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Inhibitory Activity of Flavonoids from *Prunus davidiana* and Other Flavonoids on Total ROS and Hydroxyl Radical Generation

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Since reactive oxygen species (ROS) and hydroxyl radicals (·OH) play an important role in the pathogenesis of many human degenerative diseases, much attention has focused on the development of safe and effective antioxidants. Preliminary experiments have revealed that the methanol (MeOH) extract of the stem of *Prunus davidiana* exerts inhibitory/scavenging activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, total ROS and peroxynitrites ($ONOO^-$). In the present study, the antioxidant activities of this MeOH extract and the organic solvent-soluble fractions, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH), and the water layer of *P. davidiana* stem were evaluated for the potential to inhibit ·OH and total ROS generation in kidney homogenates using 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), and for the potential to scavenge authentic $ONOO^-$. We also evaluated the inhibitory activity of seven flavonoids isolated from *P. davidiana* stem, kaempferol, kaempferol 7-*O*-β-D-glucoside, (+)-catechin, dihydrokaempferol, hesperetin 5-*O*-β-D-glucoside, naringenin and its 7-*O*-β-D-glucoside, on the total ROS, ·OH and $ONOO^-$ systems. For the further elucidation of the structure-inhibitory activity relationship of flavonoids on total ROS and ·OH generation, we measured the antioxidant activity of sixteen flavonoids available, including three active flavonoids isolated from *P. davidiana*, on the total ROS and ·OH systems. We found that the inhibitory activity on total ROS generation increases in strength with more numerous hydroxyl groups on their structures. Also, the presence of an *ortho*-hydroxyl group, whether on the A-ring or B-ring, and a 3-hydroxyl group on the C-ring increased the inhibitory activity on both total ROS and ·OH generation.

Key words: *Prunus davidiana*, Antioxidant activity, Total reactive oxygen species (ROS), Peroxynitrite ($ONOO^-$), Hydroxyl radical (·OH), Flavonoids.

INTRODUCTION

Free radicals are generated in normal and pathological cell metabolism from external factors such as foreign materials or UV radiation. When humans use oxygen for respiration and combustion, molecular oxygen reacts easily with free radicals to form reactive oxygen species (ROS), including superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2C_2), hydroxyl radical (·OH), singlet oxygen ($^1O_2$), free radicals of lipids such as alkoxyl radical (RO·), peroxyl radical (ROO·) and peroxynitrite ($ONOO^-$) as the reactive nitrogen species (RNS), formed by the *in vivo* reaction of nitric oxide (NO·) and $\cdot O_2^-$ (Singh, 1989; Aruoma, 1996; Sawa *et al.*, 2000; Balavoine and Genleti, 1999). These ROS and RNS may act as potent oxidizing and nitrating agents to damage several components in the body, such as lipids, proteins, nucleic acid, and DNA, causing inflammation or lesion on various organs (Beckman *et al.*, 1990). Also, these reactive species are likely to be involved in the pathogenesis of many human degenerative diseases as represented by cancer (Dreher *et al.*, 1996), aging (Sohal, 2002), arteriosclerosis, rheumatoid arthritis and allergy (Griffiths and Lunec, 1996; Squadrito and Pryor, 1998; Choi *et al.*, 2002).

For several years, many researchers have sought powerful, nontoxic, natural antioxidants from edible or medicinal plants to prevent these reactive species related disorders

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in humans and to replace synthetic compounds, which may be carcinogenic and harmful to the lungs and liver (Branen, 1975). In our ongoing study to identify antioxidants from natural products, we have focused on *Prunus davidiana*, which has previously been reported to have biological activities such as hypolipemic, hypoglycemic, antioxidative, and anti-inflammatory (Choi *et al.*, 1991a; 1991b; 1991c; 1992), and its isolated flavonoids such as kaempferol, kaempferol 7-*O*-β-D-glucoside, (+)-catechin, dihydrokaempferol, persiconin, isosakuranin, hesperetin 5-*O*-β-D-glucoside, naringenin and its 7-*O*-β-D-glucoside (Choi *et al.*, 1992; 1993). In addition, our previous results showed that the methanol (MeOH) extract of *P. davidiana* stem exhibited antioxidant activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, total ROS and $ONOO^-$ (Jung *et al.*, 2002).

