inhibitors such as salicylic acid acetate, indomethacin, diclofenac sodium and mefenamic acid were observed previously to suppress the photohemolysis dependently upon their inhibitory activity on cyclooxygenase.[7,23] Along with nordihydroguaiaretic acid (a inhibitor of lipoxygenase), salicylic acid suggests a possible involvement of lipoxygenase and/or cyclooxygenase in the mechanism of singet oxygen induced photohemolysis. The free radical scavenging activities of them except caffeic acid were not significant.

**Table 4.** Photohemolysis suppression and free radical scevenging activities of various phenolic compounds.

| Compound | $\tau_{50}$ (min)*1 at $\mu$M of 50 | 250 | 500 | 1000 | $SC_{50}$ ($\mu$M)*2 |
|---|---|---|---|---|---|
| *p*-coumaric acid | | 32 | 32 | 37 | >5000 |
| caffeic acid | | 35 | 46 | 64 | 11.7 |
| salicylic acid | | | 100 | 150 | >5000 |
| maltol | | 82 | | | >5000 |
| trifolin | 83 | | | | 25.4 |

*1; half time of photohemolysis (control=32 min)
*2; concentration at which the compound scavenges 50% of free radicals

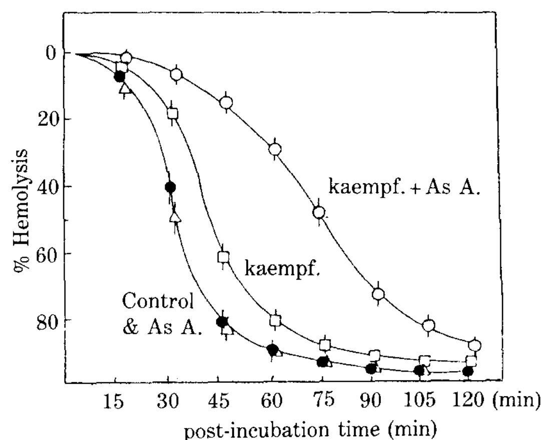


**Fig. 4.** Effects of kaempferol and L-ascorbic acid on the photosensitized hemolysis of rabbit erythrocytes (irradiation time=20 min, sensisitizer=rose-bengal).
●-●; control (no addition) △-△; 100 $\mu$M ascorbic acid □-□; 5 $\mu$M kaempferol ○-○; 5 $\mu$M kaempferol plus 100 $\mu$M ascorbic acid

### Cooperative effects of kaempferol and L-ascorbic acid on the photohemolysis

Fig. 4 shows the cooperation of kaempferol and L-ascorbic acid in the prevention of photosensitized lysis of rabbit erythrocytes. L-Ascorbic acid didn't prevent the photohemolysis in the concentration of 100 $\mu$M (But in this experimental condition 1 mM of ascorbic acid prevented photohemolysis slightly but significantly. Data not shown).[7] However, the preventive effect of kaempferol was synergistically

increased with the addition of L-ascorbic acid.

**Spectral study on the solubilization of flavonoids**
Emission spectra of kaempferol homogenized into 10% aqueous propyleneglycol with and without polyoxyethylene(12)nonylphenylether (0.2%, finally, trade name; ENP-12) were measured at 365 nm excitation wavelength (Fig. 5). ENP-12 is a nonionic surfactant and used as a solubilizer. The emission intensity of kaempferol was increased due to its solubilization, and the red shift was occurred at the emission maximum by 17 nm (from 525 nm to 542 nm). This result indicates that kaempferol can be readily infiltrated into a micelle. Quercetin, baicalein and trifolin also showed an increase of fluorescent intensity and Red shift, whereas flavonoid glycosides except trifolin did not show such properties.[7]

