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Chemiluminescent screening of quenching effects of natural colorants against reactive oxygen species: Evaluation of grape seed, monascus, gardenia and red radish extracts as multi-functional food additives

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Abstract

The quenching effects of commercial available natural colorants against reactive oxygen species (ROS) were investigated. The effectiveness was evaluated by measuring the quenching ratio of chemiluminescence (CL) intensity with each colorant on luminol or methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidaz[1,2-a]pyrazin-3-one CL induced by ROS. As a result, the grape seed extracts and polyphenolics dose-dependently quenched CL. The quenching effects of grape seed extract A at 1 mg/ml for superoxide, singlet oxygen, hydroxyl radical, hypochlorite ion and linolenic acid peroxide were 81.1 ± 0.5%, 95.7 ± 0.4%, 99.3 ± 0.3%, 27.7 ± 4.2% and 88.3 ± 1.0%, respectively ($n = 3$). Except for chalcone, the polyphenolics, such as pelargonidin, cyanidin, delphinidin and *trans*-resveratrol, also showed the high quenching effects against ROS. These results suggested that the grape seed extract might be useful as a multi-functional food additive.

Moreover, the quenching effects of several natural colorants were measured to screen new multi-functional food additives which can effectively quench ROS. Among the extracts from monascus red, gardenia yellow, blue and red radish, the red radish extract showed high quenching effects against hydroxyl radical and linolenic acid peroxide.

Keywords: Natural colorant; Grape seed extract; Polyphenolics; Monascus; Gardenia; Red radish; Reactive oxygen species; Chemiluminescent assay; Multi-functional food additive

1. Introduction

Natural colorants can be extracted from plants, animals, fungi and microorganisms,and classified by their origins or chemical structures. Several classes of natural colorants, such as anthocyanins, carotenoids and anthraquinones, are used extensively as food additives to enhance sensory response and to promote sales (Delgado-Vargas, Jimene, & Paredes-Lopez, 2000). Recently, there has been interest in using some natural colorants in foods as "multi-functional food additives" due to their additional nutritional and therapeutic effects. Especially, much attention has been focussed on their quenching effect against reactive oxygen species (ROS), because the intake of dietary ROS quenchers affords protection against some diseases. The mechanism of these effects is generally thought to be the same as that of lipid oxidation inhibition by antioxidants.

The anthocyanin-based natural colorants, with a variety of colour, ranging from orange to blue, are water-soluble and non-toxic. They are also well known as a multi-functional food additives with a quenching effect against ROS, which contributes to the prevention of coronary

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diseases (Aldini, Carini, Piccoli, Rossoni, & Facino, 2003; Bagchi et al., 2003). In this respect, a screening of natural colorants with quenching effects against ROS is very significant.

Various methods have been routinely used in order to evaluate the antioxidant properties of food extracts and their components (Arouma, 2003). An electron spin resonance technique can directly measure free radical-scavenging effects of polyphenolics (Bergman, Perelman, Dubinsky, & Grossman, 2003; Leonard et al., 2003). Espin, Soler-Rivas, Wichers, and Garcia-Viguera (2000) reported the quenching effect of anthocyanin-based natural colorants by using 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) as a free radical-producing agent (Espin et al., 2000). The thiobarbituric acid (TBA) method was used for testing antioxidant effect of polyphenolics in gerbil brain homogenate (Lee, Im, Suh, & Jung, 2003). Chemiluminescent assay with luminol or 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]-pyrazin-3-one (MCLA) was employed to evaluate an in vitro quenching effect against ROS. Using the chemiluminescent assay, phenolic antioxidants, such as probucol (Cynshi, Takashima, Katoh, & Tamura, 1995), flavonols (Selloum-Djelili, Sebihi, & Arnhold, 2004), Chinese herbal ingredients (Choi et al., 2000) and melatonin (Sariahmetoglu, Wheatley, Cakycy, & Townshend, 2003) were evaluated. In our previous reports, the quenching effects of medicines, such as flavastain and its metabolites (Nakashima et al., 2001), and non-water-soluble and water-soluble rosemary extracts (Wada et al., 2004) were examined.

