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Antioxidants from a Chinese medicinal herb – *Lithospermum erythrorhizon*

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Abstract

Seven compounds, deoxyshikonin (**1**), β,β-dimethylacrylshikonin (**2**), isobutylshikonin (**3**), shikonin (**4**), 5,8-dihydroxy-2-(1-methoxy-4-methyl-3-pentenyl)-1,4-naphthalenedione (**5**), β-sitosterol (**6**) and a mixture of two caffeic acid esters [**7** (**7a**,**7b**)] were isolated from *Lithospermum erythrorhizon* Sieb et. Zucc. and identified by spectroscopic methods. Among them, compound **5** was isolated from this plant species for the first time. The antioxidant activities of the seven compounds were compared and evaluated through Rancimat method, reducing power and radical scavenging activity. Results showed that, except compound **6**, another 6 compounds all exhibited obvious antioxidant activities against four different methods. Compounds **4** and **7** exerted much more potent antioxidant effects on retarding the lard oxidation than that of BHT and both were found to exhibit strong reducing power. Their antioxidant activities, assessed by Rancimat method and reducing power, decreased in the following order, respectively: compound **7** > **4** > BHT > **2** > **3** > **5** > **1** > **6** and compound **7** > BHT > **4** > **2** ~ **3** ~ **5** > **1** > **6**. In addition, compounds **1**–**5** all exerted very good radical scavenging activities toward $ABTS^{\cdot+}$ but showed moderate inhibition of $DPPH^{\cdot}$, while compound **7** presented as a powerful radical scavenger against both $ABTS^{\cdot+}$ and $DPPH^{\cdot}$. Thus, our results suggested that *L. erythrorhizon* could be a promising rich source of natural antioxidants.

Keywords: *Lithospermum erythrorhizon*; Antioxidant activity; Rancimat; Reducing power; Radical scavenging activity

1. Introduction

In nutritional practice, antioxidants play an important role in lengthening the shelf life of food and reducing nutritional losses and harmful substances formed. The synthetic antioxidants, however, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are now doubted for their safety (Grice, 1986; Wichi, 1988). Thus, attention is now increasingly paid to the development and utilization of more effective and non-toxic antioxidants of natural origin.

A great number of natural medicinal plants have been tested for their antioxidant activities and results have shown that raw extracts or isolated pure compounds from them were more effective antioxidants in vitro than BHT or vitamin E (Gordon & Weng, 1992; Gu & Weng, 2001; Pyo, Lee, Logendrac, & Rosen, 2004). So, medicinal plants can be a potential source of natural antioxidants.

Lithospermum erythrorhizon Sieb. et Zucc. (LE) has been used traditionally as medicine in China and as a dye for staining fabrics and food colorants (Cho, Paik, & Hahn, 1999). It possesses a wide spectrum of wound healing, anti-tumor, anti-fungus, anti-HIV and contraceptive biological activities (China Pharmacopoeia Commission of the Ministry of Public Health, 2000; Sasaki, Yoshizaki, & Abe, 2000; Yamasaki et al., 1993; Zhang, Liao, Xuan, & Guo, 2002). Previous phytochemistry studies reported that this plant

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contains mainly red pigments which were composed of naphthoquinone compounds, such as shikonin and its derivatives (Fu & Xiao, 1986; Hisamichi & Yoshizaki, 1982). Several papers have reported the antioxidant activity of LE. A recent paper describes the isolation of three naphthoquinone compounds from LE and an investigation of their antioxidant activity. It was found that shikonin, β,β-dimethylacrylshikonin and acetylshikonin acted as antioxidants in heated lard, alone or in combination with vitamin E, BHT and citric acid (Weng et al., 2000). Another research group reported that monomeric and polymeric alkannin/shikonin and *Alkanna tinctoria* root extract exerted obvious antioxidant activity in oils and radical scavenging activity against DPPH·, respectively (Assimopoulou, Boskou, & Papageorgiou, 2004; Assimopoulou & Papageorgiou, 2005). However, there are few reports addressed on the evaluation of antioxidants from LE using different methods. Moreover, apart from naphthoquinone compounds, the antioxidant activities of other kinds of components from LE have not been studied thoroughly.

Therefore, the aim of this study was to describe the antioxidant activity of the isolated compounds from LE and to compare their antioxidant effects through different methods including antioxidant activity in lard tested with a Rancimat, reducing power and radical scavenging activity toward DPPH· and $ABTS^{\cdot+}$. We also wanted to know whether there existed other kinds of fractions or compounds with strong antioxidant activity in the root of LE, apart from the naphthoquinone compounds.

2. Materials and methods

2.1. Materials

LE was purchased from Lei YunShang Drugstore, 2–18 Huashan Road, Shanghai, P.R. China, dried with ventilation at ambient temperature and stored at 4 °C until use.

2.2. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH·) was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Sigma–Aldrich. Butylated hydroxytoluene (BHT) was purchased from the Chemical Company, Shanghai, PR China. Silica gel was bought from Qingdao Ocean Chemical factory, PR China. Lard was rendered in the laboratory from fresh pig fat tissue purchased from Shanghai Slaughter House, PR China. Other chemicals and solvents in this experiment were all AR grade and from Shanghai Chemical Reagent Co.