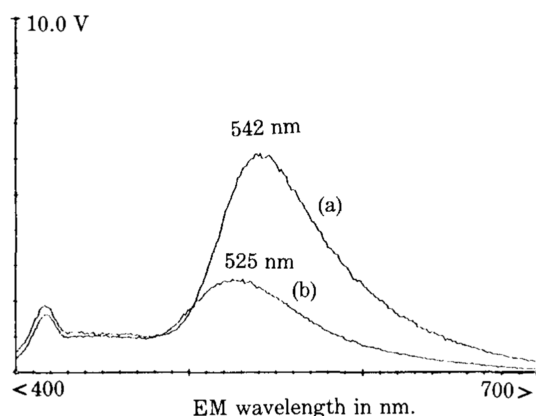


**Fig. 5.** Emission spectra of 35 $\mu$M kaempferol solubilized with ENP-12 (a) and without (b) at 365 nm excitation wavelength

**Microscopic observation of erythrocytes treated by flavonoids**

Fig. 6 shows light microscopic pictures of erythrocytes incubated for 30 min in the presence of quercetin and rutin, respectively. The erythrocytes treated with quercetin reserved its original conformation, but those treated with rutin were distorted. The effects of other flavonoid aglycones including kaempferol and baicalein were similar to that of quercetin. In the glycosides, baicalin showed a distortive effect to the membrane like rutin, but trifolin did not. The effect of trifolin was rather similar to that of kaempferol.

The fluorescent microscopic pictures of erythrocytes treated with flavonoids were also studied (pictures not shown).[7] We observed that the fluorescent intensity of erythrocytes treated with flavonoid aglycones was much higher than that of the control. Thus it was confirmable that their infiltrations into

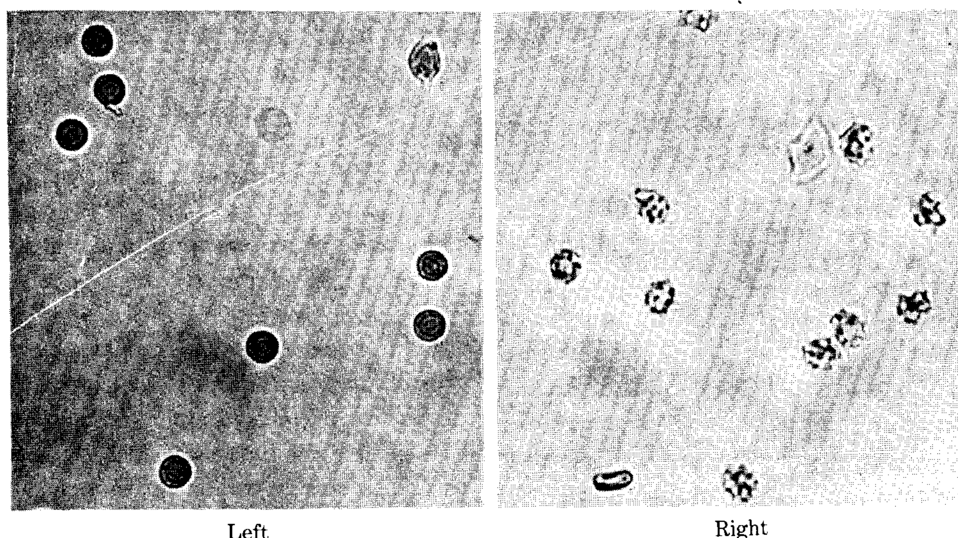


**Fig. 6.** Light microscopic pictures of rabbit erythrocytes treated 50 $\mu$M of quercetin (Left) and rutin (Reight)

hydrophobic compartments of cellular membranes are essential to their activities against photohemolysis.

**Spectral study on the reaction of kaempferol and singlet oxygen**

The reactivity of kaempferol to singlet oxygen was confirmed by the $^1O_2$-induced photodecomposition of kaempferol. Fig. 7 shows the decomposition of kaempferol by singlet oxygen generated from rose-bengal sensitization. When irradiated for 15 min with 20 Watt fluorescent lamp in 0.9% saline phosphate buffer in the presence of 1.5 $\mu$M of rose-bengal, kaempferol was decomposed by 22%. Therefore the scavenging of singlet oxygen by kaempferol was considered as an important factor for the prevention of the photohemolysis.

