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Antioxidant properties of components extracted from puccoon (*Lithospermum erythrorhizon* Sieb. et Zucc.)

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Abstract

The petroleum ether extract of puccoon has been separated with thin-layer chromatography (TLC) and three compounds have been isolated. The structures of the compounds have been identified by spectroscopic methods as β,β-dimethyl-acrylshikonin, acetylshikonin and shikonin. Their antioxidant properties in lard have been tested with the oxidative stability instrument (OSI). The raw extracts and pure compounds all have obvious antioxidant activity, and all have some synergistic effects with D,L-α-tocopherol (Ve) and butylatedhydroxytoluene (BHT). They all show antioxidant properties in lard containing Fe^{3+}, and all show synergistic effects with Ve and citric acid (CA) with different degrees. In the concentration range of 0.01–0.06%, antioxidant activity of Ve and BHT, on OSI at 100°C, increases with increase of concentration, but much less than acetylshikonin, shikonin and β,β-dimethyl-acrylshikonin.

Keywords: Puccoon (*Lithospermum erythrorhizon* Sieb. et Zucc.); Antioxidant activity; β,β-dimethyl-acrylshikonin; Acetylshikonin; Shikonin

1. Introduction

Puccoon (*Lithospermum erythrorhizon* Sieb. et Zucc.) was one of the strong antioxidant herbs screened from more than 700 Chinese medicinal plants (Weng, Ren et al., 1998). The antioxidant properties of raw extracts from puccoon were investigated (Liu, Weng et al., 1998). Puccoon is a traditional Chinese medicinal, perennial herb. It has effects in staunching bleeding, anti-inflammation, antibacteria and antivirus(Zhao, 1997). The crude pigment from puccoon can be used for treating diseases of psoriasis, plane wart, anaphylactic purpura, and infective hepatitis (Gao, 1986; Liu et al., 1998). Puccoon pigment is a kind of naphthoquinone, usually exists as ester forms, and plays an electron transportation role, accelerating or interfering with some biochemical reaction processes, and exhibiting many kinds of biological activity. In this paper, three compounds from puccoon have been separated by thin-layer chromatography and identified by mass, PMR, IR and UV spectroscopic methods. Their antioxidant activities were investigated. Up to now, there have been many reports about the medicinal effects, and isolation and identification of compounds from puccoon, but no other reports about their antioxidant activity, except one paper has reported the antagonism of shikonin to oxidative injury of liver (Zhou, Zhao, Chen, Wang & Wang, 1996). So it is possible that some new functions and uses of puccoon may be found by studying its antioxidant activity.

2. Materials and methods

2.1. Materials and reagents

Lard was rendered from fresh pig fat, purchased from Yantai Slaughter House; Ve (95%) was purchased from Merk Co., Germany; Puccoon was purchased from a traditional Chinese medicine shop in Yantai. BHT and citric acid (food grade) were purchased from Guangzhou Food Additive Shop.

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2.2. Isolation and purification of compounds from puccoon

Dry, clean puccoon roots (500 g) were powdered, then extracted by a Soxhlet extractor with petroleum ether (60–90°C) for 10 days. The solvents were removed with a rotary evaporator, then dried in a vacuum oven at 70°C for 5 h; finally 20 g purplish-red and grease-like extract was obtained.

Preparative TLC plates of silica gel G were used to isolate the compounds. The developing solvent system was petroleum ether–acetone–chloroform–acetic acid (220:7.2:4.8:1.2). Six bands were separated reasonably well on TLC plates. The R_f values of the bands were 0.34 (violet), 0.253 (violet), 0.194 (violet), 0.176 (brown), 0.118 (brown) and 0.080 (lilac). Every band was collected, and then acetone was used to elute the compounds. Bands 1–3 were the main compounds whose structures were elucidated by UV, IR, MS and PMR spectra, and mp. The compounds were coded as I, II and III. UV spectra were recorded with a Hitachi UV-3400 spectrophotometer and methanol was used as solvent. The IR spectra were recorded with a Secord 75 IR spectrophotometer and the discs were made with KBr. The mass spectra were recorded with an Incon 50 mass spectroscopic instrument. The PMR were recorded with a Cameca RMN-250 NMR spectroscopic instrument; $CDCl_3$ was used as a solvent and TMS was used as internal standard. A microscopic melting point detector was used to determine the melting points. The spectral data of three compounds were listed in Table 1.

