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Antioxidant Phenolic Constituents in Roots of *Rheum officinale* and *Rubia cordifolia*: Structure−Radical Scavenging Activity Relationships

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The phenolic constituents in the roots of *Rheum officinale* and *Rubia cordifolia* were identified with the aid of high-performance liquid chromatography and liquid chromatography−mass spectrometry and by comparison with authentic standards. A total of 17 hydroxyanthraquinones, gallic acid, and tannins were separated, and 14 of them were identified, being the main phenolic constituents present. Their antioxidant activity (Trolox equivalent antioxidant capacity) was evaluated using the improved 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt method. Hydroxyanthraquinones were the predominant antioxidant phenolic constituents in the roots of *R. cordifolia*, and tannins and gallic acid were the predominant antioxidant phenolic constituents in the roots of *R. officinale*. The structure−radical scavenging activity relationships of the tested hydroxyanthraquinones were systematically demonstrated as follows: Hydroxy groups on one benzene ring of the anthraquinone structure were essential for hydroxyanthraquinones to show activity, the *ortho*-dihydroxy structure in the hydroxyanthraquinone molecules could greatly enhance their radical scavenging effect, and glycosylation of the hydroxyanthraquinones reduced activity.

KEYWORDS: ***Rheum officinale*; *Rubia cordifolia*; phenolic constituents; radical scavenging activity; antioxidant activity; anthraquinones; hydroxyanthraquinones; structure−activity relationships**

INTRODUCTION

Anthraquinones, a group of plant phenolic compounds, normally occur in medicinal plants from two important genera, i.e., *Rheum* in the family Polygalaceae and *Rubia* in the family Rubiaceae (*1*). Both cultivated and wild species of *Rheum officinale* Bail, *Rheum palmatum* L., and *Rheum tanguticum* Maxim. ex Balf are distributed mainly in the northwestern and central regions of China. Their roots or rhizomes, called rhubarb in English or Dahuang in Chinese, have been used as an important traditional Chinese medicine for several centuries and are also herbal medicines in Japan and Korea (*2*, *3*). Chinese rhubarbs possess antibacterial, antiinflammation, antiviral, and anticancer effects. Traditionally, Dahuang is used for remedies of digestive system diseases, such as constipation (as a purgative agent), dysentery, gastritis, enteritis, gastric ulcer, and hepatitis, and also for treatment of various hemorrhages and trauma (*4*). Major active components in rhubarbs are hydroxyanthraquinones and their derivatives together with tannins. Many studies on the pharmacological action and bioactive constituents of the rhubarbs in Traditional Chinese Medicine have been reviewed (*2*).

Dahuang has also been employed in the treatment and prevention of some chronic diseases, such as inflammation, atherosclerosis (e.g., hyperlipidemia), diabetes, and cancer (*2*, *5*), which may result from damage to biomolecules (e.g., lipids, protein, and DNA) reacted with free radicals and active oxygen species. Japanese researchers reported that the methanolic extracts from five kinds of rhubarb demonstrated free radical scavenging effects (*3*). However, little information on antioxidant properties of Chinese rhubarbs has been published. In our recent studies (*6*), the methanolic extracts of *R. officinale* roots contained high levels of phenolic compounds (8.4 g gallic acid equivalent/100 g DW) and exhibited a very strong antioxidant activity (84.4 mM Trolox equivalent/100 g DW), while mean values of 112 traditional Chinese medicinal plants associated with anticancer were 3.9 g GAE/100 g DW for total phenolic content and 37.6 mM Trolox/100 g DW for total antioxidant capacity (*6*). It was also found that hydroxyanthraquinones, phenolic acids, and tannins were major phenolic compounds in this rhubarb, but these phenolic constituents have not yet been thoroughly identified.