In the present study, the antioxidant activity of its organic soluble fractions, such as dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH), and the water layer of *P. davidiana* stem was evaluated for the inhibition of ·OH and total ROS generation and the scavenging of authentic $ONOO^-$. Also, the antioxidative activity of seven flavonoids isolated from this plant was measured by the ·OH, $ONOO^-$ and total ROS systems. From our continuous research on the flavonoids isolated from this plant, we suggest that the antioxidative activity of flavonoids depends on their basic structures as well as other functional groups. To evaluate the relationship between the chemical structure and the inhibitory activity of the flavonoids on both total ROS and ·OH generation, sixteen available, structurally different flavonoids, including three active principles, kaempferol and its 7-*O*-β-D-glucoside, and (+)-catechin from *P. davidiana* stem, were used.

MATERIALS AND METHODS

Chemicals

L-Ascorbic acid, Trolox and DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid) were purchased from Sigma Chemical Company (St. Louis, MO, USA). High quality 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and DHR 123 (dihydrorhodamine 123) were purchased from Molecular Probes (Eugene, OR, USA), and $ONOO^-$ from Cayman Chemicals Co. (Ann Arbor, MI, USA).

Plant material

The *P. davidiana* used was purchased from the Chinese herb medicine shop at Pyongwha market, Pusan, Korea. The plant was identified by the botanist, Prof. J. H. Park, and a voucher specimen was deposited in the Herbarium of the College of Pharmacy, Pusan National University, Pusan, Korea.

Extraction, fractionation and isolation

Dried stems (2.2 kg) of commercially available *P. davidiana* were extracted with MeOH under reflux. The extracts were partitioned with dichloromethane, ethyl acetate, *n*-butanol, and water successively. The ethyl acetate-soluble fraction (59 g) was chromatographed over silica gel using CH_2Cl_2-MeOH mixture and further separated by Sephadex LH-20 (solvent: MeOH) to give the flavonoids which were identified by direct comparisons with authentic samples (mmp, NMR).

Measurement of the inhibition of total ROS generation

Rat kidney homogenates prepared from the kidneys of freshly killed, male Wistar rats weighing 150-200 g were mixed with or without the suspension of extracts/or compounds. The mixtures were then incubated with 12.5 mM DCFH-DA at 37°C for 30 min. A 50 mM phosphate buffer solution at pH 7.4 was also used. DCFH-DA is a stable compound that easily diffuses into cells and is hydrolyzed by intracellular esterase to yield a reduced, non-fluorescent compound, DCFH, which is trapped within cells. The ROS produced by cells oxidized the DCFH to highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF). The fluorescence intensity of the oxidized DCF was monitored using a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), with excitation and emission wavelengths of 460 and 530 nm (Label and Bondy, 1990), respectively.

Measurement of the inhibition of hydroxyl radical generation

Extracts/or compounds were added to 1 mM H_2O_2 and 0.2 mM $FeSO_4$, and incubated at 37°C for 5 min. Esterase-treated 2 μM DCFH-DA was then added and the changes in fluorescence were monitored on a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), with excitation and emission wavelengths of 460 and 530 nm, respectively, for 30 min (Label and Bondy, 1990).

Measurement of $ONOO^-$ scavenging activity

The $ONOO^-$ scavenging activity was measured by monitoring the oxidation of DHR 123 using a slight modification of the method reported by Kooy *et al.* (1994). DHR 123 (5 mM) in dimethylformamide, which was purged with nitrogen, was stored as a stock solution at 80°C. This solution was then placed in ice and kept in the dark prior to the study. The buffer used consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride at pH 7.4, and 100 μM diethylenetriaminepentaacetic acid (DTPA), each of which was prepared with high quality deionized water and purged with nitrogen.

