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Antioxidant activity and components of *Salvia plebeia* R.Br. — a Chinese herb

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Abstract

The antioxidant properties of the extracts from *Salvia plebeia* R.Br. which was screened out of over 700 species of Chinese herbs, were tested in lard at 110°C using the Oxidative Stability Instrument. The ethyl acetate extract of the herb was re-extracted by solvents (with increasing polarity) into petroleum ether-, diethyl ether-, acetone-and-ethanol-soluble fractions. The fractions obtained were then separated according to their acidic properties. From the most active sub-fractions, namely the acidic sub-fraction of the petroleum ether-soluble fraction and the weakly acidic sub-fraction of the diethyl ether-soluble fraction, three antioxidant components were isolated and identified as royleanonic acid, hispidulin and eupatorin. The royleanonic acid was a novel compound and eupatorin was isolated for the first time from the herb. Though royleanonic acid and hispidulin prolonged the induction period significantly, their antioxidant activities were much weaker than the crude extracts, implying that synergistic effects might be responsible for the high activity of the crude extracts.

Keywords: *Salvia plebeia* R.Br.; Herb; Antioxidants; Royleanonic acid; Hispidulin; Eupatorin

1. Introduction

Lipid peroxidation is one of the major factors that cause deterioration during the storage and processing of foods (Angelo, 1996). Oxidized polyunsaturated fatty acids may induce aging and carcinogenesis. Many spices possess potent antioxidant activity and examples of them are rosemary and sage. Herbs are also found to be potent sources of natural antioxidants. Some have been used for thousands of years in China and their clinical and pharmacological effects have been extensively studied from various viewpoints. Apparently, some of the clinical effects of herbs are closely related to their antioxidant activities (Gu & Weng, 1997).

Su, Osawa and Namiki (1986) screened 195 species of herbs and 22 of them were found to be as effective as α-tocopherol, including eight species which were more active than butylated hydroxyanisole (BHA). Forty-four herbs were screened, by Kim, Kim, Oh and Jung (1994) from 180 species, and found to possess significant antioxidant activity. Twenty-two of them were particularly active. However, there are in total, 4773 species of herbs recorded by the new edition of the *Herb Dictionary* (Jiangsu New Medical College, 1993), indicating that the above-mentioned screening work was far from completion. Weng, Ren, Duan, Dong and Jiang (1998) screened over 700 species of the most commonly used herbs, using the Oxidative Stability Instrument (OSI). Sixty-four herbs were found to be significantly active. Among them, *Salvia plebeia* R.Br. was identified to be a potent antioxidant plant. *Salvia plebeia* R.Br. is a biannual grass, distributed widely in many countries, in folk medicine, for its hemogenetic, hemostatic, antioncotic and anti-inflammatory effects in China. The extracts of *Salvia plebeia* R.Br. by petroleum ether and ethyl acetate were as active as α-tocopherol at a concentration of 0.02% (w/w). The extract from flowers was the most active, followed by the extract from leaves and stems. Extracts from roots possessed no activity (Weng, Cao, Dong & Duan, 1998).

Several authors have studied the active components of herbs. Zhang, Bao, Wu, Rosen and Ho (1990) isolated and identified seven quinones from *Salvia miltorrhiza* Bung. Su, Osawa, Kawakishi and Namiki (1987)

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reported the existence of tannins and flavonoids in *Oskechia chinensis* L. In this paper, the antioxidant activity and antioxidant components of *Salvia plebeia* R.Br. are investigated.

2. Materials and methods

2.1. Solvent extraction

The dry upground part of *Salvia plebeia* R.Br. was bought from the local herb store. Totally, 12 kg crude herb was minced and extracted in a Soxhlet extractor, in batches, by ethyl acetate. The extracts were combined and the solvent was removed by rotary evaporator (Tianjin instrument Inc. Tanjin, China) to yield 360 g dry extract. Extract (300 g) was dispersed in 400 ml petroleum ether. The insoluble residues were re-extracted three times each in 300 ml petroleum ether. The residue left was extracted by equal total volumes of diethyl ether (antioxidants free), acetone and ethanol, consecutively, in a similar manner as for petroleum ether.

