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HYDROXYL RADICAL SCAVENGING ACTIVITY OF FLAVONOIDS

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Key Word Index—Flavonoids; 'OH radical; scavenger; HPLC-electrochemical detection.

Abstract—The flavonoids scavenge hydroxyl ('OH) radicals generated by UV photolysis of hydrogen peroxide. Free 'OH radicals were spin-trapped by 5,5-dimethyl-1-pyrroline *N*-oxide and the adduct was detected by high pressure liquid chromatography coupled with an electrochemical detector. The scavenging activity of flavonoids decreases in the order: myricetin > quercetin > rhamnetin > morin > diosmetin > naringenin > apigenin > catechin > 5,7-dihydroxy-3',4',5'-trimethoxyflavone > robinin > kaempferol > flavone. The activity increases with the number of hydroxyl groups substituted in the aromatic B-ring. The presence of a hydroxyl at C-3 and its glycosylation does not further increase scavenging efficiency. It is suggested that the overall antioxidant effect of flavonoids on lipid peroxidation may be due to their 'OH and O_2^- scavenging properties and the reaction with peroxy radicals.

INTRODUCTION

The hydroxyl radical ('OH) has a high and indiscriminate activity and can slowly cause severe damage to susceptible biomolecules. Hydroxyl radicals can be produced in cells by a variety of processes: such as phagocytosis [1]; prostaglandin biosynthesis especially during the transformation of PGG_2 to PGH_2 [2]; by antineoplastic agents, e.g. bleomycin [3] and adriamycin [4]; fungal phytotoxins, cercosporin and dothistoromin [5]; ionizing irradiation [6, 7]; and decomposition of lipid hydroperoxides [8]. It is generally proposed that such a radical could be originated by Fenton-type reaction [9]: $H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$.

Hydroxyl radicals, being extremely reactive species, serve as both a primary toxicant and as source of secondary toxicants [10]. They are reported to mediate the lethal cell injury in cultured hepatocytes [11] and cause the cytotoxicity of alloxan [12, 13]. Hydroxyl radical generated at or near DNA may eventually result in strand breakage of DNA [6, 14] and contribute to significant biological effects such as carcinogenesis, mutagenesis and cytotoxicity [15, 16].

Damage caused by the toxic effects of 'OH radicals is often decreased by scavengers of this radical such as phenol, mannitol, soidum formate, thiourea, DMSO, benzoic acid, alcohols and analogues of methionine [17, 18]. The flavonoids widely used as therapeutic agents are also known to act as strong superoxide radical (O_2^-) scavengers [19], and singlet oxygen ($^1O_2$) quenchers [20]. Recently, we reported that flavonoids also react with peroxy radicals involving termination of chain reaction during the autoxidation of polyunsatured fatty acids [21].

The present work demonstrates that flavonoids are also capable of scavenging the OH radicals generated by photolysis of hydorgen peroxide [22]. The 'OH radicals were spin trapped by 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and measured by the HPLC-electrochemical detection method [23].

RESULTS AND DISCUSSION

It has been reported that UV photolysis of hydrogen peroxide in presence of high concentration of DMPO and low concentration of hydrogen peroxide yields DMPO-OH' adduct predominantly [24]. This indicates that 'OH is the primary radical formed during the photolysis of hydrogen peroxide in our experiment. The authenticity of 'OH was further confirmed by adding ethanol (10%) to the sample before irradiation and the peak observed for DMPO–hydroxyethyl radical was different from DMPO–OH' as shown by Pristos *et al.* [25].

Our chromatogram displayed a peak for DMPO–OH' at a retention time of eight min. The percentage of diminution of DMPO–OH' peak in presence of each flavonoid (1 mM) was depicted in Table 1. The 'OH scavenging activity of flavonoids decreased in the order: myricetin > quercetin > rhamnetin > morin > diosmetin > naringenin > apigenin > catechin > 5,7-dihydroxy-3',4',5'-trimethoxyflavone > robinin>kaempferol > flavone. The flavonoids exhibit different 'OH scavenging property depending upon structure. From our present investigations, we can infer a few generalizations. The most active compounds are flavonols. The scavenging activity seems directly related to the number of hydroxyl groups substituted at ring B, especially at C-3'. When the number of hydroxyl groups decreases the 'OH quenching ability falls rapidly, e.g. myricetin scavenges 50% of 'OH radical while kaempferol only 20%. Furthermore, the presence of a carbonyl function at C-4 position appears to play an important role: catechin devoid of a carbonyl quenched 31% of 'OH while quercetin with a carbonyl quenched 48% of 'OH. The glycosylation of this hydroxyl group does not alter the scavenging potency. The kaempferol glycoside robinin and kaempferol itself have very similar scavenging efficiencies. Similarly, we tested the quenching effect of quercetin and its 3-rutinoside, rutin. Rutin scavenges 'OH radicals even at a very low concentration but we could not compare the activity because of