The aim of the present study was to evaluate the quenching effects of natural food colorants against ROS, such as superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\cdot}OH$), hypochlorite ion (ClO^-) and linolenic acid peroxide ($LOO^{\cdot}$) by luminal chemiluminescent assay. The grape seed extracts and polyphenolics (namely, chalcone, pelargonidin, cyanidin, delphinidin and *trans*-resveratrol), shown in Fig. 1, were studied. The difference among pelargonidin, cyanidin and delphinidin is the number of hydroxyl groups on the B ring. *trans*-Resveratrol, having a well-known antioxidative property (Leonard et al., 2003), is structurally similar to pelargonidin. The relationship between the quenching effect and the structural properties of these polyphenolics was also described.

Moreover, the quenching effects of other natural food colorants, such as extracts of monascus, gardenia and red radish, were determined to screen the multi-functional food additives with quenching effect against ROS. Additionally, the relationships of quenching effect to colour value or fading ratio against ROS were also elucidated.

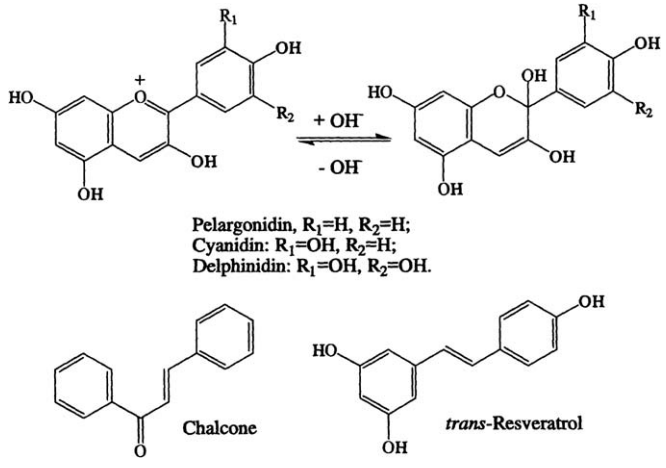

Fig. 1. Structures of polyphenolics examined.

2. Materials and methods

2.1. Materials and chemicals

Grape seed extracts A (proanthocyanin 99%) and B (proanthocyanin > 80%), red radish colour (Mitsubishi-Kagaku Foods Corporation, Kanagawa, Japan), monascus red colour (Mitsubishi-Kagaku Foods Corporation), gardenia yellow colour (Mitsubishi-Kagaku Foods Corporation) and gardenia blue colour (Mitsubishi-Kagaku Foods Corporation) were purchased from Mitsubishi Chemical Corporation (Kanagawa). The chalcone, pelargonidin, cyanidin and delphinidin used were from Funakoshi Co. (Tokyo, Japan). Xanthine oxidase from buttermilk, H_2O_2 (30%), $FeCl_2$ and NaClO solution from Wako Pure Chemicals (Osaka, Japan) were purchased. Luminol, hypoxanthine, NaBr, diethylentriaminepentaacetic acid (DETAPAC), lactoperoxidase, *N*-2-hydroxyethylpiperaine-*N*′-2-ethanesulphonic acid (Hepes), *trans*-resveratrol and linolenic acid from Sigma Chemical Corporation (St. Louis, MO, USA) were used. The MCLA used was from Tokyo Kasei (Tokyo). Water was deionized and distilled by an Aquarius GSR-500 automatic water distillation apparatus (Advantec, Tokyo).

All natural colorants and polyphenolics were dissolved in dimethyl sulfoxide (DMSO) to prepare appropriate concentrations (ranging from 0.02 to 2 mg/ml) and applied to the measurement of quenching effect against ROS. However, for the measurement of quenching effect against $^{\cdot}OH$, these compounds dissolved in dimethylformamide (DMF) were used.