2.2. Fractionation by acidic properties

The original ethyl acetate extract (50 g) and the above-mentioned petroleum ether and diethyl ether-soluble fractions were dissolved in the corresponding 700-ml of solvents, then partitioned with 200 ml 5% (w/w) $NaHCO_3$ (aqueous solution) six times. The pooled aqueous parts were combined and adjusted to pH4.0 with 2N HCl. The resulting solution was extracted with 200 ml ethyl acetate, six times, to get the acidic fractions. The original organic solutions, after 5% $NaHCO_3$ fractionation, were partitioned with 5% (w/w) Na_2CO_3 and 2% (w/w) NaOH aqueous solutions, consecutively, in the same manner, to get the weakly acidic and phenolic fractions. The substance that remained finally in the organic solution was the neutral fraction. The resulting fractions were screened for their antioxidant activity.

2.3. Chromatographic isolation

The fractions with potent activity were further separated by column chromatography on an open column packed with silica gel (100–140 mesh) of mass 300 g (50×4.0 cm i.d.). After being applied to the top of the column, the acidic sub-fraction of the petroleum ether-soluble fraction (4.07-g) was eluted with petroleum ether-ethyl acetate as eluent with increasing ethyl acetate content (98:2; 96:4; 88:12; 76:24; 50:50, each in 400 ml). The weakly acidic sub-fraction of the diethyl ether-soluble fraction (7.0 g) was eluted with chloroform-methanol with increasing methanol content (98:2; 95:5; 90:10; 75:25, each in 500 ml).

Preparative thin-layer chromatography (TLC) was performed with 20×20 cm glass plates coated in the laboratory with silica gel GF_{254} (20–40 μm) (coating of 0.5 and 0.75 mm). The solvents used for elution were as follows: system A, chloroform-methanol (90:10); system B, benzene-methanol (85:15); system C, petroleum ether-ethyl acetate-methanol (45:30:6).

The petroleum-ethyl acetate (50:50) effluents of the acidic sub-fraction of the petroleum ether-soluble fraction were collected and the volume was reduced to 300 ml by rotary evaporator. Orange needle-like crystals were formed while the eluting material was stored overnight at 4°C. The crystals were collected and crystallized twice in ethyl acetate to give compound 1. The chloroform-methanol 95:5 or 75:25 eluting parts of the weakly acidic sub-fractions of the diethyl ether-soluble fraction were further separated with preparative TLC, repeatedly, to give compounds 2 and 3, respectively.

The purity of the compounds was checked by TLC with three solvent systems and spectroscopy.

2.4. Identification

^1H, ^{13}C, Distortionless enhancement by polarization transfer (DEPT), Heteronuclear multiple-quantum coherence (HMQC) and Heteronuclear multiple-bond correlation (HMBC), nuclear magnetic resonance (NMR) spectra were run on a Bruker AM-300 spectrometer (Karlsruhe, Germany) using tetramethylsilicane as internal standard. Electron impact mass spectra (EIMS) were obtained with a Shimadzu QP-1000A mass spectrometer (Tokyo, Japan). Ultraviolet (UV) spectra were determined with a Hitachi UV-3400 spectrophotometer (Tokyo, Japan). Infrared (IR) spectra were obtained using a JASCO IR-810 spectrometer (Tsukuba, Japan). Elemental analysis was done by a CARLO ERBA 1106 elemental analyzer (Milan, Italy).

2.5. Antioxidant assay

The antioxidant activity was tested on the Oxidative Stability Instrument (Omnion Inc. Illinois, USA) at 110°C in 20 g of freshly rendered lard. The data were recorded by an AST Bro 33s computer. The instrument worked in the same way as the Rancimat and had been recommended as an alternate to the Active Oxygen Method (AOM) (AOCS, 1994). Antioxidant was added at 0.02–0.12% (w/w). To ensure its dispersion, the antioxidant was dissolved in anhydrous ethanol before addition.

