Evaluation of the Antioxidant Effects of Some Flavonylthiazolidinediones by Determining Their Effects on Lipid Peroxidation, Superoxide Anion Formation, and 2,2-Diphenyl-1-picrylhydrazyl Stable Free Radical

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The *in vitro* antioxidant effects of some flavonylthiazolidinediones (Ia—d, IIa—d) on rat liver microsomal NADPH-dependent lipid peroxidation (LP) levels were determined by measuring the formation of 2-thiobarbituric acid reactive substance. The free radical-scavenging properties of the compounds were examined *in vitro* by determining the capacity to scavenge superoxide anion formation and of the interaction with the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The compounds had no inhibitory effects on LP. However, they had variable inhibitory influence on superoxide anion production and DPPH radical in a concentration-dependent manner. The most active compound, 3-(2,5-dimethoxyphenacyl)-5-[2-phenyl-4*H*-4-oxo-1-benzopyran-6-yl)-methylenyl]-thiazolidine-2,4-dione, Id showed 98% inhibition of superoxide anion production and 95% inhibition of DPPH stable free radical at 10^{-3} M.

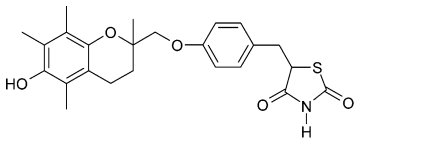
Key words antioxidant activity; flavonylthiazolidinedione; lipid peroxidation; superoxide dismutase; 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Free radicals are atoms that have at least one unpaired electron, thus making them unstable and highly reactive. Free radicals then roam throughout the body causing damage to cells by stealing stable electron partners from other cells, causing more free radicals, more instability, and more damage. This cell damage can impair the body's ability to fight illness. Flavonoids form a class of benzo-γ-pyrone derivatives include flavones, flavanes, flavonols, anthocyanidines, and catechins. They possess a wide spectrum of biological activities such as anticancer,[1)] antibacterial,[2)] antifungal,[3)] antiviral,[4)] spasmolytic,[5)] hypoglycemic,[6)] and antihistaminic.[7)] Some of these properties derive from the free radical-scavenging activities of flavonoids. Therefore there are many reports relating to the reactivities of flavonoids with active oxygen species.[8—11)] Recent interest in these substances has been stimulated by the potential health benefits arising from their antioxidant activity. The pharmacologic activities of flavonoids are assumed to result mainly from the inhibition of certain enzymes and their antioxidant activity. Many flavonoids have been reported to be potent inhibitors of enzymes such as phospholipase A2[12)] and xanthine oxidase.[13,14)] Xanthine oxidase is an important source of reactive oxygen species (ROS) in ischemia-reperfusion in endothelium. The thiazolidinediones have been reported to reduce the serum lipid peroxide (LP) concentration in mice with alloxan-induced hyperlipoperoxidemia and hyperlipidemia.[15)] It was observed that troglitazone (Fig. 1), an antidiabetic thiazolidinedione, has a scavenging effect on reactive oxygen, which is attributable to the similarity of its molecular structure to that of alpha-tocopherol (Fig. 1) produced by xanthine oxidase and generated by stimulated neutrophils.[16)] In another study, it was found that troglitazone has a potent and rapid biological inhibitory effect on ROS generation by polymorphonuclear leukocytes (PMNLs) and mononuclear cells (MNCs) and that it inhibits lipid peroxidation significantly.[17)] In our previous studies, we described the synthesis and antimicrobial evaluation of 6(4′)-flavonylthiazolidinediones.[18,19)] Ketoconazole, a well-known azole antifungal drug, was found to inhibit lipid peroxidation in both microsomal and liposomal systems. In addition, it was observed that fungistatic effect of ketoconazole on *Candida* species is associated with its membrane-stabilizing effects as indicated by inhibition of lipid peroxidation.[20)] The above-mentioned results stimulated our interest in evaluating the antioxidant capacities of some flavonylthiazolidinediones (Fig. 2). For this purpose, the antioxidant properties of compounds **Ia**—**d**, and **IIa**—**d**, that have already been shown to have antimicrobial activities,[18,19)] a typical antioxidant butylated hydroxytoluene (BHT), and an antioxidant enzyme superoxide dismutase (SOD) were investigated using three different methods.