2.2. Assay procedure for quenching effects of reactive oxygen species

The quenching effect of natural colorants against ROS was measured according to our previous method (Wada et al., 2004) as follows: for $[O_2^-]$ to 6 μl of sample in DMSO in a test tube ($\phi 12 \times 75$ mm), 600 μl of 0.8 mM hypoxanthine in 100 mM Hepes buffer (pH 7.4) and 300 μl of 1.6 mM luminol in Hepes buffer were successively added. After incubation at 37 °C for 10 min, 300 μl of 1.0 U/ml of xanthine oxidase in Hepes buffer were added to the mixture and the chemiluminescence (CL) intensity was immediately measured. For $[^1O_2]$ to 6 μl of sample in DMSO, 300 μl of 0.4% H_2O_2 in 100 mM acetate buffer (pH 4.5),

300 μl of 80 mM NaBr in acetate buffer and 0.8 mM luminol in acetate buffer were added. The mixture was incubated at 37 °C for 10 min. The CL intensity of the mixture was immediately measured after adding 300 μl of a 10 μg/ml solution of lactoperoxidase in acetate buffer. For [·OH], to 6 μl of sample in DMF, 150 μl of 1.6% H_2O_2 in 100 mM Hepes buffer (pH 7.4), 150 μl of 0.8 mM DETAPAC in Hepes buffer and 0.8 mM luminol in Hepes buffer were successively added. After incubation at 37 °C for 10 min, 300 μl of 200 μm $FeCl_2$ in Hepes buffer were added to the mixture, followed by immediate measurement of the CL intensity. For [ClO^-], 900 μl of 0.53 mM luminol in 50 mM borate buffer (pH 9.5) were added to 6 μl of sample in DMSO. After incubation at 37 °C for 10 min, 300 μl of 40 μM NaClO in borate buffer were added to the mixture and then the CL intensity was measured. For [LOO·], 5 mM linolenic acid in *n*-BuOH was aerobically oxidized in a water bath at 37 °C for 60 min. To 6 μl of sample in DMSO, 900 μl of the oxidized solution were added and then the mixture was incubated at 37 °C for 3 min. After adding 300 μl of 8 μM MCLA in *n*-BuOH, the CL intensity was measured.

The CL measurements were performed at room temperature for 1 min by a Lumatag Analyzer, Auto-250 (Berthold, UK). The concentrations of radicals generated could not be estimated. The percentage of quenching effect against the ROS was calculated from the following equation:

$$\text{Quenching effect } \% = \{(RCI_0 - RCI)/RCI_0\} \times 100$$

where RCI_0 is the CL intensity generated from blank (DMSO or DMF) and RCI is the CL intensity generated from the sample. An increased value indicates the increase of quenching effect.

The sample concentration giving 50% quenching (EC_{50}) on ROS was calculated by triplicate measurements of the dose–quenching effect curve. The data were expressed as the mean ± SD ($n = 3$).

2.3. Colour value of natural colorants

A Shimadzu UV–visible recording spectrophotometer (model: UV-265FS, Kyoto, Japan) was used to measure absorbance (Ab) of various extracts. Absorbances for red radish colour, monascus red colour, gardenia yellow colour and gardenia blue colour were measured at 520, 480, 440 and 585 nm, respectively, by using a 0.1% aqueous solution of each colorant. Colour value was calculated by the following equation:

$$\text{Colour value (10\%E)} = \text{Ab} \times 100/\text{amount of sample (g)}$$

2.4. Assay procedure for colour fading ratio of natural colorants

The colour fading ratio of natural colorants against ROS was measured as follows: For [O_2^-] to 0.1 ml of 0.1% sample, 2.4 ml of 50 mM carbonate buffer (pH 8.0), 0.1 ml of 3 mM hypoxanthine, 0.1 ml of 3 mM EDTA and 0.1 ml of 0.75 mM nitro blue tetrazolium were successively added. After pre-incubation at 25 °C for 10 min, 0.1 ml of xanthine oxidase (1.0 U/ml) was added. Then 0.1 ml of 6 mM $CuCl_2$ was added to the mixture after incubation at room temperature for 20 min. Then, absorbances of each compound were measured.

