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Scavenging Effects of Phenylpropanoid Glycosides from *Pedicularis* on Superoxide Anion and Hydroxyl Radical by the Spin Trapping Method(95)02255-4

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ABSTRACT. The scavenging activities of six phenylpropanoid glycosides, i.e. leucosceptoside A and martynoside isolated from *Pedicularis alashanica* Maxim, verbascoside, and pedicullariosides A, M and N isolated from *Pedicularis striata* Pall ssp. *arachnoidea* Franch Tsoong, on superoxide anion and hydroxyl radicals have been studied by the spin trapping method. The results demonstrated that the number of phenolic hydroxyl groups in the structures of the phenylpropanoid glycosides is related to their scavenging activities. The scavenging effects of the phenylpropanoid glycosides possessing two *o*-dihydroxyl groups were stronger than the effects of the compounds possessing two *o*-hydroxy-methoxy groups. BIOCHEM PHARMACOL 51;5:687–691, 1996.

KEY WORDS. phenylpropanoid glycoside; superoxide; hydroxyl radical; free radical; scavenging effect; iron reduction

Active oxygen species have been proposed to play an important role in a wide variety of pathologies, such as ischemia, inflammation, cancer, and aging [1]. Free radical scavengers have been used to protect biological molecules from oxygen radical damage [2]. Several phenolic compounds have been proven to be effective scavengers of superoxide anion or hydroxyl radicals [3]. Many species of *Pedicularis* are used in traditional Chinese medicine as tonics for the treatment of general debility, collapse, exhaustion, spontaneous sweating, seminal emission and senility, and to invigorate the mind and the circulation of blood [4]; they are usually called "native ginseng" by local inhibitants of the northwestern part of China. The genus *Pedicularis* contains PPG.‖ PPG extracted from other plants have been reported to have antibiotic activities [5], to inhibit platelet aggregation [6], and to inhibit leukotriene B_4 formation [7]. We have found recently that the PPG isolated from three species of *Pedicularis* can function as chain-breaking antioxidants to inhibit the peroxidation of mouse liver microsome [8], the auto-oxidation of linoleic acid in micelles [9], and the hemolysis of erythrocytes induced by radicals [10]. We also investigated the reaction of hydroxyl radical with the PPG by using the pulse radiolysis technique [11]. To examine the relationship between the antioxidative behavior and the structures of the PPG, we studied the scavenging effects of six PPG with similar structures on superoxide anion and hydroxyl radical by the spin trapping method, which has been used extensively to detect oxygen radicals [12], with DMPO as a spin trap. We also studied the abilities of these six PPG to reduce iron (III) to iron (II) by the potassium permaganate oxidation method, which has been used often to detect iron (II).

MATERIALS AND METHODS

DMPO, xanthine, xanthine oxidase, and DETAPAC were purchased from the Sigma Chemical Co. DMPO was purified by charcoal decolorization before use. Verbascoside and pedicullariosides A, M and N were extracted and purified from *Pedicularis striata* Pall ssp. *archnoidea* Franch Tsoong [13], and leucosceptoside A and martynoside from *Pedicularis alashanica* Maxim presented by Jia and Gao [13]. All PPG were dissolved in phosphate buffer solution except for pedicullarioside N and martynoside, which were dissolved with the help of dimethyl sulfoxide. All other chemicals used were of analytical grade. The structures of PPG are shown in Fig. 1.

Superoxide anion was generated from a xanthine/xanthine oxidase system (X/XO) containing 5 mM phosphate buffer, pH 7.4, 1.6 mM DETAPAC, 5.9 mM xanthine, and 0.1 U/mL xanthine oxidase. DMPO was used as a spin trapping agent at a final concentration of 80 mM. PPG were added before the addition of xanthine oxidase. The reaction mixture was transferred immediately to a capillary. EPR measurements were carried out within 5 min after mixing. To detect whether the PPG tested inhibited the X/XO superoxide-generating system

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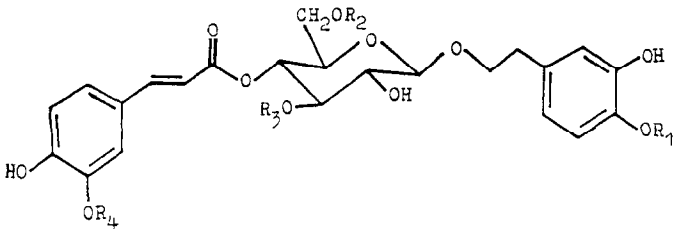
‖ *Abbreviations:* PPG, phenylpropanoid glycosides; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; and DETAPAC, diethylenetriaminepentaacetic acid.