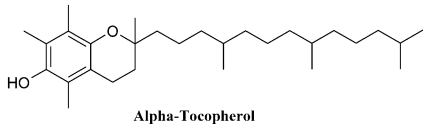
MATERIALS AND METHODS

Chemistry Compounds **Ia**—**d**, and **IIa**—**d** were obtained by Knoevenagel condensation of 6- or 4′-flavone carboxaldehyde and 3-substituted phenacyl-2,4-thiazolidinediones. The synthesis and antimicrobial activities of these compounds were reported in our previous studies.[18,19)]

Antioxidant Activity Studies. Assay of Lipid Peroxidation Male albino Wistar rats (200—225 g) were used in the experiments. Animals were fed standard laboratory rat



Troglitazone



Alpha-Tocopherol

Fig. 1. Structures of Troglitazone and Alpha-Tocopherol

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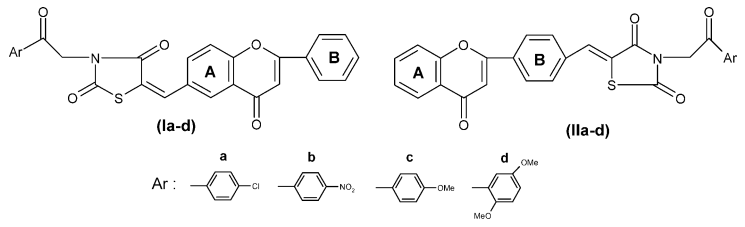


Fig. 2. Structural Formula of Compounds **Ia**—**d**, and **IIa**—**d**

chow and tap water *ad libitum*. The animals were starved for 24 h and then killed by decapitation under anesthesia. The livers were removed immediately and washed in ice-cold water, and the microsomes were prepared as described previously.[21)]

The NADPH-dependent LP level was determined using the optimum conditions determined and described previously.[21)] In this assay, control activity was regarded as the activity measured in the presence of the pure diluent for the chemicals tested (DMSO for synthesized compounds and BHT). Thus the assay was carried out only in the presence of solvent, as a control, or the indicated concentrations of compounds. The NADPH-dependent LP level was measured spectrophotometrically by estimation of thiobarbituric acid reactant substances (TBARS). Amounts of TBARS were expressed in terms of nmol of malondialdehyde (MDA)/mg protein. The assay was essentially derived from the methods of Wills[22,23)] as modified by Bishayee and Balasubramanian.[24)] A typical optimized assay mixture contained 0.2 nM Fe^{++}, 90 mM KCl, 62.5 mM potassium-phosphate buffer, pH 7.4, an NADPH generating system consisting of 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 of microsomal protein in a final volume of 1.0 ml. The reaction was started by the addition of the NADPH generating system to microsomal mixtures. Reaction was carried out at 37 °C for 30 min and trichloroacetic acid was added to stop the reaction. Then denatured proteins were removed by centrifugation. Finally the supernatant was mixed with TBA and boiled for 15 min. The absorbance was measured spectrophotometrically at 532 nm. Each experiment was performed in triplicate. Protein contents of liver microsomes were determined by the method of Lowry *et al.*[25)] with the use of bovine serum albumin as a standard.

Superoxide Radical-Scavenging Activity The capacity of some flavonylthiazolidinedione compounds (**Ia**—**d**, **IIa**—**d**) to scavenge superoxide anion formation was determined spectrophotometrically based on the inhibition of cytochrome c reduction according to the modified method of McCord and Fridowich.[26)] Superoxide anion was generated in the xanthine/xanthine oxidase system. The reaction mixture contained 0.05 M phosphate buffer, pH 7.8, xanthine oxidase 0.32 U, xanthine 50 μM, 60 mM cytochrome c, and different concentrations of synthesized compounds at 100 μl in a final volume of 1 ml. DMSO was used as a control solution and BHT as a reference compound. The absorbance was measured spectrophotometrically at 550 nm for cytochrome c reduction. Each experiment was performed in triplicate, and the results are expressed as a percentage of the control.

DPPH Free Radical-Scavenging Activity The free radical-scavenging activities of the test compounds were examined based on their ability to bleach the stable radical 2,2-diprenyl-1-picrylhydrazyl (DPPH) as described by Blois.[27)] This assay has often been used to estimate the antiradical activity of antioxidants. Because of its odd electron, DPPH gives a strong absorption band at 517 nm in visible spectroscopy. DPPH was dissolved in methanol to give a 100 μM solution. To 1.0 ml of the methanolic solution of DPPH was added 0.1 ml of the test compounds dissolved in DMSO. Absorbance at 517 nm was determined after 30 min at room temperature and the scavenging activity was 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 was calculated using the equation:

$$\text{radical scavenging activity} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

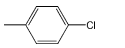
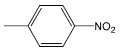
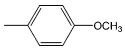
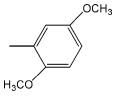
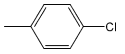
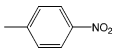
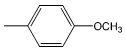
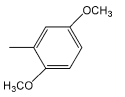
RESULTS AND DISCUSSION