3. Results and discussion

The ethyl acetate extract contributed 3.04%, on average, of the weight of the dry herb. the ethyl acetate extract was divided into four fractions with different

polarities by dissolving it in various solvents. The petroleum ether-soluble fraction and diethyl ether-soluble fraction contributed 51.7 and 32.6% of original extract, respectively. Only 8.1 and 3.7% of the extracts were acetone-soluble and ethanol-soluble (Fig. 1.).

The induction period was determined on the Oxidative Stability Instrument, in triplicate. The antioxidant activity was expressed as protection factor (PF).

$$\mathrm{PF} = \frac{\text{Induction period of the lard containing antioxidants}}{\text{Induction period of the lard without antioxidants}}$$

As shown in Fig. 2, the antioxidant activity of the original extract and its diethyl ether-soluble part increased markedly with increasing concentration. They were less active than BHA below 0.04% but superior to BHA above 0.08% in lard. The activity of the diethyl ether-soluble fraction was greater than the original extract while the petroleum ether- and acetone-soluble fractions were less active. The ethanol-soluble fraction showed the lowest activity (PF < 4). The antioxidants therefore, seem to be the most lipophilic compounds in the herb. This will facilitate their use in bulk oil.

The original ethyl acetate extract and its diethyl ether- and petroleum ether-soluble fractions showed potential antioxidant activity. They were further sub-fractionated according to their components' acidity. As seen from Fig. 3, the antioxidant activity concentrated in the acidic, weakly acidic and phenolic fractions of each extract, while the activities of neutral fractions were negligible (PF < 3.0). the acidic sub-fraction of the petroleum ether-soluble and the weakly acidic sub-fraction of the diethyl ether-soluble fraction were identified as the most active. The two fractions were subjected to chromatographic separation.

Identification of the isolated compounds was based on the NMR, MS, IR and UV spectra of the compounds. The effects of shift reagents on the UV spectra were used to confirm the positions of substituents, as described by Markham (1982).

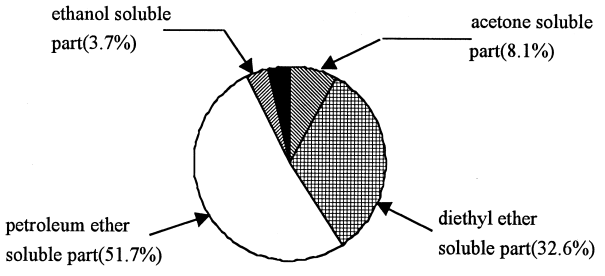

Fig. 1. Proportion of different soluble parts of the ethyl acetate extract of *Salvia plebeia* R.Br.

Compound 1: orange needles in ethyl acetate, $[\alpha]_D^{18}$ 75.3° (in acetone), mp: 232–234°C. EIMS gave the molecular ion *m/z* 346. Elemental analysis gave C 66.53%, H 7.44%. The atomic numbers were calculated as C 19.3, H 25.7. The number of carbon atoms was confirmed by ^{13}C NMR as 20, indicating that the molecular formula of this compound was $C_{20}H_{26}O_5$. UV (λ_{max}nm): 277 (MeOH); 286,365 (NaOMe); 275 ($AlCl_3$); 277 ($AlCl_3 + HCl$). EIMS (*m/z*): 346 (M^+, 12), 302 (75), 301 (12), 300 (35), 287 (18), 259 (12), 246 (18), 231 (30), 220 (15), 217 (15), 69 (30), 41 (100).