The components in bands 4–6 could not further be investigated since their quantity was limited.

The antioxidant activity of the three compounds was studied on the oxidative stability instrument at 100°C with bubbling air (20 l/h).

3. Results and discussion

Three compounds from puccoon, β,β-dimethylacrylshikonin (I), acetylshikonin (II)and shikonin (III) had identical ultraviolet-visible absorption and four peaks at about 275, 485, 520, 565 nm. This meant that they had similar conjugation systems. All showed 3100–3500 cm^{-1} absorption in the IR spectrum, but the peaks were broad, which indicated that the compounds contained hydroxy groups, and hydrogen bonds existed; strong absorption at about 1630 cm^{-1} in the IR spectra, meant that they all had quinone structures. β,β-Dimethylacrylshikonin (I), acetylshikonin (II) had strong absorptions at about 1740 cm^{-1}. This demonstrated that these two compounds had ester groups.

All three compounds had two peaks at about 12.5 ppm (2OH), 2 peaks at about 7.2 ppm (2 aromatic H), and 1 peak at 7.0 ppm (1 quinone ring H) in the PMR spectra. All these data indicated that they had the same component structure, 5,8-dihydroxy-1,4-naphthalenedione. Also, in the PMR spectra, all three compounds had two singlet peaks at about 1.6 ppm, which indicated two methyl groups with very slightly different chemical shifts, one multiplet peak at 2.5 ppm, and a methylene

Table 1
Spectral data of the compounds isolated from puccoon

MS (m/z) (relative abundance)	PMR chemical shift (ppm) (no. of peaks and H)	IR (wavenumber) (cm^{-1})	Uv (nm) (absA)	Mp (°C)
I. Purpish-red pellet crystal, β,β-dimethyl-acrylshikonin				
370 (M, 1.9), 271 (4)	1.59 (s,CH_3), 1.68 (s,CH_3)	3425, 3075, 2980	220 (2.43)	116–117
270 (23), 255 (6.9)	1.93 (s,CH_3), 2.17 (s, CH_3)	2925, 1730, 1660	278 (0.55)	
189 (1.1), 83 (100)	2.56 (m,2H), 5.13 (t, 1H)	1635, 1595, 1465	486 (0.33)	
69 (4.2), 55 (2.8)	5.78 (s,1H), 6.02 (t, 1H)	1280, 1240, 890	562 (0.28)	
	6.97 (s,1H), 7.19 (s,2H)	790	615 (0.12)	
	12.42 (s,1H), 12.6 (s,1H)			
II. Puce needle crystal, acetyl shikonin				
300 (M,1.1)	1.59 (s,CH_3), 1.68 (s,CH_3)	3470, 3080, 2995	215 (3.2)	106–107
271 (11), 270 (64)	2.59 (s, CH_3), 2.52 (m, 2H)	2920, 1745, 1625	275 (0.84)	
255 (48.6), 219(40)	5.10 (t, 1H), 6.98 (s, 1H)	1595,1470, 860	485 (0.55)	
69 (46.4), 43 (100)	7.19 (s, 2H), 12.49 (s, 1H)	800	522 (0.63)	
	12.5 (s,1H)		561 (0.39)	
III. Puce needle crystal, Shikonin				
288 (M,1)	1.68 (s, CH_3), 1.78 (s, CH_3)	3400, 3250, 2985	233 (4.36)	145–147
270 (2), 255 (5)	2.49 (m, 2H), 4.92 (m, 1H)	2920, 1625, 1595	275 (1.57)	
220 (100), 202 (1.5)	5.22 (t, 1H), 7.17 (s, 1H)	1550,1470, 1220	485 (0.90)	
191 (13), 163 (7.3)	7.19 (s 2H), 12.48 (s, 1H)	1090, 790	518 (0.96)	
69 (63)	12.58 (s, 1H)		574 (0.65),	
			609 (0.16)	