2.3. Extraction and isolation

The dried and powdered roots of LE (1 kg) were in batches extracted by a Soxhlet extractor with petroleum ether (60–90°) and chloroform successively and each for 3 days and 12 h per-day. After removal of the solvents in vacuum, 42 g petroleum ether extract and 45 g chloroform extract were obtained respectively.

Forty grams of petroleum ether extract was chromatographed on a silica gel column (200–300 mesh, 400 g, 5.0 i.d. × 90 cm), eluting with petroleum ether and dichloromethane mixtures of increasing polarity (10:1–1:10, each in 3 l). Eight fractions were obtained according to TLC analysis using a mixture of petroleum ether/dichloromethane (1:3) as the developing solvent. The spots on the TLC plates were detected with a UV detection lamp (ZF7B, Shanghai Kanghua Bio-Chem Co., China) whose wavelength was set at 365 nm. Finally, six compounds (compounds **1**–**6**) were obtained and the column chromatography separation was illustrated in Flow diagram 1.

Twenty grams of chloroform extract was added to a silica gel column (200–300 mesh, 350 g, 5.0 i.d. × 90 cm) using petroleum ether/EtoAc (99:1–80:20, each in 3 l) gradient as developing solvent to give five fractions. As shown in Flow diagram 1, a mixture of caffeic acid esters (compound **7**) was obtained from fraction 3 and β-sitosterol (compound **6**) was also obtained and purified by repeated recrystallization from fraction 2.

2.4. Elucidation of the chemical structures

EIMS spectra were recorded with an HP 5989 or an Agilent 5975 mass spectroscopic instrument. Ultraviolet (UV) spectra were recorded with a UV-260 spectroscopic instrument (Shimadzu, Japan), and ethanol was used as solvent. IR-spectra were recorded on an Avatar 370 FT-IR spectrometer (Thermo-Nicolet). Samples were prepared for IR spectroscopy by incorporating the crystals into a KBr disc. ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ on a 500 MHz Bruker AV-500 at 500 MHz and 125 MHz, respectively, and tetramethylsilane (TMS) was used as an internal standard.

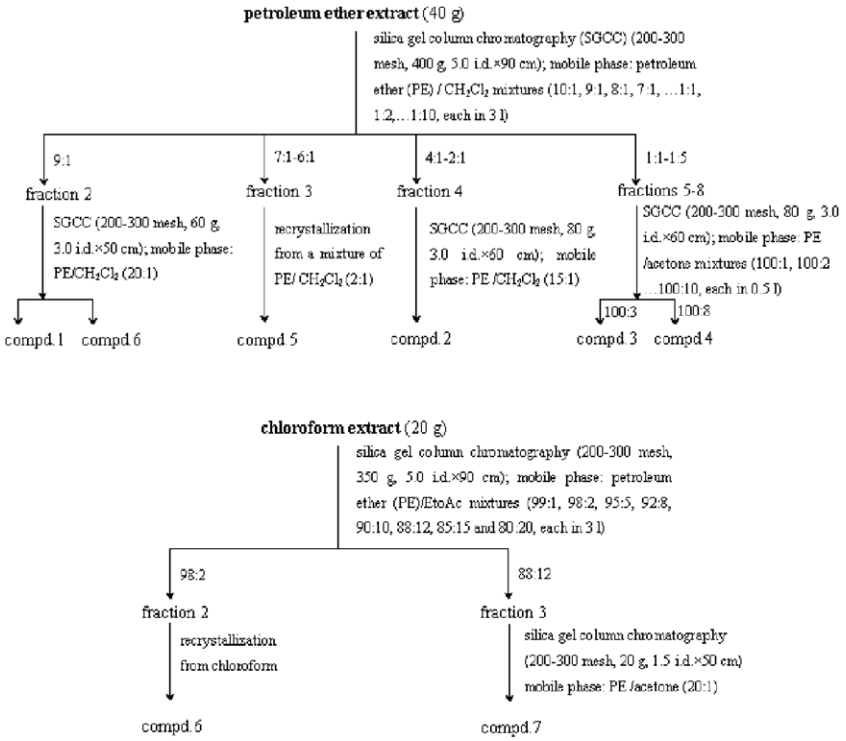
2.5. Antioxidant activity in rancimat method

Antioxidant activities in lard of the seven isolated compounds were studied with a Metrohm Rancimat Model 743, at 100 °C. The air flow rate was fixed at 20 l/h. The concentrations of the test samples, calculated on dry weight basis, were 0.02%, 0.04% and 0.06% levels (w/w). BHT was used as a comparison synthetic antioxidant.

The effect of the samples on retarding the lard oxidation, interpreted as the protection factor (*Pf*), was calculated according to the following expression:

$$Pf = IP_{\text{antiox.}}/IP_{\text{contrl.}} \quad (1)$$

where $IP_{\text{antiox.}}$ and $IP_{\text{contrl.}}$ was the induction period (*IP*) of lard oxidation with and without antioxidant, respectively.



Flow diagram 1. Column chromatography separations of petroleum ether extract and chloroform extract of *L. erythrorhizon*.

