*Biochemical Pharmacology*, Vol. 42, No. 6, pp. 1177–1179, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00


# PHENOLIC COMPOUNDS AND AN ANALOG AS SUPEROXIDE ANION SCAVENGERS AND ANTIOXIDANTS

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(*Received* 7 *January* 1991; *accepted* 20 *March* 1991)

**Abstract**—Five phenolic compounds and pyridoxine were studied for their activities as both scavengers of superoxide anions and inhibitors of lipid peroxidation. The superoxide anions were generated in a phenazin methosulfate–NADH system and were assayed by the reduction of nitroblue tetrazolium. The superoxide anion scavenging activities of verbascoside and alizarin yellow R were the strongest, followed by those of caffeic acid and phloridzin; vanillin and pyridoxine exhibited the weakest activity. The concentration values yielding 50% inhibition of lipid peroxidation in mouse liver microsomes were $10^{-5}$ M for verbascoside, $10^{-4}$ M for alizarin yellow R and caffeic acid, and $10^{-3}$ M for phloridzin; vanillin and pyridoxine had almost no antioxidative activity. The inhibition of lipid peroxidation by these individual compounds was much weaker than by butylated hydroxyanisole. The results showed that phenolic compounds and pyridoxine have more than one mechanism of action for free radicals and are able to suppress free radical processes at two stages: the formation of superoxide anions and the production of lipid peroxides.

Oxygen radicals and other oxygen-derived species including lipid peroxides have been suggested as potentially being important causative agents of aging and several human diseases [1], including cancer, multiple sclerosis, Parkinson's disease, autoimmune disease, ischemia, anemia, senile dementia and asbestosis. Phenolic compounds have been studied extensively and reported to possess both antiradical and antioxidation properties. They are expected to be promising potential drugs for combating "free radical" pathologies. Many synthetic drugs have revealed side effects. A better strategy is to look for natural substances, in our surroundings, with strong pharmacological action and less cytotoxicity. We have reported on the antioxidative and scavenging activities of seven natural hydroxylated flavonoids [2]. In the present study, we examined five phenolic compounds: caffeic acid, phloridzin, vanillin, alizarin yellow R, and verbascoside and one analog, pyridoxine, for their scavenging activities of superoxide anions and their antioxidative activities. Caffeic acid has been reported to have antiviral activity [3, 4], and vanillin to have strong antiepileptic effects [5]. Pyridoxine is vitamin $B_6$. Phloridzin can induce glycosuria [6]. Verbascoside was extracted from *Pedicularis striata* [7], which is used in folk medicine as in ascardiac tonic for the treatment of collapse, exhaustion and senility.

## MATERIALS AND METHODS

*Materials.* Phloridzin dihydrate was purchased from Carl Roth KG and pyridoxine hydrochloride from the Shanghai Chemical Company. Vanillin was produced by the Shengyang Third Chemical Co., caffeic acid by the Shanghai First Chemical Co., and alizarin yellow R by the Huangyan Shengli Chemical Co. Verbascoside was a gift from Professor Jia [7]. Alizarin yellow R was dissolved in deionized water. Butylated hydroxyanisole (BHA) was dissolved in pH 7.4 potassium phosphate-buffered saline (PBS) containing a little ethanol. The other reagents were dissolved in pH 8.0 Tris–HCl buffer or in pH 7.4 PBS.

*Lipid peroxidation in mouse liver microsomes.* Several Kunming mice weighing 18–22 g were killed by cervical dislocation. Their livers were quickly removed and homogenized followed by a 20-min 9810 *g* centrifugation at 4°. The supernatant was centrifuged at 95,850 *g* for 40 min at 4°. The pellet was stored at −20° or resuspended in ice-cold 0.15 M KCl. Protein concentration was measured by the method of Lowry *et al.* [8]. The sample consisted of 0.2 M PBS, pH 7.4, 0.1 mM ascorbate, 10 μM $FeSO_4$, and 240–400 μg of microsomal protein in a final volume of 1.0 mL; the reaction was initiated by the addition of ascorbate. After incubating in a water bath at 37° for 1 hr with shaking, the reaction was stopped by 1.0 mL of trichloroacetic acid (TCA) (20%), and 1.5 mL thiobarbituric acid (TBA) (0.67%) was added. Then 0.1 mL of different concentrations of the tested compounds dissolved in PBS, except for alizarin yellow R, was added to inhibit microsomal lipid peroxidation; 0.1 mL PBS was used for the control. TCA was added before incubation for the blank sample. Lipid peroxidation was assayed by the TBA test at room temperature and read at 532 nm against a blank sample. The percent inhibition of the generation of malonaldehyde (MDA) was plotted as a function of the concentration of phenols and then used to calculate the concentrations yielding 50% inhibition ($IC_{50}$).