Table 1. Structure and hydroxyl radical scavenging activity of flavonoids

Name	Substituent at C-3	C-5	C-7	C-2'	C-3'	C-4'	C-5'	'OH radical scavenged (%) (relative ratio to the control)
Flavonol								
Myricetin	OH	OH	OH	H	OH	OH	OH	50
Quercetin	OH	OH	OH	H	OH	OH	H	48
Rhamnetin	OH	OH	OMe	H	OH	OH	H	46
Morin	OH	OH	OH	OH	H	OH	H	40
Robinin	OGal							
	Rha	OH	O Rha	H	H	OH	H	27
Kaempferol	OH	OH	OH	H	H	OH	H	20
Flavanone								
Naringenin	H	OH	OH	H	H	OH	H	36
Flavone								
Diosmetin	H	OH	OH	H	OH	OMe	H	39
Apigenin	H	OH	OH	H	H	OH	H	34
5,7-Dihydroxy trimethoxyflavone	H	OH	OH	H	OMe	OMe	OMe	28
Flavone	H	H	H	H	H	H	H	04
Flavanol								
(+)-Catechin	OH	OH	OH	H	OH	OH	H	31
Classical scavengers								
Ethanol								25
1-Butanol								45
DMSO								62

its insolubility in acetone at millimolar concentrations. Hydroxyl scavenging property was also found to be independent of the presence of double bond between C-2 and C-3. The flavones like diosmetin and apigenin exhibited almost the same quenching power as the flavanone naringenin. The hydroxyl group at C-3 does not contribute much to the 'OH scavenging, since flavones such as apigenin and 5,7-dihydroxy-3',4',5'-trimethoxyflavone scavenge more 'OH radicals than kaempferol.

The 'OH scavenging property of flavonoids was found to be almost consistent with the earlier report of inhibitory action of flavonoids. Younes and Seigers [26] reported that antioxidative action of flavonoids on lipid peroxidation of rat liver homogenate depends upon the 3',4'-dihydroxy grouping. The presence of a C-3–OH group, its methylation or conjugation with sugar does not further increase the inhibitory action of flavonoids. From our observations, it is clear that hydroxylation is indispensible in a flavonoid for scavenging activity, since flavone lacks any significant scavenging effect.

The efficiency of classical 'OH scavengers at the same molar concentration (1 mM) was compared with that of flavonoids. Only DMSO was found to be more active than flavonoids while ethanol, a common 'OH scavenger displayed almost the same potency as that of the least effective flavonoids, kaempferol and robinin. Thus, flavonoids being strong 'OH radical scavengers may serve as better antioxidants in biological systems. They are widely present in plants and have been found to be localized in chloroplasts [27]. They have been reported to suppress lipid photoperoxidation in chloroplasts by scavenging superoxide anion and radicals formed during peroxidation [28]. Dismutation of superoxide anion in chloroplasts may lead to 'OH radical formation via Fenton-type reaction. It appears that the overall antioxidant effect of flavonoids on lipid peroxidation is the result of scavenging of 'OH and O_2^- radicals at the stage of initiation and termination of peroxyl radicals.

EXPERIMENTAL

Reagents and methods. Robinin, quercetin, morin, myricetin, apigenin, diosmetin, naringenin, rhamnetin, (+)-catechin, and 5,7-dihydroxy-3',4',5' trimethoxy flavone were purchased from Sigma and flavone (99%), kaempferol from Fluka. DMPO and H_2O_2 were supplied by Aldrich and E. Merck respectively. Prolabo (France) supplied DMSO and 1-butanol. DMPO was purified by passing through the charcoal and stored according to the method described by ref. [23].

Generation and detection of DMPO–hydroxyl radical adduct in presence of flavonoids. We generated 'OH radicals by UV photolysis of H_2O_2 [22] and allowed them to spin trap with DMPO. We placed 800 mM DMPO (40 μl), 0.06%, 2 mM H_2O_2 (20 μl), flavonoid dissolved in acetone (140 μl) in a small Petri dish (3.5 cm i.d., 2.2 cm height). To cope with the poor hydrophilicity of flavonoids, they were dissolved in acetone which apparently does not interact with the generation of 'OH radicals. UV irradiation was derived from a Desaga Duo-UV lamp (Heidelberg, F.R.G.) set at 254 nm, the lamp being placed directly above the petri dish for 10 min. The control samples having the same amount of acetone but without flavonoid were irradiated in the same way. Immediately after irradiation, 15 μl of sample was directly injected into the HPLC unit coupled with an electrochemical detector. The HPLC unit was a LDC Chromatograph equipped with a Constrametric III pump, a Valco 7000 psi injector and a pulse damper. The column elute was monitored by Metrohm 641 VA Detector consisted of Metrohm 656 Electrochemical detector cell (Metrohm, Swiss). The detector potential was set at +0.6 V vs an Ag/AgCl reference electrode, and sensitivity of 10 nA/V. Peaks were measured by Linear dual

channel recorder (Linear Instruments Corp. Calif.) set at 1 V. A spherisorb S50DS2 150 x 4.9 mm i.d. (Sopares, France) column was used to detect DMPO–OH· adduct. The eluting solvent was composed of citric acid (monohydrate), 0.03 M; anhydrous NaOAc, 0.05 M; NaOH, 0.05 M; and HOAc, 0.02 M to obtain a final pH of 5.1. The solvent was filtered through a 0.45 μm pore size Millipore filter, and was run at a flow rate of 1.0 ml/min. Each flavonoid (1 mM) was dissolved in acetone. Ethanol, 1-butanol, and DMSO were also used in acetone at the same molar concentration. The scavenging activity of flavonoid was measured by comparing the chromatograms of DMPO–OH· adduct with and without flavonoid.

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