2.6. Determination of reducing power

The reducing power of the compounds isolated from LE was determined according to the methods of Jayaprakasha, Singh, and Sakariah (2001). Briefly, a methanolic solution (0.5 ml) of the samples at various concentrations (0.1, 0.2, 0.5 mg/ml) were mixed with phosphate buffer solution (PBS, 2.5 ml, 0.2 M, pH 6.6) and $[K_3Fe(CN)_6]$ (2.5 ml, 1%, w/v). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%, w/v) was added to the mixture, which was then centrifuged at 1790 g for 10 min. The upper layer of solution (2.5 ml) was mixed with deionized water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%, w/v), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. BHT was also assayed at the same concentration for comparison purpose.

2.7. DPPH· scavenging activity

DPPH· scavenging activity of the compounds isolated from LE was carried out as described by Pyo et al. (2004) with minor modifications. 0.2 mL of different concentrations (0.02, 0.05, 0.1, 0.2, 0.5 mg/ml) of samples in ethanol was added to 2.0 mL of 1×10^{-4} M ethanol solution of DPPH·. The absorbance at 517 nm was measured after solution had been allowed to stand in dark for 60 min. Lower absorbance of the reaction mixture indicates higher DPPH· scavenging activity. DPPH· scavenging activity was calculated using the following formula:

$$\text{DPPH}^{\cdot}\text{ scavenging activity}(\%) = [1 - (S - SB)/(C - CB)] \times 100\% \quad (2)$$

where S, SB, C and CB were the absorbances of the sample, the blank sample (2.0 mL of ethanol plus 0.2 ml of sample at different concentrations), the control (2.0 ml of DPPH· solution plus 0.2 ml of ethanol), and the blank control (ethanol), respectively.

2.8. $ABTS^{\cdot+}$ scavenging activity

The radical scavenging activity of the isolated compounds against $ABTS^{\cdot+}$ was measured using the methods of Zhao et al. (2005) with some modifications. ABTS was dissolved in PBS (0.01 M, pH 7.4) to a 7 mM concentration. $ABTS^{\cdot+}$ was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixtures to stand in the dark at room temperature for 16 h before use. The $ABTS^{\cdot+}$ solution was diluted with PBS (0.01 M, pH 7.4) to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C for 30 min. An ethanolic solution (0.2 ml) of the samples at various concentrations (0.02, 0.04, 0.06, 0.08 mg/ml) was mixed with 2.0 ml of diluted $ABTS^{\cdot+}$ solution. After reaction at room temperature for 20 min, the absorbance at 734 nm was measured. Lower absorbance of the reaction mixture indicates higher $ABTS^{\cdot+}$ scavenging activity. The capability to scavenge the $ABTS^{\cdot+}$ was calculated using the formula given below:

$$\text{ABTS}^{\cdot+}\text{scavenging activity}(\%) = [1 - (S - SB)/(C - CB)] \times 100\% \quad (3)$$

where S, SB, C and CB were the absorbances of the sample, the blank sample (2.0 ml of PBS plus 0.2 ml of the sample at different concentrations), the control (2.0 ml of diluted $ABTS^{\cdot+}$ solution plus 0.2 ml of ethanol), and the blank control (2.0 ml of PBS plus 0.2 ml of ethanol), respectively.

2.9. Statistical analysis

All tests were performed in triplicate. The results were given as means ± SD. Analysis of variance and significant differences among means were tested by one-way ANOVA, using SPSS (Version 13.0 for Windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Elucidation of the chemical structures of isolated compounds

The spectroscopic data for each compound isolated from LE were listed in Table 1. The chemical structure confirmation of these components was accomplished by comparing the UV, IR, mass, ^1H and ^{13}C NMR spectral data obtained in the laboratory to those published. Their structures are shown in Fig. 1.

Compound **1** was isolated and rechromatographed on a silica gel column with petroleum ether/dichloromethane (20:1) as bright-red lamellar crystal. Its detailed spectral data from IR, MS, ^1H and ^{13}C NMR listed in Table 1 agreed well with the reported compound, deoxyshikonin (Cho et al., 1999; Lu, Xiang, & Zhu, 1983).

Compound **2** was isolated from petroleum ether/dichloromethane (15:1) fraction and recrystallized from hexane as purplish-red pellet crystal. Its detailed spectral data listed in Table 1 agreed well with the data reported (Fu & Xiao, 1986; Weng et al., 2000). This compound was identified as β,β-dimethylacrylshikonin.

Compound **3** was isolated from petroleum ether/acetone (100:3) fraction and recrystallized from hexane as puce needle crystals. This compound was identified as isobutylshikonin by comparing the obtained data with those reported (Cho et al., 1999).

Compound **4** was isolated from petroleum ether/acetone (100:8) fraction and recrystallized from petroleum ether as puce needle crystals. Its detailed spectra data, listed in Table 1, were identical with the data reported for shikonin (Cho et al., 1999; Fu & Xiao, 1986; Weng et al., 2000).