The UV spectrum profile was similar to that of *para*-quinone (Huang & Yu, 1988). The IR (KBr) absorptions at ν3403, 1646 and 1632 cm^{-1} were characteristic for hydroxyl-*para*-benzoquinone. The ^1H NMR spectra suggested the compound belongs to the royleanone type of diterpenoids (Hernandez, 1988; Michavila, 1986; Sukh & Renuka, 1986). Two double signals of methyl δ 1.22 (3H, d, $J = 7$Hz) and δ 1.18 (3H, d, $J = 7$Hz), exhibited by ^1H NMR spectra, were assigned to positions 16 and 17 on the isopropyl group, which were split into double peaks by the coupling of hydrogen atoms at position 15 (δ3.19m) of the diterpenoid. The assignments of these hydrogens were confirmed by the correlation between them and C-13 (δ 125.3) in HMBC. The other two CH_3 signals, each in singlet, might belong to positions 18, 19 or 20. The benzene rings contained in the molecule were all substituted, judging by the absence of signals of hydrogen attached to it. The absorption in IR spectra at 3000–3500 cm^{-1} and 1700 cm^{-1} suggested the existence of a carboxyl group. This was confirmed by ions *m/z* 300 and 302 in EIMS. The molecular ion lost CO_2 to form *m/z* 302 and lost carboxyl group and a hydrogen atom to form *m/z* 300. It was possible for the carboxyl group to locate at positions 18, 19 or 20. Since the ion *m/z* of 69 in EIMS was highly characteristic for royleanone type diterpenoids without modification on ring A (Chong, 1987), the carboxyl group was assigned to position 20. This was further supported by correlation between C-20 (δ 188.4) and H-1 α β (δ 2.40, 2.71) in HMBC.

The structure of the compound was elucidated by careful analysis of DEPT, HMQC and HMBC spectra. The signals were interpreted and assigned as shown in Table 1. The compound was given the name royleanonic acid. The structure is shown in Fig. 4a.

Compound 2: pale yellow cubic crystals in methanol, mp: 191–193°C. Positive to HCl-Mg reaction. UV (λ_{max}nm): 276,342 (MeOH); 275,375 (NaOMe); 275,342 (NaAc); 275,342 ($NaAc + H_3BO_3$); 276,365 ($AlCl_3$); 275,365 ($AlCl_3 + HCl$). IR (KBr) ν_{max} (cm^{-1}): 3450,1655,1605,1465,1375,1280,1130. EIMS (*m/z*): 345 ($M + H$,32), 344 (M^+,100), 329 (M-15,94), 315 (M-29,14), 301 (M-43,25), 181 (7), 153 (25). ^1H NMR (DMSO-d_6), δ: 3.82, 3.95, 3.98 (each 3H, s, OCH_3); 6.68 (1H, s, H-8); 6.79 (1H, s, H-3); 7.14 (1H, d,

$J = 9$Hz, H-5′); 7.51 (1H, d, $J = 2$Hz, H-2′); 7.58 (1H, dd, $J = 9$Hz, $J = 2$Hz H-6′). These spectral data were identical to those of eupatorin (Adams, 1976). It was isolated for the first time from the herb.

Compound 3: yellow needles in methanol, mp: 285–287°C. Positive to HCl-Mg reaction. UV (λ_{max}nm): 275,334 (MeOH); 275,327,391 (NaOMe); 276,298 (shoulder), 349 (NaAc); 276,334 ($NaAc + H_3BO_3$); 301,360 ($AlCl_3$); 301,357 ($AlCl_3 + HCl$). IR (KBr) ν_{max} (cm^{-1}): 3400,1660,1605,1575,1497,1460,1382,1250. EIMS (m/z): 301 ($M + H$, 18), 300 (M^+,100), 285 (M-15,60), 257 (M-43,55). ^{1}H NMR (DMSO-d_6), δ: 3.76 (3H, s, OCH_3); 6.58 (1H, s, H-8); 6.78 (1H, s, H-3); 6.94 (2H, d, $J = 6$Hz, H-5′, H-3′); 7.94 (2H, d, $J = 6$Hz, H-2′ H-6′);