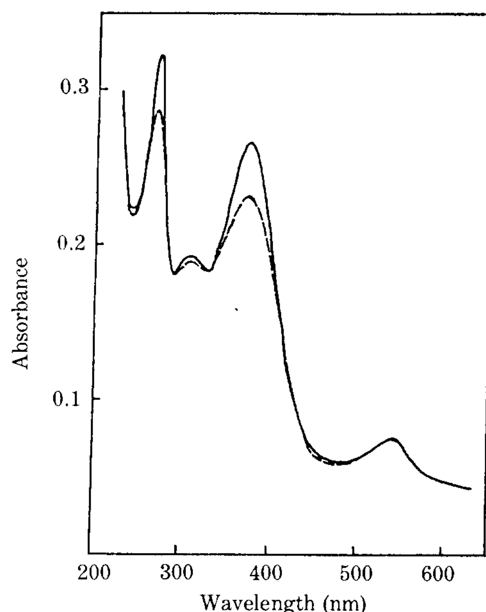


**Fig. 7.** Elesctronic absorption spectrum of kaempfed before (—) or after (---) photodecomposition oxygen

## Discussion

Autooxidation of polyunsaturated fatty acids which are commonly distributed in biomembranes is a free radical chain reaction. Damage on erythrocyte cellular membrane caused by $^1O_2$ may accelerate the homolysis with secondary generation of active oxygen species and organic free radicals.[7] Thus the lipid peroxidation and hemolysis by $^1O_2$ can be suppressed by $^1O_2$ quenchers or radical scavengers at the initiation step, or terminated by peroxy radical scavengers such as phenolic compounds.[24-25] We previously reported the pronounced effects of curcuminoids, which are phenolic compounds from *Curcuma longa* L., on the cellular membranes against active oxygen species.[26]

Flavonoids also appeared to prevent the photohemolysis strongly (Table 1) by quenching singlet oxygen (Fig. 7) and scavenging peroxy radical involving termination of radical chain reaction (Table 2). However, there was a poor correlationship between the free radical scavenging activity and the practical protective effect against $^1O_2$-induced hemolysis. Although the flavonoid glycosides have similar free radical scavenging activities to their corresponding aglycones (Table 2), they are not so effective as the latters in the protection against the photohemolysis (Table 1). It suggests that not only the suppression of the lipid peroxidation by scavenging active oxygen species or free radicals but also the solubilization of them into lipid bilayer of cellular membrane should be the important factor for practical protection of cellular membrane against $^1O_2$. Ascorbic acid increased the protective effect of kaempferol synergistically (Fig. 4). This result indicates that the membrane-binding kaempferol can function as a carrier of oxidizing equivalents from erythrocyte membranes to the water soluble L-ascorbic acid.

The most specific result obtained in this study was that trifolin, a flavonoid glycoside isolated from Ginseng leaves was rather protective against the photohemolysis in dose-dependent manner, unlikely the other glycosides tested. The unique property of trifolin which overcame the insolubility was studied further. In the light and fluorescent microscopic study, we could observe that trifolin, similarly to flavonoid aglycones stabilized the cellular membranes.

From the obtained results, it was confirmable that flavonoids may prevent the photohemolysis by

scavenging free radicals and $^1O_2$ in the interior or at the surface of cellular membranes.

Additionally the results reminded us that certain membrane bound enzymes such as lipoxygenase and cyclooxygenase which are inhibited by flavonoids may be possibly involved in the photohemolysis mechanism. The inhibitory activities of flavonoids against the lipoxygenase, one of the enzymes which function over the dynamic actions of cellular membrane, were also assayed but the data were not helpful to correlate with the protective effects. But *in vivo*, the phenomena might be different and thus it should not be excluded that lipoxygenase inhibition may be one of the factors in the protective effects against $^1O_2$-induced damage.

Throughout the study, the photohemolysis experiment was ranked confirmly as a screening method for antioxidants at the cellular level. The method was considered as a reproducible diagnostic tool for measuring the protective effects of various natural products on the cellular membranes against reactive forms of oxygen.

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