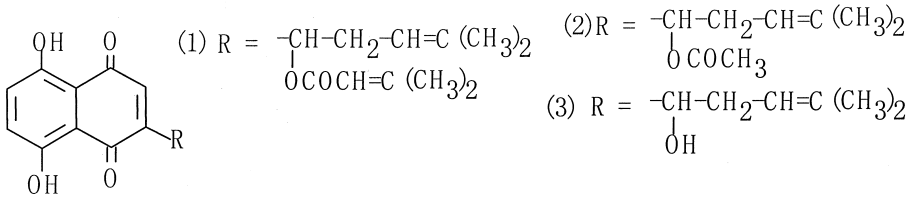

Fig. 1. The structures of: (1) β,β-dimethyl-acrylshikonin (I); (2) acetylshikonin (II); and (3) shikonin (III).

group, triplet peak (1H on double bond carbon) at about 5.1 ppm. β,β-Dimethylacrylshikonin (I) and acetylshikonin (II) had one triplet peak (1H) at 6.02 ppm. But the peak of shikonin (III) was shifted to high field, at 4.92 ppm because of the hydroxy group in compound I and substituted ester group in compounds II and III. This data meant that all three compounds had the group,-$CH(X)CH_2CH{=}C(CH_3)_2$. The mass spectra of all three compounds had 270 *m*/*z* peak which also confirmed that this structure lost XH (Fig. 1).

The PMR spectra of compound I had two singlet peaks at about 2 ppm, which indicated two methyl groups with 0.24 ppm different chemical shifts, and one singlet peak at 5.78 ppm. The molecular weight of this compound, from its mass spectra was 370, the molecular weight of XH lost from the compound was 100 (370–270). The X therefore had to be the group –$OOCCH{=}C(CH_3)_2$. The melting point (mp) of this compound was 116–117°C. The mass, PMR, IR spectra and melting point (mp) agreed well with those reported by Lin, Chai, Wang, Guo, Lu and Xiang (1980) and Liu (1981).

The PMR spectra of the compound II had a singlet peak (methyl group) at 2.16 ppm. The molecular weight of this compound was 330. The molecular weight of XH lost from the compound was 60 (330–270). The X had to be-$OOCCH_3$. The mp of the compound was 106–107°C. The mass, PMR, IR spectra and mp agreed well with those reported by Lin et al. (1980) and Lu, Xiang and Zhi (1983).

The molecular weight of compound III was 288. After loss of water molecule, it was 270. Since the hydroxy group formed a hydrogen bond with the quinone carbonyl oxygen, the chemical shift of H on the quinone ring moved to lower field (0.22 ppm). The mp of the compound was 145–147°C. The mass, PMR, IR spectra and mp agreed well with those reported by Lin et al. (1980) and Lu et al. (1983).

The antioxidant properties of the three compounds, β,β-dimethylacryl-shikonin, acetylshikonin and shikonin, were investigated and compared with those of Ve, BHT, and petroleum extract of puccoon (pp) (Table 2).

The antioxidant activity of β,β-dimethylacryl-shikonin (Pf = 1.33), acetylshikonin (1.95) and shikonin (1.73) was much lower than that of Ve (3.72) and BHT (2.08) at low concentration (0.01%). But the antioxidant activity of the three compounds from puccoon increased significantly with increase of their concentration, nevertheless the Ve increase was very limited. Acetylshikonin had a much stronger antioxidant activity (Pf = 7.04) than Ve (4.59) and BHT (4.26) at the concentration 0.06%. The antioxidant activity (4.95) of shikonin became similar to that of Ve and BHT at the concentration 0.06%.

The antioxidant synergies between Ve, BHT and components from puccoon are compared in Table 3. The three compounds (0.02%) had different antioxidant synergistic effects with Ve and BHT (0.02%) in lard on OSI at 100°C. The antioxidant synergistic effects (SE) were calculated and listed in Table 2 according to the modified calculation formula referring to Weng (1991) and Hudson and Lewis (1981).

$$\mathrm{SE}\% = \frac{\{Pf_{(0.02\%\mathrm{A}+0.02\%\mathrm{B})} - 1/2(Pf_{0.04\%\mathrm{A}+Pf0.04\%\mathrm{B}})\}}{\{1/2(Pf_{0.04\%\mathrm{A}} + Pf_{0.04\%\mathrm{B}})\}} \times 100$$

Table 2
The antioxidant protection factor (Pf) pf various antioxidants in lard on OSI at 100°C[a]