The final concentration of DHR 123 was 5 μM. The background and final fluorescent intensities were measured 5 min after treatment with and without the authentic $ONOO^-$. DHR 123 was oxidized rapidly by the authentic $ONOO^-$, and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of the oxidized DHR 123 was measured using a microplate fluorescence reader FL 500 (Bio-Tek Instruments Inc.) at excitation and emission wavelengths of 480 and 530 nm, respectively. The results are expressed as the mean±standard error (n = 3) for the final fluorescence intensity minus background fluorescence. The effects are expressed as the percent inhibition of DHR 123 oxidation.

Statistical analysis

The data is expressed as the mean ± standard error of three or five experiments.

RESULTS AND DISCUSSION

It is well known that free radicals and ROS or RNS, including H_2O_2, $\cdot O_2^-$, ·OH, NO· and $ONOO^-$, play a role in the etiology of a vast variety of human degenerative diseases (Pincemail, 1995; Beckman *et al.*, 1990). These reactive species are formed in the body as a consequence of aerobic metabolism, and damage all intracellular components, such as nucleic acids, proteins and lipids. ROS are also implicated in both aging and various degenerative disorders (Sagar *et al.*, 1992; Ames *et al*, 1993).

In this study, we investigated the general antioxidant effects, for the potential to inhibit ·OH and total ROS and to scavenge authentic $ONOO^-$, of the MeOH extract along with its solvent soluble fractions, as represented by CH_2Cl_2, EtOAc, and *n*-BuOH, and the water layer derived from *P. davidiana* stem. As summarized in Table I, the MeOH extract and several solvent partitioned fractions, CH_2Cl_2, EtOAc, and *n*-BuOH, except for the water layer derived from *P. davidiana* stem, exhibited strong antioxidant activities on ·OH, total ROS generation and the $ONOO^-$ systems. The inhibition percent of the MeOH extract and its solvent soluble fractions on ·OH generation increased in the order EtOAc > MeOH > *n*-BuOH > CH_2Cl_2 > H_2O at the concentration of 40 μg/mL, comparable to that of the well-known antioxidant, L-ascorbic acid. In the total ROS system, the stem of *P. davidiana* showed increasing inhibition in the order MeOH ≥ EtOAc > CH_2Cl_2 > *n*-BuOH at the concentration of 40 μg/mL, with values of 83.78±1.31, 83.18±3.30, 70.39±3.70 and 56.71±3.42%, respectively. The IC_{50} values of the MeOH extract, the CH_2Cl_2, EtOAc and *n*-BuOH fractions, except for the water layer from this plant, on total ROS generation were 18.00±1.92, 6.77± 0.08, 6.61±0.59 and 33.02±3.01 μg/mL, respectively, comparable to that of the positive control Trolox (16.64±0.26 μg/mL). The scavenging activity of the MeOH extract, the EtOAc and *n*-BuOH fractions from the stem of *P. davidiana* on authentic $ONOO^-$ was stronger than that of the positive control penicillamine (74.14±0.47%) at concentration of 10 μg/mL, with values of 92.84±0.16%, 95.56±0.23% and 84.77±0.34%, respectively.

Table II shows the antioxidative activities of seven flavonoids isolated from the active EtOAc fraction of *P. davidiana* on the total ROS, ·OH and $ONOO^-$ systems. Of the seven flavonoids, three compounds, kaempferol, kaempferol 7-*O*-β-D-glucoside and (+)-catechin, were isolated as active principles, while four inactive components, dihydrokaempferol, hesperetin 5-*O*-β-D-glucoside, naringenin and its 7-*O*-β-D-glucoside, were isolated in the total ROS system. However, these isolated flavonoids did not show

Table I. Antioxidant activity of the MeOH extract and its solvent soluble fractions from *P. davidiana* stem