For [·OH], the mixture of 4.5 ml of 0.1% sample solution and 0.5 ml of 30% H_2O_2 was incubated at room temperature for 30 min. Then, absorbances of each compound were measured.

For [ABTS], 2,2′-azobis(2-amidinopropane)dihydrochloride (ABTS, 1 mM, 0.5 ml) was added to 4.5 ml of 0.1% sample solution. After incubation at room temperature for 30 min, the absorbances for each sample were measured. Wavelengths of each compound measured are summarized in Table 1. The average decreasing ratio of absorbance for gardenia yellow was shown as a colour fading ratio. The data were expressed as averages of triplicate measurements.

Table 1
Wavelengths of each compound for measurement of colour fading ratio

Sample	Wavelength, nm		
	O_2^-	OH	ABTS
Monascus red	497	497	497
Red radish	514	514	514
Gardenia yellow	438,462	438,462	438,462
Gardenia blue	595	595	595

3. Results and discussion

3.1. Quenching effects of grape seed extracts and polyphenolics against ROS

The quenching effects of grape seed extracts A and B, and polyphenolics, namely chalcone, pelargonidin, cyanidin, delphinidin and *trans*-resveratrol, were studied. The test solutions were prepared at 0.02–2 mg/ml, and applied to the measurement of quenching effect. CL induced by ROS was quenched dose-dependently by grape seed extracts A and B, pelargonidin, cyanidin, delphinidin and *trans*-resveratrol.

For [O_2^-], Fig. 2 shows the quenching effects of grape seed extracts and polyphenolics against O_2^-. The EC_{50} values of grape seed extract A and B were 0.24 ± 0.01 and 0.28 ± 0.02 mg/ml, respectively. Chalcone and pelargonidin did not demonstrate quenching effects. The quenching effect order of polyphenolics was: delphinidin (EC_{50}: 0.10 ± 0.01) > cyanidin (0.49 ± 0.03) > *trans*-resveratrol (>2.0 mg/ml).

For [$^1O_2$], CL produced by luminol and $^1O_2$ was inhibited by all samples except chalcone, as shown in Fig. 3. Both grape seed extracts showed higher quenching effects (EC_{50}: A, 0.11 ± 0.01; B, 0.12 ± 0.02 mg/ml). The quenching effect order of polyphenolics was: delphinidin (EC_{50}:

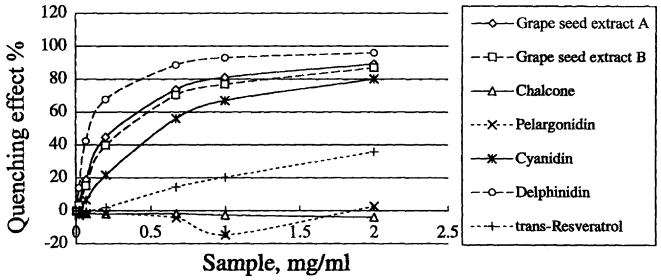

Fig. 2. Quenching effects of grape seed extracts and polyphenolics against superoxide anion. Sample concentrations: 0.02–2 mg/ml. Experimental details are shown in Section 2.

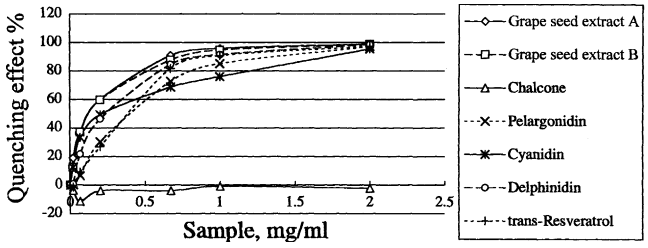

Fig. 3. Quenching effects of grape seed extracts and polyphenolics against singlet oxygen. Sample concentrations: 0.02–2 mg/ml. Experimental details are shown in Section 2.

0.19 ± 0.01) = cyanidin (0.19 ± 0.01) > *trans*-resveratrol (0.26 ± 0.01) > pelargonidin (0.28 ± 0.04 mg/ml).