Concentration of additives	0.01%	0.02% Pf[b]	0.04%	0.06%
pp[c]		1.40 ± 0.27	2.61 ± 0.19	
I	1.33 ± 0.21	1.60 ± 0.15	2.05 ± 0.16	2.95 ± 0.37
II	1.95 ± 0.26	2.08 ± 0.15	4.40 ± 0.43	7.04 ± 0.29
III	1.73 ± 0.31	2.46 ± 0.52	2.91 ± 0.15	4.94 ± 0.15
Ve	3.72 ± 0.25	4.04 ± 0.28	4.39 ± 0.16	4.59 ± 0.23
BHT	2.08 ± 0.10	2.98 ± 0.20	3.99 ± 0.52	4.26 ± 0.05

[a] M ± range of values, $n = 2$.
[b] Pf = IP (sample with antioxidant)/IP (control).
[c] PP, petroleum extract of puccoon.

Table 3
Antioxidant synergistic effects (SE) between components from puccoon and BHT or Ve in lard on OSI at 100°C[a]

Additives	Pf	SE%
0.02%pp + 0.02%Ve	4.40 ± 0.12	25.7 ± 3.44
0.02% I + 0.02%Ve	5.84 ± 0.35	81.4 ± 10.84
0.02% II + 0.02%Ve	6.76 ± 0.20	52.7 ± 3.92
0.02% III + 0.02%Ve	8.49 ± 0.06	132.6 ± 1.65
0.02%pp + 0.02%BHT	4.56 ± 0.42	38.2 ± 12.71
0.02% I + 0.02%BHT	3.61 ± 0.12	19.5 ± 4.01
0.02% II + 0.02%BHT	4.19 ± 0.59	0.0 ± 13.93
0.02%III + 0.02%BHT	4.53 ± 0.10	31.3 ± 2.90

[a] M ± range of values, $n = 2$.

Table 4
The antioxidant effects of PP and its three compounds on the stability of lard containing 4 ppm Fe^{3+} [a]

Sample	Pf
0.04% pp	1.99 ± 0.25
0.02% I	1.62 ± 0.27
0.02% II	1.04 ± 0.16
0.02% III	1.07 ± 0.27

[a] (M ± range of values, $n = 2$).

Table 5
Synergistic effects of I, II and III with CA and Ve in lard containing 4 ppm Fe^{3+} [a]

Additives	Pf
0.02% I + 0.02 CA	1.44 ± 0.05
0.02% II + 0.02% CA	2.55 ± 0.08
0.02% III + 0.02% CA	2.30 ± 0.00
0.04% CA	0.93 ± 0.14
0.02% I + 0.02% Ve	2.8 ± 0.63
0.02% II + 0.02% Ve	7.67 ± 0.52
0.02% III + 0.02% Ve	9.39 ± 0.56
0.04% Ve	4.60 ± 0.03

[a] M ± range of values, $n = 2$.

The components from puccoon had much stronger antioxidant synergy with Ve than BHT. Shikonin had a very distinctive synergistic interaction (SE% = 132.6) with Ve. But acetylshikonin had no antioxidant synergistic effect (SE% = 0.0) with BHT (Table 3).

On adding 4 ppm Fe^{3+} to lard, the induction period of the lard decreased from 4.94 to 3 h. Table 4 indicates that 4 ppm Fe^{3+} depressed the antioxidant activity of β,β-dimethyl-acrylshikonin, acetylshikonin and shikonin greatly. It is well known that ferric iron is a very powerful free radical inducer and can efficiently catalyze lipid autoxidation (Gordon & Weng, 1992).

Citric acid (CA) is a good chelator of ferric iron (Weng, 1991). Table 5 shows that 0.02% CA made β,β-dimethyl-acrylshikonin, acetylshikonin, shikonin and Ve effectively act as a primary antioxidant when 4 ppm Fe^{3+} was added to the samples at the same time.

However, 0.02% acetylshikonin and 0.02% shikonin, separately with 0.02% Ve together added to lard containing 4 ppm Fe^{3+} but without CA, demonstrated very strong antioxidant activity. This meant that these two compounds from puccoon have strong ability to chelate ferric iron.

The results of the antioxidant properties (Pf) and synergistic effects (SE) were calculated from IPs. All the IPs were duplicates, and their reproducibility was reasonably good (Tables 2–5).

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