Samples	·OH[a]	Total ROS[b]		$ONOO^-$[c]
	Inhibition ratio (%) Mean ± SE*	IC_{50} (μg/mL) Mean ± SE*	Inhibition ratio(%) Mean ± SE*	Inhibition ratio (%) Mean ± SE*
MeOH	87.95 ± 2.17	18.00 ± 1.92	83.78 ± 1.31	92.84 ± 0.16
CH_2Cl_2	70.48 ± 7.69	6.77 ± 0.08	70.39 ± 3.70	70.73 ± 0.55
EtOAc	92.77 ± 4.78	6.61 ± 0.59	83.18 ± 3.30	95.56 ± 0.23
n-BuOH	76.51 ± 2.76	33.02 ± 3.01	56.71 ± 3.42	84.77 ± 0.34
H_2O	43.98 ± 9.56	ND*	0.88 ± 1.98	33.51 ± 1.61
L-ascorbic acid	55.20 ± 5.50			
Trolox		16.64 ± 0.26	73.56 ± 0.31	
Penicillamine				74.14 ± 0.47

[a]·OH is the inhibition percent of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$ at the concentration of 40 μg/mL. [b]Total ROS is the inhibitory activity of total ROS generation in kidney postmicrosomal fraction (IC_{50}: μg/mL and the inhibition percent at 40 mg/mL). [c]$ONOO^-$ is the inhibition percent of authentic peroxynitrite at the test concentration of 10 μg/mL. *Values of ·OH, total ROS and $ONOO^-$ are expressed as the mean±standard error of three or five experiments. *ND represents no inhibitory effect at 40 mg/mL.

Table II. Antioxidant activity of isolated compounds from *P. davidiana* stems

Compounds	Total ROS[a]	$ONOO^-$[b]	$\cdot OH$[c]
	Inhibition ratio (%)	Inhibition ratio (%)	Inhibition ratio (%)
	Mean ± SE[d]	Mean ± SE[d]	Mean ± SE[d]
Kaempferol (Kp)	54.02 ± 2.64	82.13 ± 0.59	25.54 ± 0.96
Kp 7-*O*-β-D-glucose (populnin)	27.33 ± 2.35	45.77 ± 1.35	27.71 ± 0.35
Dihydrokaempferol (aromadendrin)	-0.91 ± 5.52	14.31 ± 5.87	17.90 ± 0.21
Hesperetin 5-*O*-β-D-glucose	6.99 ± 1.68	57.59 ± 4.26	5.35 ± 0.11
Naringenin (Na)	6.68 ± 0.54	22.00 ± 2.05	12.10 ± 0.04
Na 7-*O*-β-D-glucose (pruning)	0.05 ± 2.24	3.89 ± 0.90	9.87 ± 0.03
Catechin	30.10 ± 4.43	73.14 ± 2.00	44.01 ± 1.00
Trolox	73.56 ± 0.31		50.80 ± 0.84
Penicillamine		74.14 ± 0.47	

[a]Total ROS is the inhibition percent of total ROS generation in kidney postmicrosomal fraction at the concentration of 10 μM. [b]$ONOO^-$ is the inhibition percent of peroxynitrite at the test concentration of 10 μM. [c]$\cdot OH$ is the inhibition percent of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$ at the concentration of 4 μM. [d]Values of total ROS and $ONOO^-$ are expressed as the mean ± standard error of three or five experiments.

good activity in $\cdot OH$ systems. In the $ONOO^-$ system, only four flavonoids, kaempferol, kaempferol 7-*O*-β-D-glucoside, (+)-catechin, and hesperetin 5-*O*-β-D-glucoside, showed scavenging activity. Our previous study suggested that these flavonoids exhibited scavenging activity on authentic $ONOO^-$ (Choi *et al.*, 2002).