For [$^{\cdot}$OH], all samples showed higher quenching effects among the ROS tested (Fig. 4). CL was largely quenched by concentrations greater than 0.2 mg/ml. Particularly, the quenching effects of grape seed extracts, cyanidin and delphinidin, reached 91–97% at a concentration of 0.2 mg/ml. Chalcone indicated very low quenching effect (EC_{50}: > 2.0) and *trans*-resveratrol was moderate (0.28 ± 0.03 mg/ml).

For [ClO^-], Fig. 5 shows the quenching effects of grape seed extracts and polyphenolics against ClO^-. All samples showed lower quenching effects for ClO^- than those for the other ROS. The quenching effects of grape seed extracts A and B at 1.0 mg/ml were $27.7 \pm 4.2\%$ and $22.0 \pm 3.7\%$, respectively. The quenching effect order of polyphenolics at 1.0 mg/ml was: delphinidin > cyanidin > *trans*-resveratrol > pelargonidin. Chalcone had no quenching effect.

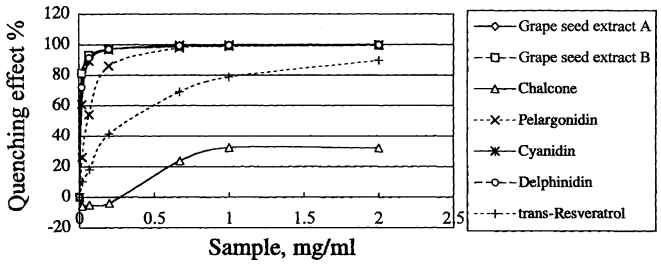

Fig. 4. Quenching effects of grape seed extracts and polyphenolics against hydroxyl radical. Sample concentrations: 0.02–2 mg/ml. Experimental details are shown in Section 2.

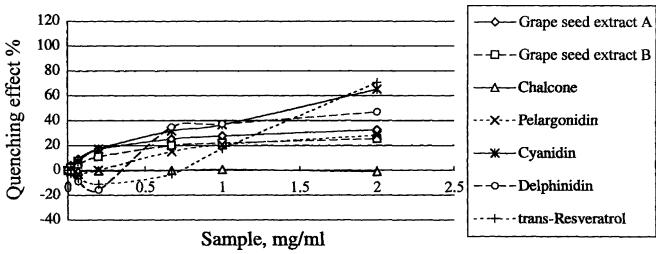

Fig. 5. Quenching effects of grape seed extracts and polyphenolics against hypochlorite ion. Sample concentrations: 0.02–2 mg/ml. Experimental details are shown in Section 2.

For [LOO$^{\cdot}$], the quenching of MCLA CL induced by linolenic acid peroxide was examined (Fig. 6). The order of effectiveness of polyphenolics was: delphinidin (EC_{50}: 0.03 ± 0.002) > cyanidin (0.04 ± 0.004) > *trans*-resveratrol (0.20 ± 0.01) > pelargonidin (0.23 ± 0.01 mg/ml). The quenching effects for grape seed extracts A and B at 1.0 mg/ml were $88.3 \pm 1.0\%$ and $88.3 \pm 0.9\%$, respectively. The different kinetic curves are shown in Figs. 2–6. The differences might due to the reactivities of ROS to samples and the effect of medium pH.

The quenching effect of each sample was also expressed as the equivalency value to delphinidin (Table 2), which was defined as the ratio of each slope of dose–quenching effect curves for the sample and delphinidin. The slope of delphinidin was taken as 1.00. Both grape seed extracts showed relatively high delphinidin equivalency values against most ROS (ranging from 0.39 to 1.78). For all ROS, grape seed extract A showed higher equivalency value than did grape extract B. These results corresponded to the amount of proanthcyanin indicated.