The roots of *Rubia* species are not only used as herbal medicines (e.g., roots of *Rubia cordifolia* L.) but also as a source of natural dyes (e.g., madder, roots of *Rubia tinctorum* L.) (*2*, *7*). The roots of *R. tinctorum* L. have been used to dye textiles in many parts of the world since ancient times and are also

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used as food colorants in certain countries. *R. cordifolia* L., called Qiancao in Chinese, is cultivated mainly in northern China. The roots of *R. cordifolia* are used as a traditional Chinese herbal medicine mainly to treat hemorrhages, chronic bronchitis, trauma, and certain cancers (*2*, *5*). It has been reported that hydroxyanthraquinones are major bioactive compounds in *R. cordifolia* roots (*5*, *8*).

The antioxidant activity of the phenolic constituents is related to their structures. However, the phenolic constituents in the roots of *R. officinale* and *R. cordifolia* have not been identified completely. The structure−antioxidant activity relationships of the phenolic constituents from the roots of *R. officinale* and *R. cordifolia* have not yet been investigated, especially the structure−activity relationships of many kinds of hydroxyanthraquinones. The objectives of this study were (i) to identify and elucidate chemical structures of major phenolic constituents from the roots of *R. officinale* and *R. cordifolia* by comparison with authentic standards and with the aid of high-performance liquid chromatography photodiode array (HPLC-PDA) and liquid chromatography−mass spectrometry (LC-MS) and (ii) to investigate their structure−activity relationships on the basis of radical scavenging activity assay using the improved 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt ($ABTS^{\bullet+}$) method.

MATERIALS AND METHODS

Plant Material. Roots of *R. officinale* Bail (Dahuang) and *R. cordifolia* L. (Qiancao) were purchased (200 g per sample) in a well-known market for Chinese herbal medicines in Qichun, Hubei, China. These medicinal plants were traditionally harvested and processed and naturally dried in 2002.

Chemicals and Regents. ABTS and potassium persulfate were purchased from Sigma/Aldrich (St. Louis, MO), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and bovine serum albumin (BSA) were purchased from Fluka Chemie AG (Buchs, Switzerland), and HPLC grade organic reagents and formic acid were purchased from BDH (Dorset, England). Authentic standards of gallic acid, anthraquinone, and different hydroxyanthraquinone aglycones (emodin, aloe-emodin, rhein, chrysophanol, physcion, alizarin, purpurin, pseudopurpurin, 1,4-dihydroxyanthraquinone, 2,3-dihydroxyanthraquinone, 2,6-dihydroxyanthraquinone, 1,5-dihydroxyanthraquinone, etc.) were obtained from Sigma/Aldrich and Fluka.

Extraction and Sample Preparation of Phenolic Compounds. The dried roots of *R. officinale* and *R. cordifolia* were ground to a fine powder. Extraction, isolation, and purification of the tested phenolic compounds followed previous methods (*6*) with little modification. The ground samples were extracted with 80% methanol at 35 °C for 24 h in a water bath shaker (Techne, United Kingdom). Primary separation was conducted on a 100 cm × 2.5 cm i.d. Sephadex LH-20-100 column. The final purification was carried out by preparative HPLC with different chromatographic conditions. The completely purified phenolic compound samples were freeze-dried in a Heto FD3 freeze-dryer (Heto-Holten A/S, Denmark, United States) and used for the antioxidant activity assay.

HPLC System and Conditions. The HPLC apparatus consisted of a HP 1100 HPLC System (Hewlett-Packard, Waldbronn, Germany), equipped with a binary pump and a diode array detector. Preparative HPLC was conducted on a 250 mm × 9.4 mm i.d., 5 μm, Zorbax SB-C18 column (Agilent Technologies, Palo Alto, CA). Chromatographic conditions were as follows: solvent A, 2.5% aqueous formic acid, and solvent B, MeOH. Different elution gradients were used as follows. *R. officinale*: 0 min, 95% A; 15 min, 70% A; 80 min, 0% A (100%, B). *R. cordifolia*: 0 min, 50% B; 40 min, 100% B. The injection volume was 100 μL, and a flow rate of 3.5 mL/min was used. The separations were monitored at 280 and 435 nm. Analytical HPLC was conducted on a 250 mm × 4 mm i.d., 5 μm, Nucleosil 100-5 C18 column with 4 mm × 4 mm i.d., 5 μm, Nucleosil 5 C18 guard column (Agilent Technologies). Detailed chromatographic conditions have been described in a previous report (*6*).