On the basis of the above results, the relationship between flavonoid structure and inhibitory activity on total ROS generation was further evaluated. Flavonoids, a family of diphenylpropanes (C_6-C_3-C_6), are naturally occurring compounds and are widely distributed in vegetables, fruits and beverages such as tea and red wine. The structural diversity in each flavonoid is determined by the number and the arrangement of hydroxyl groups and by the glycosylation (Rice-Evans *et al.*, 1997). In addition, there are reports of a variety of biological activities of flavonoids, including anti-carcinogenic, anti-inflammatory, anti-radical and antioxidant (Harborne and Williams, 2000; Burda and Oleszek, 2001). In particular, flavonoids have been found to possess their antioxidant activity by virtue of both excellent metal chelating and radical scavenging properties (Morel *et al.*, 1993; Salah *et al.*, 1995; Van Acker *et al.*, 1998; Rice-Evans *et al.*, 1996). The chemical structures of the sixteen flavonoids used in the present study are presented in Fig. 1. As shown in Table III, the number of hydroxyl groups on their structures influenced the inhibitory activity in the total ROS system. The inhibitory activity of quercetin with five hydroxyl groups was stronger than that of four hydroxyl group-substituted flavonoids such as fisetin, kaempferol and luteolin, and of the three hydroxyl group-substituted flavonoid, baicalein, on total ROS generation. Also, the presence of an *ortho*-hydroxyl group on the B-ring and a 3-hydroxyl group on the C-ring was associated with lower IC_{50} values for inhibitory activity on total ROS generation. In the case of the catechol skeleton of the B-ring, the IC_{50} value in the total ROS system of quercetin (0.16 μM) was lower than that of morin (0.58±0.13 μM). When the hydroxyl groups at the positions C-3 and C-7 were substituted by a glycoside or when there was no hydroxyl group at these positions, the antioxidant activity decreased. The IC_{50} value of kaempferol, at 0.72±0.04 μM, was reduced by the glycosylation at the positions C-3 and C-7, as presented by its 3-*O*-β-D-glucoside (astragalin) and its 7-*O*-β-D-glucoside (populnin) values of 8.87±0.45 μM and 9.88±0.02 μM, respectively. These above results suggest that the inhibitory activities of flavonoids on total ROS are dependent on the structure of the agents. In conclusion, the strength of the inhibitory activity on total ROS generation increases with the rising number of hydroxyl groups on their structures. Also, the presence of an *ortho*-hydroxyl group on the B-ring and a 3-hydroxyl group on the C-ring plays an important role on the antioxidant activity in the total ROS system. These results were similar to those of other research on the structure-antioxidant activity of flavonoids on the modified ROS assay (Krishnamachari *et al.*, 2002; Lee *et al.*, 2002; Cos *et al.*, 1998; Nagao *et al.*, 1999).

On the other hand, not all tested flavonoids exhibited good inhibitory activity in the $\cdot OH$ generation model system by comparison with the positive control, Trolox. Regardless of the number and position of hydroxy groups, the IC_{50} values of the tested flavonoids were higher than that of the positive control, Trolox, in the $\cdot OH$ model system. Among the flavonoids tested, flavone (i.e. luteolin), flavonol (i.e. quercetin) and flavan (i.e. catechin), which all have *o*-