Table 1
NMR chemical shifts assignments of compound 1 (300MHz, acetone-d_6, TMS as internal standard)[a]

position	^{13}C	DEPT	^{1}H and HMQC (J in Hz)
1 β	27.0	CH_2	2.71 ddd (J 1 β,1 α = 18.0 J 1 β,2β = 6.1 J 1 β,2 α < 1.0)
1 α			2.40 ddd (J 1 α,1 β = 18.0 J 1 α,2 β = 12.5 J 1 α,2 α = 6.7)
2 α	18.0	CH_2	2.06 m
2 β			1.88 m
3 β	41.9	CH_2	1.54 dd (J 3 β,2 β = 6.9 J 3 β,2 α < 0.5)
3 α			1.29 dd (J 3 α,2 β = 12.5 J 3 α,2 α = 6.7)
4	34.5	C	
5 α	53.4	CH	1.43 dd (J 5 α,6 β = 12.5 J 5 α,6 α = 1.0)
6 α	20.8	CH_2	2.36 m
6 β			1.52 m
7 β	35.1	CH_2	3.05 ddd (J 7 β,7 α = 13.0 J 7 β,6 β = J 7 β,6 α = 2.0)
7 α			1.10 ddd (J 7 α,7 β = 13.0 J 7 α,6 β = 12.5 J 7 α.6 α < 0.3)
8	147.5	C	
9	143.8	C	
10	47.5	C	
11	176.0	C	
12	153.2	C	
13	125.3	C	
14	184.1	C	
15	25.1	CH	3.19 m (J 15,16 = J 15,17 = 7.0)
16	20.6	CH_3	1.22 d
17	20.5	CH_3	1.18 d
18	33.1	CH_3	0.96 s
19	20.2	CH_3	0.87 s
20	188.4	C	7.37 s (merged with OH)

[a] NMR, nuclear magnetic resonance; TMS, tetramethylsilicane; DEPT, distortionless enhancement by polarization transfer; HMQC, heteronuclear multiple-quantum coherence.

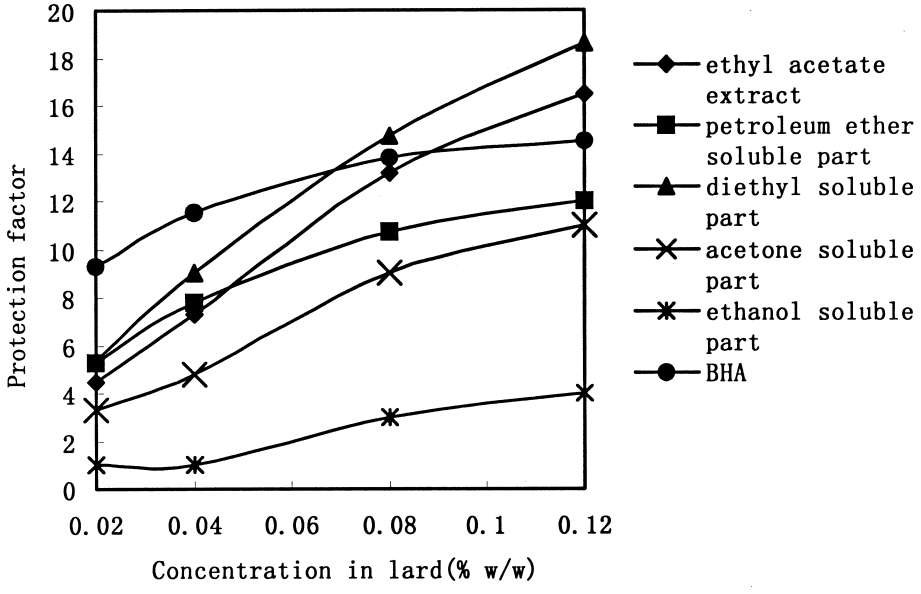

Fig. 2. Antioxidant activity of ethyl acetate extract of *Salvia plebeia* R.Br. and its different soluble parts compared with butylated hydroxyanisole (BHA).

13.06 (1H, s, OH-5). These spectral data were found to be identical to those of hispidulin (Kartnig, 1977).