Compound **5** was isolated from petroleum ether/dichloromethane (7:1–6:1) fraction and recrystallized from petroleum ether/dichloromethane (2:1) mixture as puce needle crystals. Its UV spectrum was similar to those recorded for compounds **1**–**4** and was characterized by maxima at 224, 284, 494, 527 and 566 nm. This meant that it had similar conjugation system to them. Its ^1H NMR spectra was almost the same as shikonin (**4**), but a singlet peak (3H) at 3.35 ppm indicated that a methoxyl group (–OCH_3) existed in this compound instead of the hydroxyl group on the side chain of shikonin (**4**). This was confirmed by its mass spectra with ion peaks at m/z 302 (molecular ion peak) and 270 (also presented at the spectrums of compounds **1**–**4**). The molecular weight of the group lost from the compound was 32 (302–270) and the group therefore should be –OCH_3. Thus, the structure of this compound was elucidated as 5,8-dihydroxy-2-(1-methoxy-4-methyl-3-pentenyl)-1,4-naphthalenedione which was once synthesized and tested for wound healing and antiallergic activities (Takeshi, Shigeru, Junichi, & Hideo, 1984), but it was here isolated from LE for the first time.

Compound **6** was obtained from petroleum ether/dichloromethane (20:1) as colorless needle crystal. It had the same R_fs with the standard of β-sitosterol in TLC (petroleum ether/EtoAc 8:2, $R_f = 0.35$; hexane/acetone 8:2, $R_f = 0.48$; chloroform/methanol 19:1, $R_f = 0.56$). Its spectral data agreed well with those of β-sitosterol standard (Wei, Liang, Zhao, & Zhang, 1997).

Compound **7** (**7a** and **7b**) was isolated from petroleum ether/acetone (20:1) fraction as white powder crystal. Its detailed spectra data, listed in Table 1, agreed well with data reported (Wang et al., 2003) that led to its identification as a mixture of caffeic acid octadecyl ester (**7a**) and caffeic acid eicosyl ester (**7b**). Hisamichi, Yoshizaki, and Kondo (1982) reported that a mixture of caffeic acid esters of various alcohols including **7a** and **7b** had been isolated from LE and caffeic acid docosyl ester was synthesized and tested for its anti-inflammatory effect. However, though there are many reports on the antioxidant activity of caffeic acid, few reports have addressed the antioxidant activities of caffeic acid esters.

3.2. Antioxidant activity in rancimat method

The Rancimat method is commonly used to evaluate the antioxidative properties of various antioxidants and is based on the increase of electrical conductivity due to the formation of volatile dicarboxylic acids as a result of lipid oxidation (Yen, Chang, & Su, 2003). As shown in Table 2, the isolated compounds from LE presented different effects on retarding the lard oxidation. Among the eight samples tested (including BHT), compound **7** was found to have extremely potent antioxidant activity in lard since its *Pf* values at 0.02% and 0.04% levels were, respectively three times and four times more than that of BHT. Compound **4** appeared to be the compound with the next strongest antioxidant activity in lard and compound **2** was less active than compound **4**. Compounds **3** and **5** began to show antioxidant activity at 0.04% level and both displayed intermediate potency in retarding the lard oxidation, while compound **1** showed a slight antioxidant activity. Thus, the order of their antioxidant activities in lard decreased as, compound **7**, followed by compound **4**, BHT, compounds **2**, **3**, **5**, **1** and **6**, respectively. The order of activity