In the present study, we have investigated the antioxidant activity of the synthesized compounds on lipid peroxidation, superoxide anion radical formation, and DPPH radical-scavenging activity. The inhibitory effects of synthesized compounds on lipid peroxidation are presented in Table 1. The results showed that none of the synthesized compounds had a marked inhibitory effect on malondialdehyde formation. Moreover, two (**IIa**, **c**) had slightly stimulatory effects on LP levels. The scavenging activities of superoxide anion radicals are presented in Table 2. The results showed that all synthesized compounds at a concentration of 10^{-3} M had superoxide anion-scavenging activity, and the scavenging rates were in the range of 7—98%. The most active compounds were found **Ia**—**d**, and **IIa** at a concentration of 10^{-3} M. They inhibited superoxide anion formation in the range of 94—98%, which was greater than the inhibitory rate of BHT (48%) and SOD (89%). Substitutions on the A ring instead of the B ring of the flavone ring system play a role in radical-scavenging activities. Compounds **Ic**, **IIa**, and **IIb** had a biphasic effect on superoxide anion formation. These compounds induced superoxide anion formation at a concentration of 10^{-4} M, whereas they inhibited it at 10^{-3} M. This differing effect was also reported in the literature: an increase in superoxide anion formation may occur at lower concentrations, whereas inhibition results at higher concentrations.[28,29)]

As seen in Table 2, compound **Id** had very potent DPPH-scavenging activity (95%), followed by compound **IIb** (37%). Compound **Id** also had the highest scavenging activity among all the compounds tested.

In the present study, the effects of the compounds on superoxide radical-scavenging activity, lipid peroxidation, and

Table 1. Effects of the Tested Compounds on Liver LP Levels[a)]

Compound	Ar	Concentration in incubation medium (M)	LP (nmol MDA/mg protein)	% of Control
Control[b)]			16.25±1.45	100
Ia		10^{-3}	16.05±1.16	99
Ib		—	—	—
Ic		—	—	—
Id		10^{-3}	15.85±2.62	97
IIa		10^{-3}	21.02±1.77	129
IIb		10^{-3}	17.70±1.18	109
IIc		10^{-3}	19.15±4.97	118
IId		10^{-3}	14.82±1.15	91
BHT		10^{-3}	5.68±0.22	35

a) Each value represents the mean±S.D. of 2—4 independent experiments. *b*) Dimethylsulfoxide only, control for compounds. —, Not tested.

Table 2. Effects of the Tested Compounds on Superoxide Anion Production and Reduction of DPPH Radical[a)]

Compound	Concentration in incubation medium (M)	Superoxide anion ($O_2^{\cdot-}$) production (% of control)	DPPH free radical-scavenging activity (% of control)
Control[b)]		100	100
Ia	10^{-4}	100±8	105±1
	10^{-3}	6±1	95±1
Ib	10^{-4}	105±5	—
	10^{-3}	4±1	
Ic	10^{-4}	115±5	—
	10^{-3}	6±1	
Id	10^{-4}	77±8	70±3
	10^{-3}	2±1	5±1
IIa	10^{-4}	128±5	99±2
	10^{-3}	6±1	102±1
IIb	10^{-4}	125±5	87±2
	10^{-3}	93±3	63±1
IIc	10^{-4}	103±7	100±1
	10^{-3}	86±6	98±1
IId	10^{-4}	99±4	108±2
	10^{-3}	75±3	96±1
Control[c)]		100±2	
SOD	30 IU	24±2	
	45 IU	11±1	
BHT	10^{-4}	103±3	31±1
	10^{-3}	52±6	9±1

a) Each value represents the mean±S.D. of 2—4 independent experiments. *b*) Dimethylsulfoxide only, control for compounds. *c*) Distilled water, control for SOD. —, Not tested.

DPPH free radical-scavenging activity were variable. Such contradictory results have been reported previously.[30—32)] Therefore the observation of different effects of synthetic compounds on LP levels, superoxide anion radical formation, and DPPH free radical-scavenging activity is not surprising since the mechanisms of production of oxidative stress using these methods are different.[33—35)]

Superoxide anion radicals have been implicated in several pathophysiologic processes due to their transformation into more reactive species such as hydroxyl radicals that initiate lipid peroxidation. Superoxide has also been observed to initiate lipid peroxidation directly.[36)] It has also been reported that the antioxidant properties of some herbal plants are effective mainly *via* scavenging of superoxide anion radicals.[13,37)] Thus the greater inhibitory effects of the synthetic compounds on superoxide anion formation noted here possibly render them promising antioxidants.

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