Verbascoside	$R_1 = R_2 = R_3 = R_4 = H$			
Pedicularioside A	$R_1 = R_4 = H$	R_2 = rhamnosyl	R_3 = apiosyl	
Pedicularioside M	$R_1 = H$	R_2 = rhamnosyl	R_3 = apiosyl	$R_4 = CH_3$
Leucosceptoside A	$R_1 = R_2 = H$	R_3 = rhamnosyl	$R_4 = CH_3$	
Martynoside	$R_1 = R_4 = CH_3$	$R_2 = H$	R_3 = rhamnosyl	
Pedicularioside N	$R_1 = R_4 = CH_3$	R_2 = rhamnosyl	R_3 = apiosyl	

FIG. 1. Structures of PPG used in this paper.

in the concentration ranges used, the activity of xanthine oxidase was expressed as consumption of O_2 (mg/L • min) measured by an oxygen electrode at constant temperature (25 ± 0.2°).

Fenton's reaction was used to generate hydroxyl radical. The reaction mixture consisted of 3.1 mM phosphate buffer, pH 7.4, 0.39 M H_2O_2, 0.14 mM ferrous ammonium sulfate, and 89 mM DMPO. PPG were added before the addition of H_2O_2. EPR spectra were measured within 5 min after mixing.

When detecting the scavenging effects on superoxide anion and hydroxyl radical, the PPG were added at different concentrations to the above two systems. EPR spectra were recorded at room temperature using a Brucker ESP-300 spectrometer operating at 9.42 GHz with 100 KHz field modulation. A scan range of 20 mT, a modulation amplitude of 0.28 mT, a microwave power of 10 mW, a receiver gain of 5×10^5, time constants of 160 msec, and a scan time of 200 sec were used for all spectra.

The reduction mixture consisted of 3.1 mM phosphate buffer, pH 7.4, 0.14 mM ferric chloride, and the corresponding PPG. After reaction for 10 min, sulfuric acid and phosphoric acid were added to final concentrations of 1 and 0.2 mM, respectively. The total volume was 3 mL. Then 0.01 N potassium permanganate (which had been standardized with sodium oxalate) was used to titrate iron(II) in the above system. The iron(II) normality was calculated as follows:

$$N = 0.01 \times V_0/3$$

where V_0 is the consumption volume of the potassium permanganate for titrating. The percent reduction (R) (see Table 3) of PPG on iron(III) was calculated as:

$$R = [N/0.14] \times 10^3 \times 100\%$$

where 0.14×10^{-3} M is the iron(III) concentration in the reduction mixture.

The scavenging activities on superoxide anions were expressed as SC_{50}—the concentration of PPG at which 50% of the superoxide anions could be scavenged.

RESULTS AND DISCUSSION

Superoxide anions generated from the xanthine/xanthine oxidase system were trapped by DMPO instantly; the corresponding EPR spectrum is shown in Fig. 2A, while Fig. 2B–D are the spectra in the presence of verbascoside. According to former reports [12, 13], spectrum A in Fig. 2 is a typical spectrum of the spin adduct DMPO-OO(H) with a 12-line hyperfine splitting. The splitting constants are: A_N = 1.42 mT, A_H^β = 1.17 mT, and A_H^γ = 0.12 mT. The percent radical scavenging (S) was calculated as follows:

$$S = [(h_o - h_x)/h_o] \times 100\% \quad (1)$$

where h_o is the height of the first peak in the control spectrum, and h_x is the height of the first peak in spectra B–D.

According to the S-concentration curve, the SC_{50} of PPG on superoxide anion can be calculated (Table 1).