Table 1
Spectral data of the compounds isolated from *L. erythrorhizon*

Compound	Data of spectra				
	^{1}H NMR (500 MHz) TMS as int. standard	^{13}C NMR (125 MHz) TMS as int. standard	EIMS (*m*/*z*)	IR (cm^{-1}) (KBr)	UV (nm) (EtOH)
1	1.60(s, 3H), 1.70(s, 3H), 2.30(q, 2H), 2.63(t, 2H), 5.14(t, 1H), 6.84(s, 1H), 7.19(s, 2H), 12.46(s, 1H), 12.62(s, 1H)	17.77, 25.64, 26.56, 29.67, 111.70, 111.93, 122.37, 130.83, 131.15, 133.61, 134.49, 151.45, 162.31, 162.97, 182.97, 182.99	272(13), 229(6), 216(7), 204(22), 91(6), 69(100), 53(6), 41(46)	3444, 1606, 1570, 1457, 1239, 1213, 804	215, 278, 485, 515, 554
2	1.58(s, 3H), 1.68(s, 3H), 1.94(d, 3H), 2.16(d, 3H), 2.48(m, 1H), 2.62(m, 1H), 5.15(t, 1H), 5.78(t, 1H), 6.01(m, 1H), 6.98(s, 1H), 7.18(s, 2H), 12.44(s, 1H), 12.60(s, 1H)	17.95, 20.36, 25.74, 27.55, 32.93, 68.65, 111.62, 111.90, 115.30, 118.03, 131.62, 132.45, 132.60, 135.82, 149.05, 158.87, 165.25, 166.30, 166.84, 177.49, 178.97	270(8), 100(5), 84(6), 83(100), 69(6), 55(16), 44(8), 41(10)	3450, 1715, 1647, 1616, 1572, 1456, 1414, 1224, 1140, 1078, 858, 763	215, 275, 488, 521, 560
3	1.21(d, 6H), 1.59(s, 3H), 1.69(s, 3H), 2.48(m, 1H), 2.64(m, 2H), 5.12(t, 1H), 6.02(dd, 1H), 6.97(s, 1H), 7.18(s, 2H), 12.41(s, 1H), 12.58(s, 1H)	17.95, 18.85, 18.95, 25.74, 32.99, 34.06, 69.04, 111.59, 111.85, 117.82, 131.37, 132.66, 132.83, 135.95, 148.57, 166.84, 167.38, 175.74, 176.84, 178.35	271(6), 270(23), 255(10), 220(6), 71(93), 69(14), 43(100), 41(32)	3455, 1738, 1615, 1572, 1459, 1211, 1155, 777	215, 275, 488, 521, 560
4	1.66(s, 3H), 1.76(s, 3H), 2.36(m, 1H), 2.64(m, 1H), 4.92(dd, 1H), 5.21(t, 1H), 7.17(s, 1H), 7.19(d, 2H), 12.49(s, 1H), 12.60(s, 1H)	18.11, 25.97, 35.69, 68.36, 111.56, 112.01, 118.47, 131.89, 132.32, 132.41, 137.43, 151.47, 164.91, 165.53, 179.82, 180.62	270(3), 220(95), 219(39), 191(16), 69(100), 55(12), 53(17), 41(75)	3446, 1611, 1573, 1453, 1203, 1066, 778	215, 281, 485, 515, 554
5	1.55(s, 3H), 1.70(s, 3H), 2.31(m, 1H), 2.54(m, 1H), 3.35(s, 3H), 4.58(m, 1H), 5.20(t, 1H), 7.12(s, 1H), 7.19(d, 2H), 12.50(s, 1H), 12.61(s, 1H)	17.98, 25.77, 34.14, 57.69, 77.01, 111.63, 112.03, 118.89, 132.16, 132.30, 132.65, 134.87, 149.96, 166.28, 166.80, 178.62, 179.29	302(weak), 270(3), 233(100), 190(13), 77(12), 69(34), 53(15), 41(52)	3439, 1607, 1454, 1410, 1276, 1202, 1107, 836, 765	224, 284, 494, 527, 566
6	0.70(s, 3H), 0.79(s, 3H), 0.80(s, 3H), 0.81(s, 3H), 0.83(s, 3H), 1.01(s, 3H), 3.53(m, 1H), 5.36(s, 1H)		414(100), 396(45), 381(32), 255(88), 145(81), 213(79), 159(72), 105(68)	3445, 2959, 2936, 1639, 1464, 1379,	
7	0.90(t, 3H), 1.30(brs, 34H), 1.70(m, 2H), 4.20(t, 2H), 5.77(s, 1H), 6.00(s, 1H), 6.28(d, 1H), 6.88(d, 1H), 7.02(dd, 1H), 7.12(s, 1H), 7.58(d, 1H)	14.14, 22.71, 25.99, 28.72, 29.31, 29.38, 29.56, 29.61, 29.68, 29.70, 29.72, 31.95, 64.94, 114.47, 115.53, 115.77, 122.41, 127.61, 143.73, 144.82, 146.27, 167.96	460(6), 432(15), 180(100), 163(50), 136(15), 97(12), 83(12), 69(10)	3482, 3319, 2919, 2849, 1685, 1603, 1279, 1180	219, 247, 298, 330

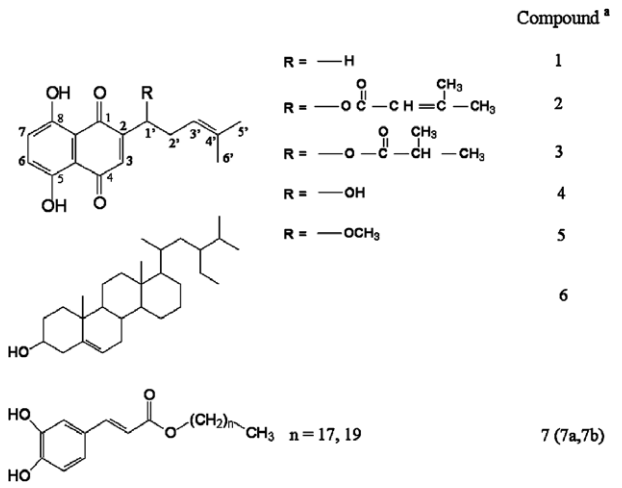


Fig. 1. The structures of the compounds isolated from *L. erythrorhizon*. [a]**1**, deoxyshikonin; **2**, β,β-dimethylacrylshikonin; **3**, isobutylshikonin; **4**, shikonin; **5**, 5,8-dihydroxy-2-(1-methoxy-4-methyl-3-pentenyl)-[1,4]-naphthalenedione; **6**, β-sitosterol; **7**, caffeic acid ester (**7a**, caffeic acid octadecyl ester; **7b**, caffeic acid eicosyl ester).

of the two compounds, shikonin (**4**) and β,β-dimethylacrylshikonin (**2**), was in agreement with the report of Weng et al. (2000). In addition, it was interesting that, although compounds **1**–**5** possessed the same naphthoquinone parent moiety, they exhibited different antioxidant capacities in lard ($P < 0.05$). Shikonin (**4**) showed much stronger antioxidant activity than its derivatives, compounds **1**, **2**, **3** and **5**. The reasons might be that: (i) the hydroxyl group at 1′-position makes shikonin readily undergo reactions shown in Scheme 1. More resonance structures can form to stabilize the free radical of shikonin, and its oxidized products (such as product I in Scheme 1) can also exert antioxidant activity in further lard oxidation processes. While compounds **1**, **2**, **3** and **5** are very difficult to undergo these reactions due to deoxylation, esterfication or methoxylation of the 1′-hydroxyl group and (ii) the molar concentration of shikonin was higher. Since the test concentration was expressed as mg sample/g lard (w/w), and the molecular weight of shikonin was less than that of compounds **2**, **3** and **5**. For example, a 0.04% level corresponds to 0.4 mg/g lard for all the compounds, but 1.39 μmol/g lard of shikonin, which is higher than 1.08, 1.12 and 1.32 μmol/g lard for compounds **2**, **3** and **5**, respectively.