All the isolated compounds were tested in the Oxidative Stability Instrument at 110°C in lard. Royleanonic acid and hispidulin at 0.04% (w/w) significantly prolonged the induction period of oxidation in the presence or absence of 10 ppm ferric ions. No significant antioxidant effect was observed with eupatorin (Table 2). Contrary to the original extract, the activity of hispidulin showed almost no increase at concentrations from 0.02 to 0.12% (w/w). Though the crude extracts of *Salvia plebeia* R.Br possessed potent antioxidant activity, comparable to BHA and rosemary extracts (Weng, Ren, Duan, Ding & Jiang, 1998), the isolated pure phenols were much less active than the mixture. The protection factor was 2.0 for royleanonic acid and 1.2 for hispidulin, compared with 8.7 and 7.5 for the original extracts at 0.04% (w/w). Several researchers reported that, in some instances, the extracted mixtures had greater antioxidant activities than individual components (Gordon & An, 1995; Saucier & Waterhouse, 1999). As had been reported by Gordon and An (1995), some fractions of the chloroform extract of licorice were more active than α-tocopherol. But the protection factor of the most active pure compound (3-arylxoumarin) among the eight isolated compounds was 2.7, compared to 6.2 for α-tocopherol at 175 ppm tested on the Rancimat. Saucier and Waterhouse (1999) discovered that the antioxidant activities of phenols in grape could be enhanced by synergistic activity with other antioxidants. Though it may be possible that the compounds isolated were not those with the greatest antioxidant activity, synergistic effects may also greatly contribute to the high activities of the mixtures.

Table 2
antioxidant activity of isolated compounds at 0.04% (w/w) in lard[a]

Samples	Induction period (h) ± S.D.	
	Lard	Lard added with 10 ppm ferric ion
Control	2.1±0.2a	0.6±0.1a
Royleanonic acic	4.3±0.3b	0.9±0.1b
Hispidulin	3.3±0.2c	0.9±0.1b
Eupatorin	2.2±0.2a	0.6±0.1a

[a] Values with the same letter in the same column are not significantly different at $P < 0.05$. Ferric ions were in the form of ferric stearate.

The plant of the *Salvia* genus contains various diterpenoid antioxidants (Gu & Weng, 1997). The structure of royleanonic acid is similar to that of carnosic acid, which also possesses a carboxyl group on position 10 of the deterpenoid skeleton. Carnosic acid is the main

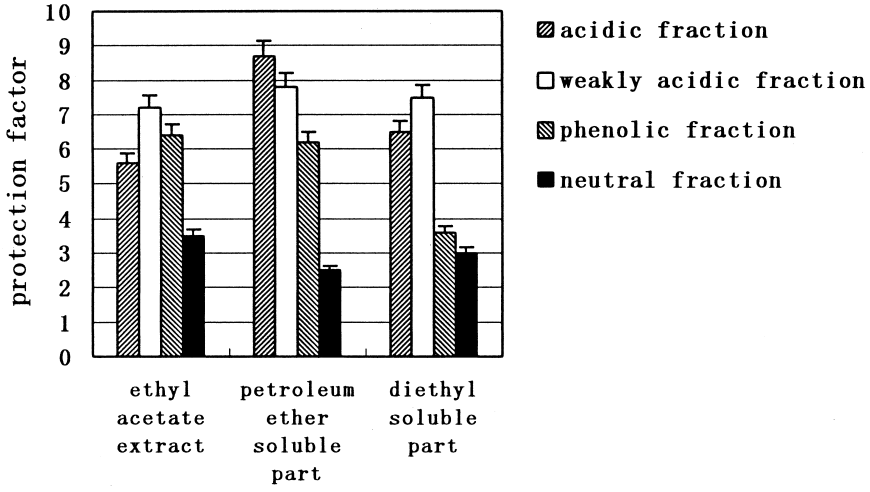

Fig. 3. Antioxidant activity of different acidic fractions of the extracts at 0.04% (w/w) in lard tested with Oxidative Stability Instrument.

