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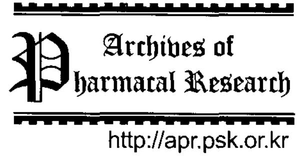

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Antioxidant Properties of Flavone-6(4')-Carboxaldehyde Oxime Ether Derivatives

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The *in vitro* antioxidant properties of some flavone-6(4)-carboxaldehyde oxime ether derivatives (**Ia-f, IIa-f**) were determined by their effects on the rat liver microsomal NADPH-dependent lipid peroxidation (LP) levels by measuring the formation of 2-thiobarbituric acid reactive substances. The free radical scavenging properties of the compounds were also examined *in vitro* by determining their capacity to scavenge superoxide anions and interact with the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The most active compounds, **IIb** (Flavone-4'-carboxaldehyde-*O*-ethyl oxime) and **Id** (Flavone-6-carboxaldehyde-*O*-[2-(1-pyrolidino) ethyl] oxime), caused 98 and 79% inhibition of superoxide anion production and DPPH stable free radical at 10^{-3} M, respectively.

Key words: Flavone, Oxime ether, Lipid peroxidation, Superoxide dismutase, DPPH, Antioxidant

INTRODUCTION

Free radicals, including the superoxide, hydroxyl, hydrogen peroxide and lipid peroxide radicals, have been implicated in a number of degenerative diseases, such as brain dysfunction, cancer, heart diseases and immune system decline (Rice-Evans and Diplock, 1991). These reactive oxygen species (ROS) are produced as a normal consequence of biochemical processes in the body and due to increased exposure to environmental and/or dietary xenobiotics. It is an imbalance in these oxidants versus antioxidant processes (oxidative stress) that is thought to cause the subsequent cellular damage that leads to the above mentioned disease processes (Griffits *et al.*, 1998). Neurodegenerative diseases, such as Alzheimers disease, are also linked to damage from ROS as a result of an imbalance between the rates of radical generation and scavenging (Richardson, 1993). Antioxidant systems, including superoxide dismutase, catalase and glutathione, should keep the oxidative processes in balance. However, deficiencies of nutritional antioxidants (flavonoids, vitamin A, C, E, the minerals selenium and zinc, coenzyme Q10, lipoic acid) and/or overwhelming oxidant stress can overload these systems (Nordmann, R). The biological activities of flavonoids have been extensively reviewed, and some have been found to possess antibacterial (Mori *et al.*, 1987), anticancer (Deschner *et al.*, 1991; Elangovan *et al.*, 1994), antihistaminic (Amella *et al.*, 1985), anti-inflammatory (Krishnaveni *et al.*, 1997; Sala *et al.*, 2003), antiischemic (Rump *et al.*, 1995), antiviral (Wleklik *et al.*, 1988) and hypoglycemic (Bozdağ *et al.*, 2001) activities. Flavonoids have also been found to inhibit a wide range of enzymes involved in oxidation systems, such as phospholipase A_2 (Chang *et al.*, 1994), 5-lipoxygenase, cyclooxygenase (Robak *et al.*, 1988) and xanthine oxidase (Chang *et al.*, 1993; Cos *et al.*, 1998), and these activities are related to their antioxidant properties. Flavonoids can exert their antioxidant properties by scavenging radicals, binding metal ions and inhibiting enzymatic systems.

In our previous studies, the synthesis and antimicrobial evaluation of 6- or 4'-flavone carboxaldehyde oxime ether derivatives have been described (Ayhan-Kılcıgil *et al.*, 1999; Tunçbilek *et al.*, 1999). The fungustatic effect of ketoconazole, an azole antifungal drug, associated with its membrane stabilizing effects on *Candida* species is indicated by inhibition of lipid peroxidation (Wiseman *et*

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al., 1991). Therefore, the antioxidant properties of some flavone 6- or 4'-carboxaldehyde oxime ether derivatives, already known to have antimicrobial activities, were investigated (Ayhan-Kılcıgil *et al.*, 1999; Tunçbilek *et al.*, 1999) by comparing with butylated hydroxytoluene (BHT) and superoxide dismutase (SOD), a well known antioxidant and an antioxidant enzyme, respectively.

MATERIALS AND METHODS

Chemistry

The compounds **Ia-f** and **IIa-f** (Fig. 1) were synthesized, starting with 6- or 4'-flavone carboxaldehyde, by treatment with the appropriate *O*-substituted hydroxyl amine derivatives (Ayhan-Kılcıgil *et al.*, 1999; Tunçbilek *et al.*, 1999). All reagents were purchased from Sigma (Taufkirchen, Germany).

Antioxidant activity studies

Assay of lipid peroxidation

Male albino Wistar rats (200-225 g) were used in the experiments. The animals were fed a standard laboratory rat chow and allowed tab water ad libitum. The animals were starved for 24 h prior to sacrifice, and then killed by decapitation under anesthesia. The livers were removed immediately, washed in ice-cold distilled water and microsomes prepared, as described previously (Iscan *et al.*, 1984).