Polyphenolics examined in this study were tentatively classified into three groups with regard to the quenching effect, as shown in Fig. 7: (1) low quenching effect (chalcone), (2) moderate quenching effect (pelargonidin and *trans*-resveratrol) and (3) high quenching effect (cyanidin and delphinidin). Considering groups (2) and (3), the number of hydroxyl groups in the B ring played a decisive role in increasing the quenching effect. Moreover, the quenching effect of flavonoids depended mainly on the electron-donating properties of the B ring and was enhanced by an increase in the number of hydroxyl groups (Choi

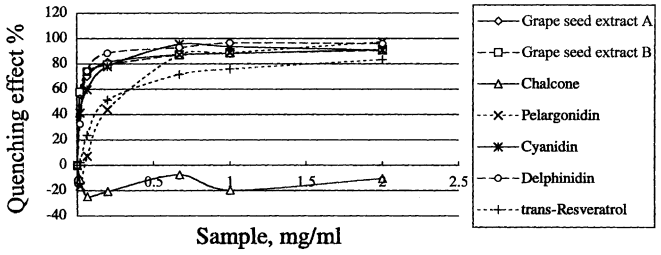

Fig. 6. Quenching effects of grape seed extracts and polyphenolics against linolenic acid peroxide. Sample concentrations: 0.02–2 mg/ml. Experimental details are shown in Section 2.

Table 2
Delphinidin equivalent value of grape seed extracts and polyphenolics against ROS

Sample	Delphindin equivalency value				
	O_2^-	$^1O_2$	·OH	ClO^-	LOO^-
Grape seed extract A	0.46	1.78	0.86	0.64	0.43
Grape seed extract B	0.39	1.56	0.54	0.59	0.40
Chalcome	n.d.[a]	n.d.[a]	4.40×10^4	n.d.[a]	n.d.[a]
Pelargonidin	n.d.[a]	0.69	0.04	0.20	0.15
Cyanidin	0.22	1.03	0.57	1.02	0.99
Delphinidin	1.00	1.00	1.00	1.00	1.00
trans-Resveratrol	0.03	0.75	0.01	1.63	0.17

[a] n.d., not detectable.

et al., 2000; Selloum et al., 2004). Our results were also supported by these facts.

3.2. Quenching effects of natural colorants on ROS

Quenching effects of other natural food colorants, such as extracts of monascus, gardenia and red radish, were determined to screen multi-functional food additives and compared with that of the grape seed extract A. These colorants have been used as food additives in Japan. In Fig. 8, the quenching effects of extracts at 1.0 mg/ml are shown. Among all samples, grape seed extract A showed the highest quenching effects against O_2^-, $^1O_2$, ·OH and ClO^-. The significant differences (on all ROS) were detected between grape seed extract and other colorants ($p < 0.001$). Red radish, gardenia yellow and blue had relatively high quenching effects against hydroxyl radical (>73%). Moreover, red radish also quenched linolenic acid peroxide by 91%. Gardenia yellow and blue quenched ·OH and LOO·. The monascus red had very low quenching effects against

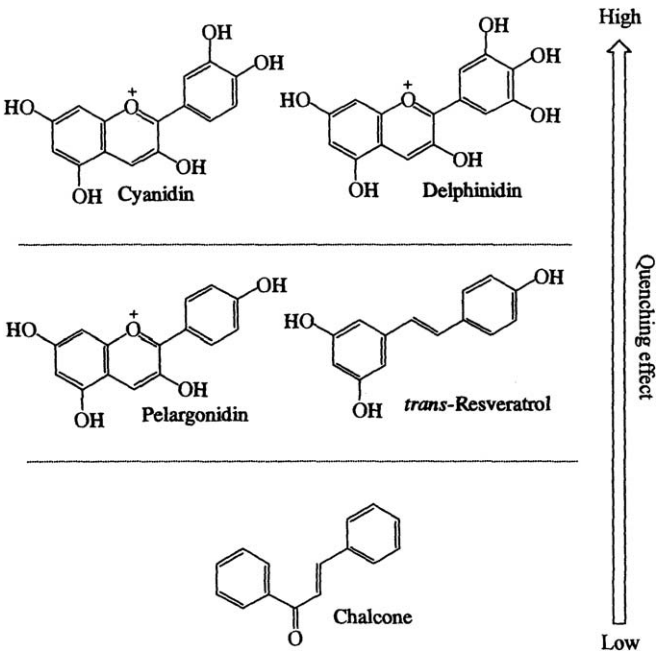

Fig. 7. Quenching effects of polyphenolics against ROS and their structural properties.