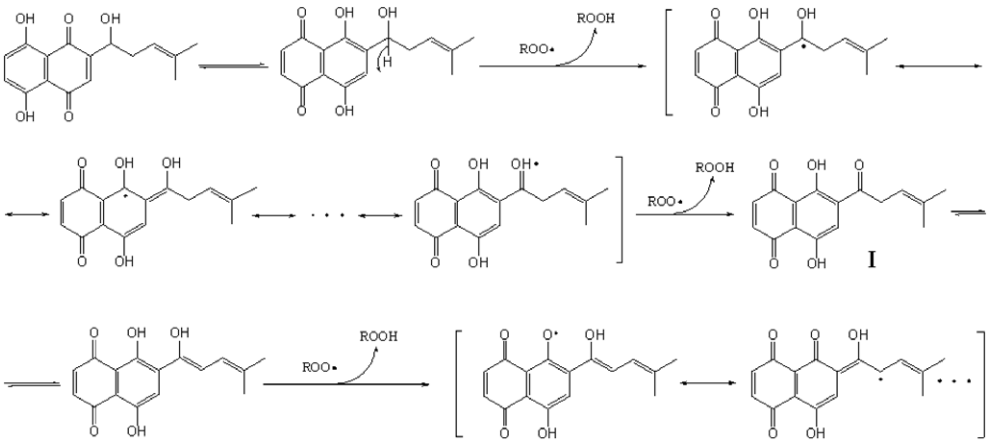
3.3. Determination of reducing power

Some previous studies have reported that the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Jeong et al., 2004). Thus, it appears necessary to determine the reducing power of the compounds isolated from LE to evaluate their antioxidant potentials. Fig. 2 demonstrated the reducing power of the test samples using the potassium ferricyanide reduction method. A strong reducing power was noted for compounds **4**, **7** and BHT. Much lower reducing power was found for compounds **1**, **2**, **3** and **5**. It was notable that,

Table 2
Antioxidant effects (*Pfs*) of compounds isolated from *L. erythrorhizon* investigated using Rancimat method[A]

Level (w/w) (%)	Compound							
	1	**2**	**3**	**4**	**5**	**6**	**7**	BHT
0.02	1.36 ± 0.05^{gB}	2.23 ± 0.02^{d}	1.84 ± 0.04^{e}	3.94 ± 0.01^{b}	1.66 ± 0.04^{f}	1.00 ± 0.04^{h}	10.23 ± 0.06^{a}	3.03 ± 0.07^{c}
0.04	1.55 ± 0.01^{f}	2.93 ± 0.02^{d}	2.26 ± 0.09^{e}	5.34 ± 0.14^{b}	2.02 ± 0.06^{e}	1.06 ± 0.05^{g}	15.16 ± 0.31^{a}	3.50 ± 0.13^{c}
0.06	1.84 ± 0.02^{g}	3.67 ± 0.01^{d}	2.67 ± 0.08^{e}	6.80 ± 0.09^{b}	2.25 ± 0.01^{f}	1.03 ± 0.03^{h}	$>18.88^{a}$	4.02 ± 0.06^{c}

[A] Data are means ± SD.
[B] Data bearing different superscript letters in the same line are significantly different ($n = 3$, $P < 0.05$).



Scheme 1. Explanation of why shikonin possesses much stronger antioxidant activity than its derivatives in lard.

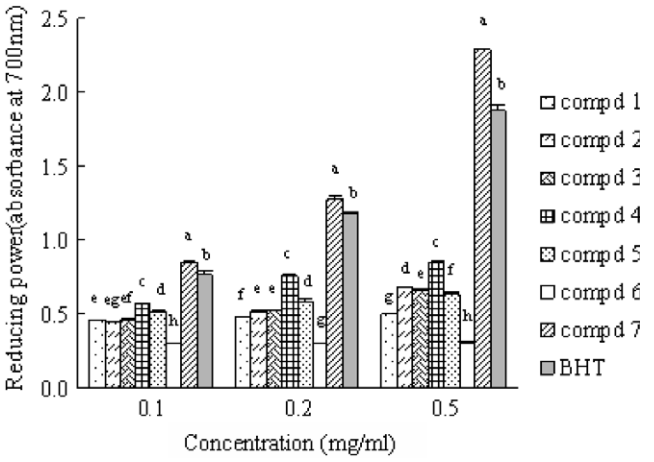


Fig. 2. Reducing power (absorbance at 700 nm) of compounds isolated from *L. erythrorhizon*. Vertical bars represent the standard deviation for each dada point. Locations for each concentration of the samples marked by different letters are significantly different ($n = 3$, $P < 0.05$).