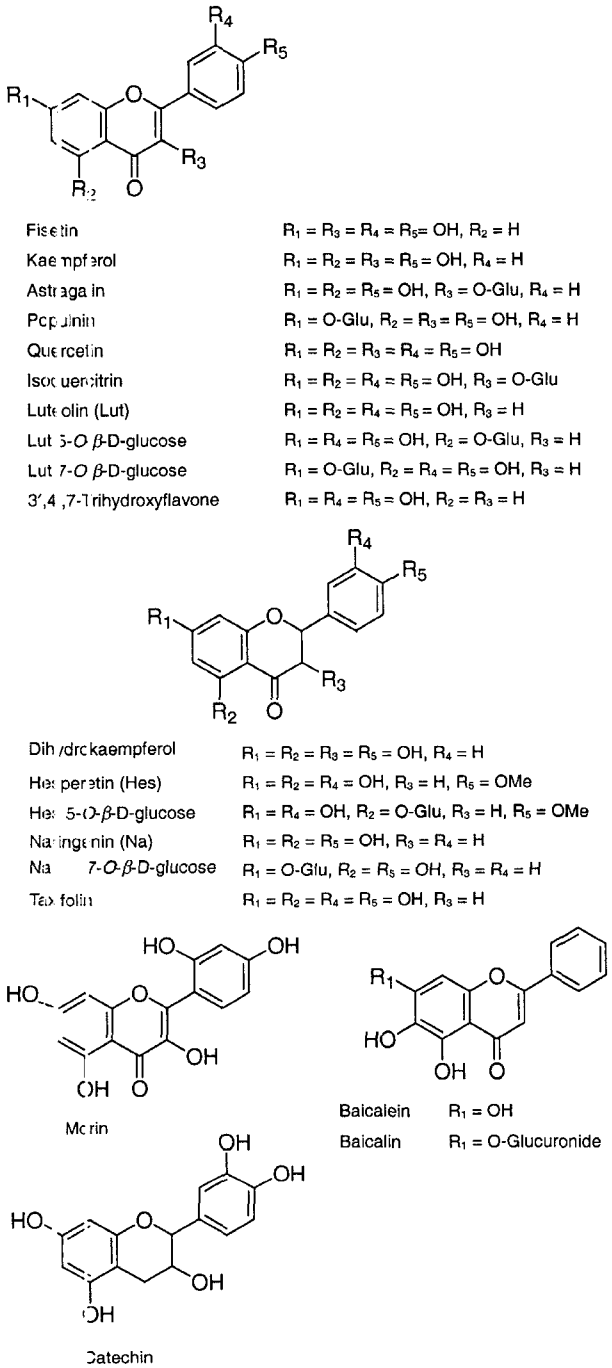

Fig. 1. Structures of flavonoids used in the total ROS and the ·OH system

dihydroxy functional groups at C-3' and C-4', showed significant activity. In addition, flavone baicalein (or baicalin) with a 5,6-dihydroxyl group was also found to have an inhibitory activity similar to that of the 3',4'-dihydroxylated flavonoics. This indicates that *ortho*-dihydroxyl substitutions, whether on the A-ring or B-ring, are an important feature for the inhibitory activity of flavonoids in the ·OH mode system. Our results are in agreement with those

Table III. Inhibitory activity of flavonoids on total ROS and ·OH generation

Compounds	Total ROS[a]	·OH[b]
	IC_{50} (μM)	IC_{50} (μM)
	Mean± SE[c]	Mean± SE[c]
Quercetin (Qc)	0.16± 0.00	7.87± 1.17
Fisetin	0.27± 0.01	10.69± 0.86
Morin	0.58± 0.13	4.03± 0.61
Kaempferol (Kp)	0.72± 0.04	10.03± 1.12
Luteolin	0.77± 0.07	5.66± 0.46
Baicalein	0.88± 0.08	8.46± 0.80
Catechin	2.11± 0.20	4.58± 0.37
Luteolin 7-O-β-D-glucose	3.25± 0.16	10.76± 1.87
Hesperetin	3.59± 0.12	6.37± 0.97
Taxifolin	3.79± 0.04	5.57± 1.24
Luteolin 5-O-β-D-glucose	3.88± 0.11	15.41± 2.62
3',4',7-Trihydroxy flavone	4.87± 0.27	10.15± 2.39
Qc 3-O-β-D-glucose (isoquercitrin)	6.17± 0.27	4.88± 0.97
Baicalin	7.67± 0.84	6.54± 1.05
Kp 3-O-β-D-glucose (astragalin)	8.87± 0.45	14.87± 2.91
Kp 7-O-β-D-glucose (populnin)	9.88± 0.02	7.85± 0.18
Trolox	3.55± 0.07	3.87± 0.08

[a]Total ROS is the inhibitory activity of total ROS generation in kidney postmicrosomal fraction (IC_{50}: μg/mL). [b]·OH is the inhibition percent of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$ (IC_{50}: μg/mL). [c]Values of total ROS and ·OH are expressed as the mean±standard error of three or five experiments.

previously reported on the inhibition of ·OH (Ueda *et al.*, 1996).

The present work supports the view that the antioxidant capacities of the MeOH extract, its various fractions and the components of the *P. davidiana* stem may be useful for the treatment of oxidative damage. Furthermore, we demonstrated that the compounds, as well as some of the other flavonoids available, exhibited a potent inhibitory effect on total ROS/·OH generation. It will be interesting to further investigate the antioxidative activity of these natural compounds in preventing various radical-mediated injuries in *in vivo* pathological situations. Investigations of further antioxidant principles and of the biochemical mechanism of the flavonoids in the inhibitory effect on the ROS/·OH system will be elucidated in a future study.

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