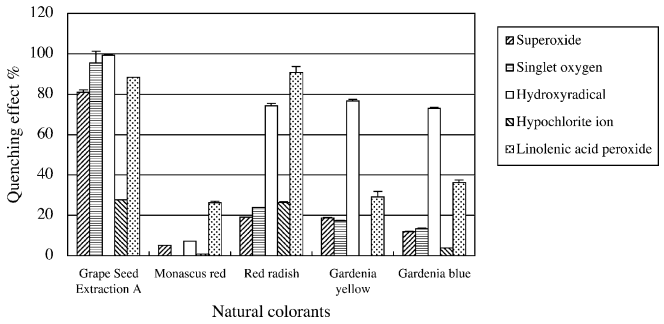

Fig. 8. Quenching effects of natural food colorants at 1.0 mg/ml against ROS. Experimental details are shown in Section 2.

$^1O_2$ and ClO^-, but a moderate effect against LOO·. In other words, this colorant was stable against these ROS. These results indicate that red radish extract, gardenia yellow and blue might be useful as multi-functional food additives.

Grape seed extract was constituted of proanthocyanin derivatives (Delgado-Vargas et al., 2000). In Fig. 9, the basic chemical structures of proanthocyanidin of the grape extracts, except proanthocyanins, are shown. Procyanidin trimer and more highly polymerized procyanidins are contained in the grape seed extract (Shi, Yu, Pohorly, & Kakuda, 2003). Red radish extract consisted of acylated pelargonidin glucosides (Otsuki, Matsufuji, Takeda, Toyoda, & Goda, 2002). Malien-Aubert, Dangles, and Amiot (2001) reported that acylated anthocyanin, which is stable due to intramolecular copigmentation, is mainly contained in the red radish. This property might affect the quenching effect of ROS.

Crocetin and crocin have been known to be the main components of gardenia yellow (Watanabe & Terabe, 2000). The antioxidant activities of these compounds were detected by the thiocyanate method and TBA method (Pham, Cormier, Farnworth, Tong, & Van Calsteren, 2000). Furthermore, a study of the antioxidant properties of carotenoids, such as β-carotene, against lipid peroxidation was reported by Toyosaki (2002). Genipin, an iridoid derivative, is the component of gardenia blue (Paik, Lee, Cho, & Hahn, 2001). This compound was more stable at alkaline pH than neutral and acidic pH. Recently, Tanaka,

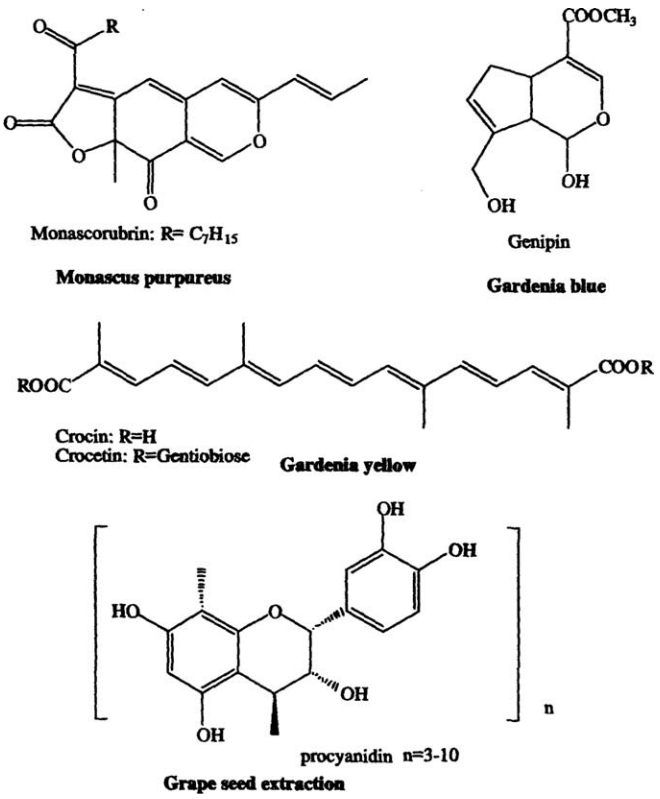

Fig. 9. The structures of constituents of grape seed extract and natural colorants.