among these isolated compounds, compound **7** was the only one which showed stronger reducing power than that of the comparison standard BHT. As concentration increased from 0.1 to 0.5 mg/ml, the reducing power of compound **7** increased rapidly from 0.85 to 2.23, while compounds **1**, **2**, **3** and **5** increased in a moderate way. Like the test in lard, shikonin (**4**) manifested stronger antioxidant activity than its derivatives (compounds **1**, **2**, **3** and **5**) again in reducing power assay, which suggested that the hydroxyl group in the side chain of the shikonin (**4**) seemed to be beneficial to its reducing power, whereas deoxylation of the hydroxyl group of the side chain may obviously depress the reducing power, since the reducing power of deoxyshikonin (**1**) was poor and increased little with increased concentration. Compounds **2**, **3**, and **5** exerted similarly intermediate reducing power, while compound **6** did not show any reducing power ($P > 0.05$, compared against the control). Thus, at 0.5 mg/ml, reducing power of test samples decreased in the following order: compound **7** > BHT > **4** > **2** ~ **3** ~ **5** > **1** > **6**, which was similar to the observation for the antioxidant activity in lard. These results suggested that the antioxidant activities of these compounds in lard were probably due to their reducing capacities. In other words, these compounds are electron donors, and can reduce the oxidized intermediates of lipid peroxidation processes, so they can act as primary and secondary antioxidants and therefore inhibit lipid peroxidation (Ordoñez, Gomez, Vattuone, & lsla, 2006).

3.4. DPPH· scavenging activity

The DPPH· is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant (Lu & Yeap Foo, 2001). Since the DPPH· assay can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentration, the DPPH· scavenging activity has been widely used to evaluate the antiradical activity of various samples (Piao et al., 2004; Yu et al., 2002). Fig. 3 shows the DPPH· scavenging activity of the compounds isolated from LE. As established, compound **7** showed valuable high radical scavenging activity. A sharp increase, 39–85%, of its DPPH· scavenging activity was observed, as its concentration increased from 0.02 to 0.05 mg/ml. Concentrations of 0.1, 0.2, and 0.5 mg/ml, the scavenging effects were the same (91.5%), which suggested that it had scavenged the whole amount of DPPH· and products of the reaction between compound 7 and DPPH· gave colour giving similar absorbance at 517 nm. On the other hand, the activities of scavenging DPPH· were moderate in the case of compounds **1**–**5**. At 0.5 mg/ml, compounds **1**–**5** scavenged DPPH· 59.4%, 62.6%, 65.4%, 60.5% and 55.8%, respectively. It should be noted that shikonin (**4**) did not show superior antioxidant activity to its derivatives in this assay. This result, though the absolute values for DPPH· scavenging activity of shikonin (**4**) in the present study were lower than those of the paper reported (about 95% DPPH· inhibition at 500 ppm) (Assimopoulou & Papageorgiou, 2005), was accordant with the same paper which found that the DPPH· scavenging activity of alkannin/shikonin (A/S) and their derivatives was attributed mainly to the naphthoquinone/naphthazarin moiety and possibly the side chain of A/S played a minor role on DPPH· scavenging activity. The duration of the reaction between test samples and DPPH· is likely to be one of the factors affecting values of radical scavenging activity. Thus, the lower radical scavenging activity of the corresponding compounds in this study is partly due to the short reaction time (60 min).

3.5. $ABTS^{\cdot+}$ scavenging activity

The $ABTS^{\cdot+}$ scavenging assay, which employs a specific absorbance (734 nm) at a wavelength remote from the visible region and requires a short reaction time, can be used

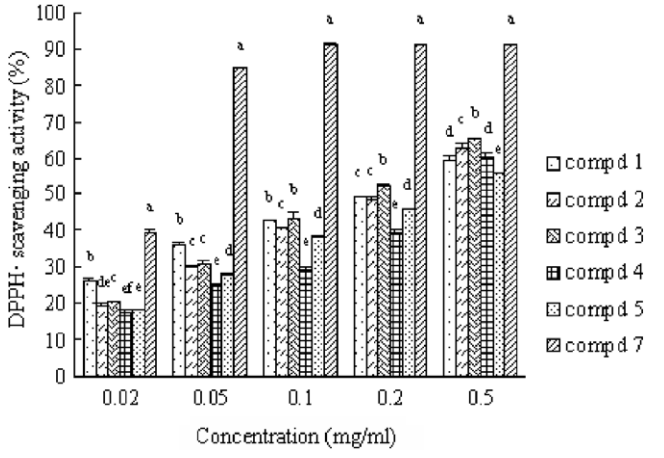


Fig. 3. DPPH· scavenging activities (%) of compounds isolated from *L. erythrorhizon*. Vertical bars represent the standard deviation for each dada point. Locations for each concentration of the samples marked by different letters are significantly different ($n = 3$, $P < 0.05$).