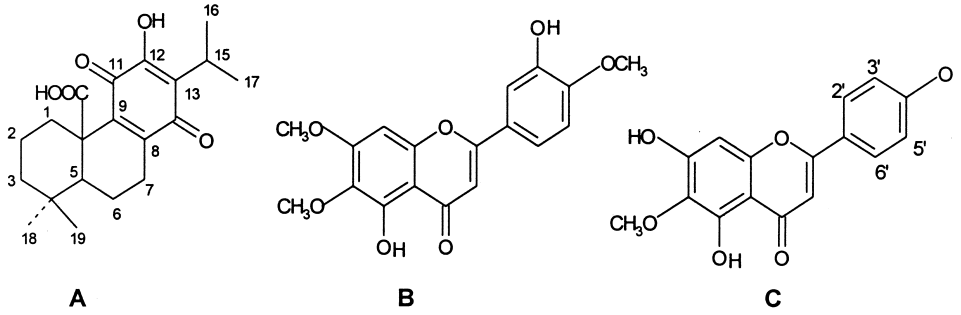

Fig. 4. Structures of isolated compounds: (A) royleanonic acid; (B) eupatorin; (C) hispidulin.

antioxidant component in rosemary and sage (Wenkert, Fuchs & McChesney, 1965). Its antioxidant activity was superior to BHA in bulk oil (Gu and Weng, 1997). Carnosic acid possessed *ortho*-dihydroxyl groups on the aromatic ring compared with hydroxy-*para*-benzoquinone in royleanonic acid. It is generally accepted that phenols inhibit the oxidation of the lipid by donating hydrogen atoms to scavenge free radicals. The *ortho*-dihydroxy-phenols could form more stable radicals after donating hydrogen atoms, so were more active than the *mono*-hydroxy-phenols as antioxidants (Zhang, 1998). Danshenxinkun B, which was isolated from *Salvia miltiorrhiza* mung, possessed an identical hydroxy-*para*-benzoquinone moiety to royleanonic acid. The antioxidant activity of Danshenxinkun B is shown by the protection factor of 1.8 obtained with the Rancimat (Zhang et al., 1990). It was rather weak and comparable to that of royleanonic acid. The hydroxy-*para*-benzoquinone could form a stable intramolecular hydrogen bond between the hydrogen of a hydroxyl and the oxygen of a neighboring carbonyl group. This bond makes the donation of hydrogen difficult and is adverse to antioxidant activity according to Zhang (1998).

Flavonoid antioxidants have been studied intensively in recent years for their multiple health-promoting properties. The relationship between structure and antioxidant activity of flavonoids has been studied by several groups (Das & Dereira, 1990). Flavonoids with the *ortho* 3′ 4′-dihydroxyl group were the most active. Hydroxyl groups on positions 3 or 7 contribute to activity more than at positions 5 or 8. Metal chelation is an important mechanism in the antioxidant action of *ortho*-dihydroxyflavonoids and 3 or 5 hydroxyl flavonoids. So it is reasonable that hispidulin exhibits significant antioxidant activity while eupatorin does not. The activity of royleanonic acid and hispidulin in lard challenged with ferric ions could be explained partly by their chelating effects. It is noteworthy that the antioxidant activity of flavones depends on the testing method used. In the evaluation of antioxidant activity of phenols, varied results can be obtained with different lipid substrates and with various measuring methods at different stages of lipid oxidation according to Hopia, Huang, Schwarz, German and Frankel (1996). The flavones seem not to be potential antioxidants when tested by accelerated oxidation methods such as OSI and Rancimat. Dziedzic and Hudson (1983) tested the antioxidant activity of apigenin (5,7,4′-trihydroxyflavone), daidzein (7,4′-dihydroxyisoflavone) and genistein (5,7,4′-trihydroxyisoflavone), using the Rancimat, in lard at 100°C. No activity was detected for apigenin and daidzein at up to the high concentration of 0.10% (w/w). Genistein showed weak activity (PF = 1.7 at 0.05% w/w). However, the antioxidant activities of daidzein and genistein were fairly good in linoleic acid emulsions and comparable to α-tocopherol in liposomes or LDL systems at a concentration of 200 μM (Gu, 2000). For the convenience of comparison, the antioxidant activities of our extracted mixtures and isolated compounds were based on the OSI method. As implied above, the antioxidant activities of the phenols tested with OSI or Rancimat did not reflect their effects in heterogeneous hydrophilic food or biological systems. The antioxidant activity of the isolated compounds, especially the novel compound royleanonic, in various systems, will be published elsewhere.

Acknowledgements

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