Nishikawa, and Ishimaru (2003) reported that iridoid glycosides in *Cornus capitata* adventitious root culture exhibit very low quenching effects against DPPH, O_2^- and LOO·. These results correlated well with our results. To date, few papers have been published on the quenching effect of monascus red. Monascorubrin is one of the constituting compounds of monascus red (Martinkova et al., 1999). On the other hand, Obon, Castellar, Cascales, and Fernandez-Lopez (2005) reported that the antioxidant activity of red synthetic colorant was detected by using ABTS radical. Six kinds of colorants, such as azorubine amaranth and ponceau 4R, demonstrated a 35 times lower antioxidant activity than that of trolox. The structures of the main constituents of gardenia yellow, blue and monascus red are shown in Fig. 9.

Next, the relationship of quenching effect of colorants with colour values was studied. The colour values for monascus red, red radish, gardenia yellow and blue were 71, 60, 365 and 265, respectively. However, a relationship between the quenching effects and colour values was not found.

The colour fading ratio of natural colorants was determined against O_2^-, ·OH and ABTS radical. The averages of triplicate measurements of fading ratio are summarized in Table 3. The absorbances of quality controls of each colorant are relatively stable in this study and rapid decreasing of absorbance was not observed. The red radish extract was mostly affected by O_2^- (22.1) and ·OH (73.0%). Colour

Table 3
Colour fading ratio of natural colorant against ROS

Sample[a]	Colour fading ration%[b]		
	O_2^-	·OH	ABTS
Monascus red	n.d.[c]	14.1	48.9
Red radish	22.1	73.0	54.2
Gardenia yellow	3.3	−9.2	99.0
Gardenia blue	1.8	−5.3	19.9

[a] Sample concentration = 1.0 mg/ml.
[b] Average of triplicate measurements.
[c] n.d. means that a significant difference was not detected against the value of the blank.

fading of anthocyanin is initiated by the attack of oxidant at the C2 position, which results in a colourless hemiketal or chalcone form (Malien-Aubert et al., 2001). On the other hand, the conditions in this study (pH 8) might affect the colour fading ratio of red radish colorant. Clifford (2000) reported that the pH value affects the degree of anthocyanin transformation, as shown in Fig. 1. Chigurupati, Saiki, Gayser, and Dash (2002) examined the stability of anthocyanin at different pH values and temperatures. More than 80% decrease in Ab at 536 nm was observed over a period of 10 days at 37 °C. Therefore, the pH value might also be one of the major causes for the colour fading in this study as well as ROS.

Compared to other ROS, ABTS radicals decomposed colorants to a much higher degree. A colorant having high quenching effect against ROS showed relatively high fading ratio. Gardenia yellow was almost fully decomposed by ABTS radicals (fading ratio of 99.0%). Gardenia blue, which showed high quenching effects against ·OH and ·LOO, could not be easily faded by ROS. On the other hand, monascus red, having a very low quenching effect against ROS, could be faded by ABTS radicals.

Although an obvious relationship between quenching effect of natural colorants and their colour fading ratios could not be found, the findings in this study may be useful for quality control of natural colorants in foods.

4. Conclusions

The quenching effects of natural colorants against ROS were studied by a chemiluminescent assay. Grape seed and red radish extracts showed high quenching effects against ROS. The structural properties for quenching effect were compared with structurally similar polyphenolics. Moreover, the potential of natural colorants as multi-functional food activities was elucidated. An obvious relationship between quenching effects of extracts and their colour properties, such as the colour value and the colour fading ratio, could not be found. However, the radical species were suspected of inducing the decomposition of colorants. The findings obtained in this study might be useful for the quality control of colorants in foods.

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