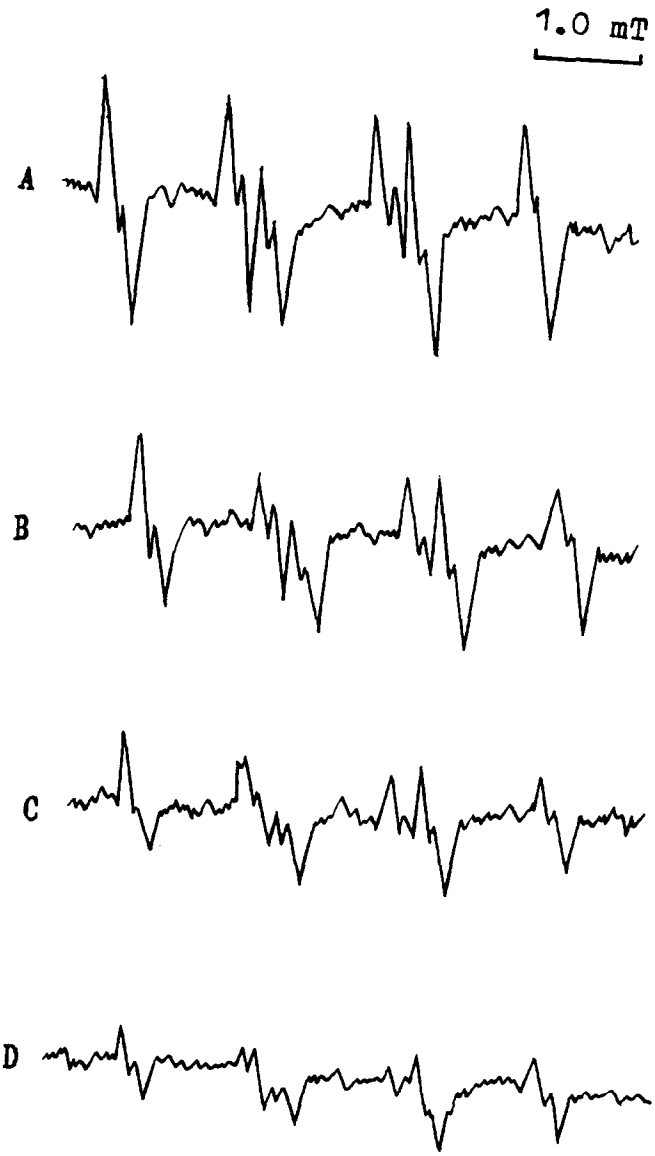


FIG. 2. Scavenging effect of verbascoside on superoxide anion. EPR spectra were obtained from a xanthine/xanthine oxidase system containing 5 mM phosphate buffer, pH 7.4, 1.6 mM DETAPAC, 5.9 mM xanthine, 0.1 U/mL xanthine oxidase and 80 mM DMPO. (A) control. (B–D) Same as A, except that verbascoside was added at final concentrations of 0.014, 0.048 and 0.138 mM, respectively.

TABLE 1. Half-scavenging concentrations (SC_{50}) of PPG on superoxide anion

Phenylpropanoid glycosides	No. of Ph-OH	SC_{50} (mM)
Verbascoside (3)*	4	0.063
Pedicularioside A (3)	4	0.132
Pedicularioside M (3)	3	0.161
Leucosceptoside A (2)	3	0.294
Martynoside (4)	2	1.070
Pedicularioside N (3)	2	1.188

*Number of experiments is given in parentheses.

Table 2 showed that verbascoside and pedicularioside A cannot inhibit the activity of xanthine oxidase in the concentration ranges used in the above superoxide-generating system. The other four PPG (not determined because the supply of chemicals was exhausted) probably had the same tendency because all PPG have similar structures. Therefore, the decline of the EPR signal in the presence of PPG is due to their superoxide-scavenging activity rather than the xanthine oxidase being inhibited by PPG.

In Fenton's reaction, hydroxyl radicals are produced from the reduction of hydrogen peroxide. EPR spectra derived from this system are shown in Fig. 3; spectrum A, consisting of four lines with an intensity ratio 1:2:2:1, is a typical spectrum of the spin adduct DMPO-OH [12, 13]. The splitting constants were A_n = 1.49 mT and A_H^β = 1.49 mT.