NADPH-dependent lipid peroxidation (LP) was determined using the optimum conditions, as previously determined and described (Iscan *et al.*, 1984). In this assay, the control activity has been regarded as the activity measured in the presence of the pure diluent for the chemicals tested (Dimethylsulfoxide (DMSO) for synthesized compounds and BHT). Thus, the assay has been carried out in the presence of only solvent, as a control, or at the indicated concentrations of compounds. NADPH-dependent LP was measured spectrophotometrically by estimation of thiobarbituric acid reactant substances (TBARS). The amounts of TBARS were expressed in terms of nmol malondialdehyde (MDA)/mg protein. The assay was essentially derived from the methods of Wills (Wills, 1966; Wills, 1969), as modified by Bishayee (Bishayee and Balasubramanian, 1971). A typical optimized assay mixture contained 0.2 nM Fe^{++}, 90 mM KCl and 62.5 mM potassium-phosphate buffer, pH 7.4, and the NADPH generating system (0.25 mM $NADP^{+}$, 2.5 mM $MgCl_2$, 2.5 mM glucose-6-phosphate, 1.0 U glucose-6-phosphate dehydrogenase and 14.2 mM potassium phosphate buffer pH 7.8), and 0.2 mg microsomal protein, in a final volume of 1.0 mL. The reaction was initiated by the addition of the NADPH generating system to the microsomal mixtures. The reaction was carried out at 37°C for 30 minutes, and trichloroacetic acid added to stop the reaction. The denatured proteins were then removed by centrifugation. Finally, the supernatant was mixed with thiobarbituric acid (TBA) and boiled for 15 minutes. The absorbance was measured spectrophotometrically at 532 nm. Each experiment was performed in triplicate. The protein contents of liver microsomes were determined by the method of Lowry *et al.* (Lowry *et al.*, 1951), using bovine serum albumin as a standard.

Superoxide radical scavenging activity

The capacity of compounds to scavenge superoxide anions was determined spectrophotometrically on the basis of the inhibition of cytochrome c reduction, according to the modified method of McCord et al (McCord and Fridowich, 1969).

Superoxide anions were generated in the xanthine/xanthine oxidase system. The reaction mixture contained 0.05 M phosphate buffer, pH 7.8, 0.32 U xanthine oxidase, 50 μM xanthine, 60 mM cytochrome c and 100 μL of different concentrations of the synthesized compounds in a final volume of 1 mL. DMSO and BHT were used as the control solution and reference compound, respectively. The absorbance was measured spectrophotometrically at 550 nm to determine the cytochrome c reduction. Each experiment was performed in triplicate, and the results expressed as a percentage of the control.

Ia-f **IIa-f**

	a	b	c	d	e	f
R	$-CH_3$	$-C_2H_5$	$-CH_2CH_2N(CH_3)_2$			

Fig. 1. General formula of the compounds

DPPH free radical scavenging activity

The free radical scavenging activities of these compounds were tested by their ability to bleach the stable radical DPPH, as described by Blois (Blois, 1958). This assay has often been used to estimate the antiradical activity of antioxidants. Because of its odd electronic structure DPPH gives a strong absorption band at 517 nm in its visible spectrum. DPPH was dissolved in methanol to give a 100 μM solution. 0.1 mL of the test compounds and BHT, dissolved in DMSO, was added to 1.0 mL of the methanolic DPPH solution. The absorbance at 517 nm was determined after 30 min at room temperature, and the scavenging activities calculated as a percentage of the radical reduction. Each experiment was performed in triplicate. DMSO was used as a control solution and BHT as a reference compound. The radical scavenging activity

Table I. Effects of the compounds on liver superoxide anion production and reduction of DPPH radical [a]

Compound	R	Concentration in incubation medium (M)	Superoxide anion ($O_2^{\bullet -}$) production percent of control	LP Percent of control	DPPH free radical scavenger activity (percent of control)
Control[b]			100	100	100
Ia	CH_3	10^{-4} 10^{-3}	106±4 31±6	80	103±3
Ib	C_2H_5	10^{-4} 10^{-3}	178±9 4±1	-	-
Ic	$CH_2CH_2N(CH_3)_2$	10^{-4} 10^{-3}	97±2 31±2	-	-
Id		10^{-4} 10^{-3}	46±2 42±2	131	21±1
Ie		10^{-4} 10^{-3}	88±6 8±4	221	91±1
If		10^{-4} 10^{-3}	103±3 37±4	124	102±1
IIa	CH_3	10^{-4} 10^{-3}	231±8 31±6	44	105±2
IIb	C_2H_5	10^{-4} 10^{-3}	97±2 2±2	58	98±3
IIc	$CH_2CH_2N(CH_3)_2$	10^{-4} 10^{-3}	99±4 101±6	138	82±3
IId		10^{-4} 10^{-3}	99±4 106±3	141	55±1
IIe		10^{-4} 10^{-3}	78±4 64±2	128	83±4
IIf		10^{-4} 10^{-3}	114±2 62±3	102	84±3
BHT		10^{-4} 10^{-3}	103±3 52±6	65	9±1
Control[c]		Water	100±2		
SOD		30 IU 45 IU	24±2 11±1		