in both organic and aqueous solvent systems (Wu et al., 2006) and can also be an index reflecting the antioxidant activity of the test samples. Hence, the isolated compounds from LE were measured and compared to their capacities to scavenge $ABTS^{\cdot+}$. As seen from Fig. 4, except β-sitosterol (**6**) (data were not shown), all other compounds were found to be very effective scavengers against $ABTS^{\cdot+}$ and their activities increased in a concentration dependent manner. At 0.02 mg/ml, compounds **1**, **3** and **4** exerted moderate $ABTS^{\cdot+}$ scavenging activities of 38%, 45% and 37%, respectively, whereas compounds **2**, **5** and **7** exhibited lower activities of 29%, 15% and 24%, respectively. However, quite strong $ABTS^{\cdot+}$ scavenging activities were observed for all of the six compounds (**1**–**5** and **7**) at 0.08 mg/ml for their values of antiradical activities ranged from 95% to 99%. Thus, with regard to scavenging activity toward radicals, the five naphthoquinone compounds (**1**–**5**) all were excellent scavengers against $ABTS^{\cdot+}$, but showed moderate scavenging activities against $DPPH^{\cdot}$ (Figs. 3 and 4). One of the probable causes of this difference was that the kinetic constants of reactions between $ABTS^{\cdot+}$ and naphthoquinone compounds were generally higher than that for the reactions between $DPPH^{\cdot}$ and naphthoquinones. In addition, different systems used to measure the scavenging abilities toward the two radicals may also affect the values of radical scavenging activities. Moreover, ANOVA showed there were no significant differences ($P > 0.05$) for scavenging $ABTS^{\cdot+}$ activities among the naphthoquinones compounds (**1**–**5**) at 0.08 mg/ml, which also leads to the conclusion that it is the hydroxynaphthoquinone moiety that is responsible for the high $ABTS^{\cdot+}$ scavenging activity, whereas the side chains of the compounds **1**–**5** do not affect the radical scavenging activity.

Thus, though the above assays are based on different principles and represented different aspects of the antioxidant potential, the data report in this study indicates that the isolated compounds from LE are effective antioxidants,

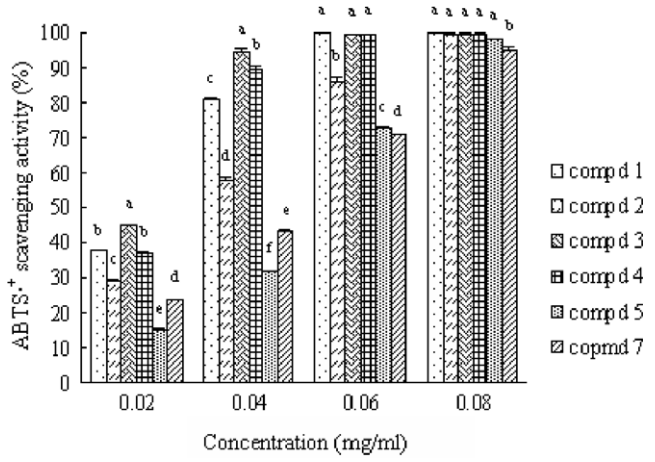


Fig. 4. $ABTS^{\cdot+}$ scavenging activities (%) of compounds isolated from *L. erythrorhizon*. Vertical bars represent the standard deviation for each dada point. Locations for each concentration of the samples marked by different letters are significantly different ($n = 3$, $P < 0.05$).

which are able to inhibit lard peroxidation, scavenge free radicals and also participate in redox reactions.

Since the literature pertaining to the antioxidant activity of phenol compounds is voluminous and it is reported that radicals add to quinones to form stabilized radicals, allowing some quinones to be inhibitors in free radical chain reactions (Weng & Gordon, 1992), so, it may be that the hydroxynaphthoquinone moiety (Fig. 1) endows compounds **1**–**5** with obvious antioxidant activities in the present study, which may be also responsible for some of their pharmaceutical effects, such as wound healing, anti-inflammatory effect, antitumor and antibacterial effects (Gao, Kakuma, Oka, Sugino, & Sakurai, 2000; Sekine, Masumizu, Maitani, & Nagai, 1998). However, it should be noted that, though compounds **1**–**5** had the *para*-dihydroxy-phenolic moiety which was known as a strong antioxidant functional group, they were shown to be less effective antioxidants when compared to compound **7**. This was because at the *ortho*-position of each phenolic hydroxyl group of compounds **1**–**5**, there was a carbonyl group substituted, which was a strong electron-withdrawing group and therefore depressed the antioxidant activities of the hydroxynaphthoquinones to some extent in this study. In addition, the above assays also have shown that the antioxidant activity of LE is not limited to naphthoquinone compounds. Activity also comes from other kinds of compounds, such as compound **7**, a mixture of caffeic acid esters, which carries a caffeic moiety in its structure (Fig. 1) and was the most outstanding in its antioxidative potential among these test compounds. It is generally accepted that phenols are effective antioxidants for their good abilities to donate hydrogen atoms. Also, the main reasons why compound **7** has such strong antioxidant activity should be: (i) the *ortho*-dihydroxy-phenolic moiety make it more facile to donate hydrogen atoms to active free radicals to form the neutralized phenoxyl radical of the compound (Weng & Wang, 2000), (ii) there is a –CH=CH–COOR group directly connecting the phenol in the *para*-position of the phenolic hydroxyl group. The double bond extends the conjugation system and participates in stabilizing the phenoxyl radical by resonance (Cuvelier, Richard, & Berset, 1992), and therefore strengthens the antioxidant activity of the compound **7**.

Hence, this study furnishes additional data to the literature about compounds with antioxidant activity from LE and suggests that this traditional Chinese medicinal herb could be considered as a promising source of natural antioxidants for food.

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