Similarly, the percent scavenging (Table 3) of PPG on hydroxyl radicals was calculated from equation (1), where h_0 is the height of the second peak in the control spectrum, and h_x is the height of the second peak in spectra B–D.

By comparing the results shown in Tables 1 and 3, it can be found that the percent scavenging of the PPG on superoxide and hydroxyl radicals is affected by their structures and concentrations. Verbascoside and pedicularioside A, which possess four phenolic hydroxyl groups, are the most effective scavengers of superoxide and hydroxyl radical. Martynoside and pedicularioside N, with two phenolic hydroxyl groups, are relatively weaker scavengers of superoxide and hydroxyl radical. The scavenging effects of leucosceptoside A and pedicularioside M, with three phenolic hydroxyl groups, rank in the middle. Therefore, the number of phenolic hydroxyl groups in the structures of the PPG may play an important role in their scavenging activities. This result is in agreement with our previous studies about the antioxidant activities of PPG [8–11]. In addition, in most cases, the scavenging effects of PPG decrease, more or less, with a decrease of the concentration used. However, pedicularioside M and N and martynoside exhibited a scavenging effect on hydroxyl radical at high concentrations, but promoted the generation of hydroxyl radical at middle or lower concentrations. It has been reported that many antioxidants can have pro-oxidant actions [14]. Some phenolic chain-breaking antioxidants are capable of binding iron(III) and reducing it to iron(II), which can result in a pro-oxidant action under certain circumstances. The balance between anti- and pro-oxidant may be a function of their concentrations; it is most common for the antioxidant effects to dominate at high concentrations and the pro-oxidant effects at lower concentrations. According to our results, it can be proposed that the pro-oxidant actions of pedicularioside M and N and martynoside in Fenton's system stem from their participation in redox cycles and acceleration of the produc-

TABLE 2. Effect of PPG on xanthine oxidase activity

	Concn of PPG (mM)	O_2 consumption (mg/L · min)
X/XO		1.99 ± 0.09
X/XO + verbascoside	2.5	1.94 ± 0.11
	0.5	1.97 ± 0.12
X/XO + pedicularioside A	2.5	1.90 ± 0.08
	0.5	2.04 ± 0.15

Values are means ± SD of triplicate determinations. X/XO = xanthine/xanthine oxidase.