[a] Each value represents the mean ± S.D. of 2-4 independent experiments
[b] Dimethylsulfoxide only, control for compounds and BHT
[c] Distilled water, control for SOD
– : not tested

was obtained from the equation: Radical scavenging activity % = $\{(OD_{control} - OD_{sample}) / OD_{control}\} \times 100$

RESULTS AND DISCUSSION

The inhibitory effects of the compounds on the NADPH-dependent lipid peroxidation levels were determined using rat liver microsomes by measuring the formation of 2-thiobarbituric acid reactive substances. Only two of the compounds (**IIa** and **IIb**), at a concentration of 10^{-3} M, showed marked inhibition (56 and 42%, respectively) of the lipid peroxidation. These inhibitions were close to those obtained with 10^{-3} M BHT (65%), a well known antioxidant, but the others had no effects on the level of lipid peroxidation (Table I).

The superoxide anion radical scavenging activities of the compounds were investigated using the xanthine/xanthine oxidase system, and the results are presented in Table I. The most active compounds were found: **IIb** (98%), **Ib** (96%), and **Ie** (92%) at a concentration of 10^{-3} M. The -N-OEt derivatives especially were more potent than the others. The compounds appeared to have stronger inhibitory effects on superoxide anion formation than that of the BHT (48% at 10^{-3} M) that was used as the positive control. In addition, they had similar inhibitory effects on superoxide anion formation to that of SOD (89% inhibition at 45 IU), the antioxidant enzyme used for comparison. Compounds **IIc** and **IId** had no effect on superoxide anion formation. The scavenging rates of the rest of the compounds were in the range of 36-69%. In general, substitutions on the A ring of the flavone ring system results in an improvement of the superoxide radical scavenging properties, with the exception of compound **IIb**. The structure-activity correlation within the 6- and 4'- substituted derivatives is not straightforward; there is a difference in activity between the aminoalkyl substituted member of each series and its counterpart. Compounds **Ib**, **IIa,** and **IIf** showed biphasic effects on superoxide anion formation. These compounds increased and decreased the superoxide anion formation at concentrations of 10^{-4} and 10^{-3} M, respectively. This pattern of differing effects can be seen in biological assays, where increases may occur at lower concentrations, whereas inhibition results at higher concentrations (Iscan, 1984; Al-Assadi *et al.*, 1992). Biphasic effects of other chemicals, such as thiazolidinedione/imidazolidinedione (Dündar *et al.*, 2002) and hydroxychalcones (McCord and Fridowich, 1969; Kornbrust and Maris, 1980; Parke *et al.*, 1991; Dix and Aikens, 1993) on the formation of superoxide anions have been well established in various *in vitro* systems.

The free radical scavenging properties of the compounds were also examined by the interaction with the stable free radical, DPPH. As seen in Table I, compounds **Id** and **IId**, which bear a pyrolidino moiety, as an R substituent, were found to have some marginal DPPH scavenger activities (79 and 45% respectively) at concentrations of 10^{-3} compared with BHT (91%). The compounds **Ie, IIc, IIe**, and **IIf** showed slight inhibitions of the DPPH stable free radical, whereas the others were ineffective.

There are two mechanisms by which an antioxidant can scavenge DPPH. First, a direct H-atom abstraction process (eq 1), and second, a proton concerted electron-transfer process (eq 2) (Litwinienko and Ingold, 2003).

$$DPPH^{\bullet} + RXH \rightarrow DPPHH + RX^{\bullet} \quad (1)$$

$$DPPH^{\bullet} + RXH \rightarrow DPPH^{-} + RXH^{\bullet +} \rightarrow DPPHH + RX^{\bullet} \quad (2)$$

Since some theoretical methods, especially density functional theory, have been successfully used to investigate and elucidate the structure-activity relationship (SAR) of antioxidants, this would also be helpful in clarifying our compounds, and thus in the design of novel antioxidants with better pharmacological effects.

The activity patterns of compounds on LP, superoxide anion formation and DPPH radical scavenging activity were dissimilar. Distinct antioxidant effects of chemicals have also been previously noted in different *in vitro* assay systems (Dündar *et al.*, 2002; Ölgen and Coban, 2003). Thus, herein, observation of the distinct effects of some compounds on the assay systems utilized was not surprising, as the mechanisms of production of oxidative stress in these assays are different (Kornbrust and Mavis, 1980; Parke *et al.*, 1991; Dix and Aikens, 1993).

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