*Generation of superoxide anions.* Superoxide anions were generated in samples that contained

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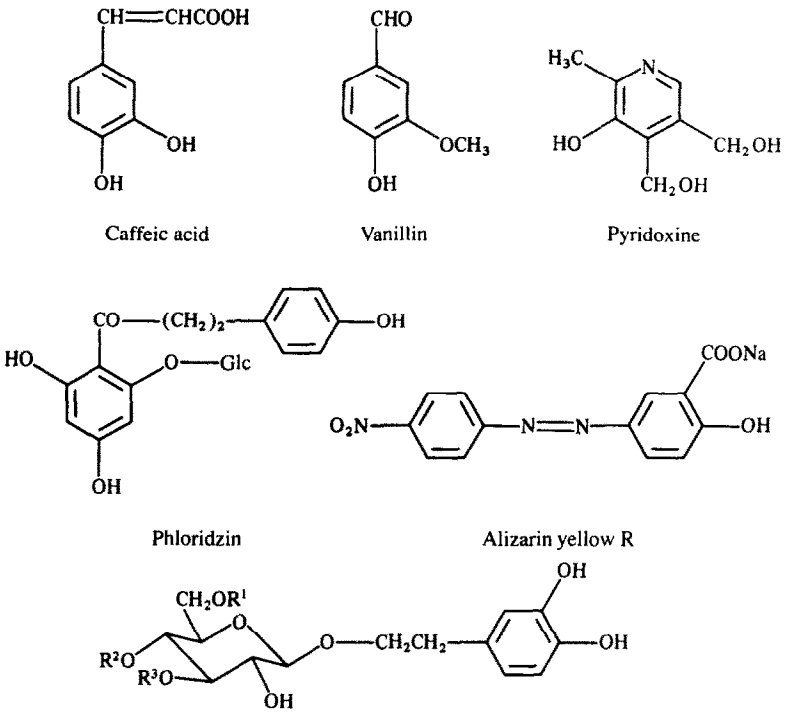


Fig. 1.

Table 1. Half-inhibition concentrations ($IC_{50}$, $\mu M$) of the tested compounds on superoxide anions ($O_2^{\cdot-}$) and on lipid peroxidation (LP)

| Compounds | $IC_{50}$ ($\mu M$) | |
|---|---|---|
| | Scavenging of $O_2^{\cdot-}$ | Inhibition of LP |
| Verbascoside | 51.8 ± 12.5 | 65.3 ± 9.6 |
| Caffeic acid | 258 ± 66 | 438 ± 51 |
| Phloridzin | 645 ± 262 | 1,040 ± 140 |
| Alizarin yellow R | 63.1 ± 5.8 | 299 ± 11 |
| Vanillin | 2,945 ± 247 | Inactive at 3,285 |
| Pyridoxine | 3,945 ± 1,421 | Inactive at 29,120 |
| Superoxide dismutase | 1.69 unit | |
| Butylated hydroxyanisole | | 1.63 ± 0.15 |

Values are means ± SD, N = 3.

73 $\mu M$ NADH (Sigma), 50 $\mu M$ nitroblue tetrazolium (Shanghai Qianjing Chemical Co.) and 15 $\mu M$ phenazine methosulfate (Shanghai Biochemical Co.). All tested compounds except for alizarin yellow R were dissolved in 0.016 M Tris–HCl buffer, pH 8.0. The blank sample contained no NADH. After absorbance at 560 nm reached a maximum and became stable in the control tube, the color was read [9]. Every test had three replicates. The $IC_{50}$ values of the generation of superoxide anion by the tested compounds were calculated as described above.

## RESULTS AND DISCUSSION

The $IC_{50}$ values of the phenolic compounds and an analog on superoxide anion ($O_2^{\cdot-}$) and malonaldehyde (MDA) are presented in Table 1. Both their scavenger activities for $O_2^{\cdot-}$ and their inhibition of lipid peroxidation were concentration dependent.

The inhibition of lipid peroxidation by the six compounds was much weaker than that by butylated hydroxyanisole. Verbascoside, alizarin yellow R, caffeic acid and phloridzin were effective in $O_2^{\cdot-}$ scavenging and in inhibition of lipid peroxidation as well. Since vanillin and pyridoxine demonstrated weak scavenging activity of $O_2^{\cdot-}$, they are inefficient antioxidants. This does not mean that there was a correlation between the scavenging activities for $O_2^{\cdot-}$ and antioxidative activity. Because the rank of antiradical and antioxidative activities for an antioxidant depends on what oxidant stress is imposed, what target is used to detect oxidant stress, and what method is adopted to measure antioxidative activity, different experimental conditions will produce different "hierarchies" of antioxidants [10].

Pyridoxine is not a phenol but a pyridine, which still possesses a $p$-$\pi$ conjugated hydroxyl group as do phenolic compounds, and still was able, even weakly, to scavenge $O_2^{\cdot-}$. The results show that phenolic compounds and an analog have more than one mechanism of action for free radicals and are able to suppress free radical processes at two stages: the formation of $O_2^{\cdot-}$ and the production of lipid peroxides.

*Acknowledgement*—This project was supported by the National Natural Science Foundation of China.

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