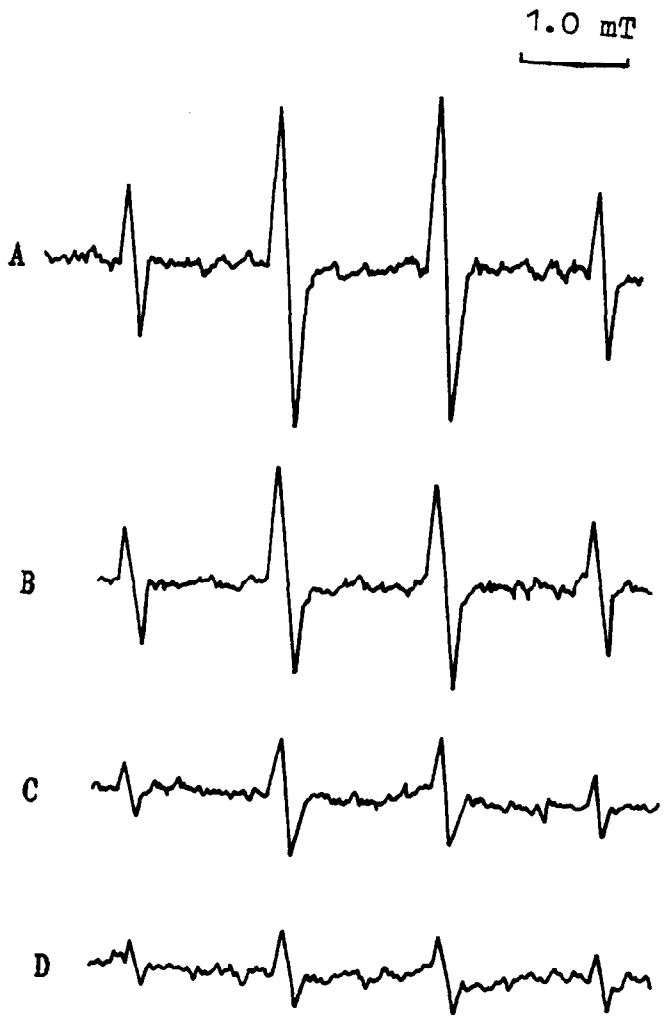


FIG. 3. Scavenging effect of verbascoside on hydroxyl radical. EPR spectra were obtained from the system of 3.1 mM phosphate buffer, pH 7.4, 0.39 M H_2O_2, 0.14 mM ferrous ammonium sulfate, and 89 mM DMPO. (A) control. (B–D) Same as A, except that verbascoside was added at final concentrations of 0.15, 0.54 and 1.53 mM, respectively.

TABLE 3. Percentage of scavenging of PPG on hydroxyl radical and percent reduction of PPG on iron (III)

Phenylpropanoid glycosides	No. of Ph-OH	Concn (mM)	% Radical scavenging	SC_{50} (mM)	% Iron reduction*
Verbascoside (3)†	4	1.53	79.4 ± 2.1		
		0.54	55.7 ± 1.1	0.434	NR‡
		0.15	40.2 ± 2.1		
		0.054	NS§		
Pedicularioside A (3)	4	1.50	77.8 ± 2.8		
		0.52	67.5 ± 1.4	0.518	NR
		0.15	45.9 ± 4.2		
		0.052	13.9 ± 8.3		
Pedicularioside M (5)	3	1.73	55.1 ± 1.7		23.57 ± 1.43
		0.61	−88.7 ± 18.2		14.93 ± 2.14
		0.17	26.2 ± 1.2		0.23 ± 0.4
		0.061	NS		NR
Leucosceptoside A (4)	3	1.57	17.5 ± 4.1		17.86 ± 2.86
		0.55	27.8 ± 2.1		4.29 ± 0.71
		0.157	18.6 ± 9.3		3.64 ± 0.07
		0.055	25.8 ± 6.2		NR
Martynoside (3)	2	1.50	28.3 ± 1.3		17.01 ± 2.14
		0.52	13.9 ± 7.9		6.76 ± 3.57
		0.15	−33.2 ± 7.9		5.25 ± 0.14
Pedicularioside N (3)	2	1.56	33.0 ± 1.2		22.56 ± 0.57
		0.55	21.6 ± 5.7		10.86 ± 0.36
		0.156	−30.7 ± 10.2		6.23 ± 0.71
		0.055	NS		NR

Values are means ± SD.
* Means of triplicate determinations.
† Number of experiments is given in parentheses.
‡ NR, no reducing effect was observed.
§ NS, no scavenging effect was observed.

tion of hydroxyl radical. However, verbascoside and pedicularioside A were not able to reduce iron(III) to iron(II); thus, both PPG could not exhibit pro-oxidation via Fenton's reaction. With regard to leucosceptoside A, why it reduces iron(III) but does not exhibit pro-oxidation activity remains unclear (Table 3).

It has been demonstrated that polyphenol compounds are attacked by superoxide or hydroxyl radical predominantly at the *o*-dihydroxy site [15]; the semiquinone radicals formed from the reaction of the *o*-dihydroxy group with superoxide or hydroxyl radical are quite stable, probably because of the presence of hydrogen bonding. There are two *o*-dihydroxy structures in each verbascoside or pedicularioside A molecule, which possess four phenolic hydroxyl groups, so they react with superoxide or hydroxyl radical more readily, and exhibit strong scavenging effects. However, for a phenolic hydroxyl group with a methoxy substituent at the ortho position, the aroxyl radical derived from its reaction with superoxide or hydroxyl radical is less stable owing to the absence of intramolecular hydrogen bonding. There are two such *o*-hydroxymethoxy structures instead of *o*-dihydroxy in each martynoside or pedicularioside N molecule, so the reaction for them is relatively slow, and the scavenging effects observed are weak.

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