LC-MS Instrumentation and Conditions. An LC-MS-2010A system (Shimadzu) consisting of a LC binary pump (LC-10ADvp), autosampler (SIL-10Avp), column oven, PDA, central controller, and single quadrupole MS detector with ESI (electrospray) interface was employed. A 250 mm × 2.0 mm i.d., 5 μm, C_{18} ODS-VP column (Nomura Chemical Co. Ltd., Seto, Japan) was used. LC conditions were as follows: solvent A, 0.1% formic acid, and solvent B, MeOH with 0.1% formic acid. A gradient elution was carried out as follows: 0 min, 20% B; 50−70 min, 100% B; 71−80 min, 20% B. The total flow rate of the mobile phase was 0.2 mL/min. The LC elute was introduced directly into the ESI interface without flow splitting. The ESI voltage was 4.5 kV in the positive mode and 3.5 kV in the negative mode. A nebulizing gas of 1.5 L/min and a drying gas of 10 L/min were applied for ionization (nitrogen in both cases).

Estimation of Phenolic Content. Relative contents of individual hydroxyanthraquinones and gallic acid were determined using HPLC and expressed as a percentage of peak area. Estimation of total tannins was conducted by the protein (BSA) precipitation assay method as described in our previous study (*6*). The total phenolic content was estimated by the Folin−Ciocalteu colorimetric method (*6*, *9*).

Radical Scavenging Activity Assay. The free radical scavenging activity assay was conducted using the improved $ABTS^{\bullet+}$ method (*6*, *10*). All samples of the tested phenolic compounds were diluted with 80% ethanol so as to give 20−80% inhibition of the blank absorbance with 0.1 mL of sample. $ABTS^{\bullet+}$ solution (3.9 mL; absorbance of 0.70 ± 0.05) was added to 0.1 mL of the tested samples and mixed thoroughly. After 6 min, the absorbance of the reactive mixture was immediately recorded at 734 nm. Trolox standard solution (final concentration 0−15 μM) in 80% ethanol was prepared, and its activity was determined under the same conditions. The absorbance of the resulting oxidized solution was compared to that of the calibrated Trolox standard. Results were expressed in terms of TEAC (Trolox equivalent antioxidant capacity) (mM). Assays of all samples were conducted in triplicate and averaged. All results were calculated as means ± standard deviation (SD). Differences between means of data were compared by least significant difference (LSD) calculated using the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Differences at $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Identification of Phenolic Constituents in the Tested Roots. Bioactive constituents from the two genera *Rheum* and *Rubia* are quite complex. The chemical structures of many constituents from these two genera are known (*1*, *5*, *7*). In this study, RP-HPLC and LC-MS were mainly applied to identify phenolic constituents from the roots of *R. officinale* and *R. cordifolia*. Hydroxyanthraquinones and their derivatives were major phenolic constituents identified in the tested roots of *R. officinale* and *R. cordifolia*. Identification of major hydroxyanthraquinones was ascertained by cochromatography with authentic samples, by comparison with literature data, and by LC-MS measurements. **Figures 1** and **2** display typical HPLC profiles of methanolic extracts from the roots of *R. officinale* and *R. cordifolia*, respectively. Major hydroxyanthraquinones separated in the tested roots are listed in **Table 1**. The chemical structures of identified hydroxyanthraquinones are summarized in **Table 1**.

According to the UV/vis spectra, chromatographic profiles, and MS analysis and by comparison with anthraquinone standards, the peaks 8−12 isolated from *R. officinale* (**Figure 1**) were identified as aloe-emodin (8), rhein (9), emodin (10), chrysophanol (11), and physcion (12), which are common hydroxyanthraquinone aglycones in the plants of the family Polygalaceae. The peaks 15−18 isolated from *R. cordifolia* (**Figure 2**) were identified as pseudopurpurin (15), munjistin

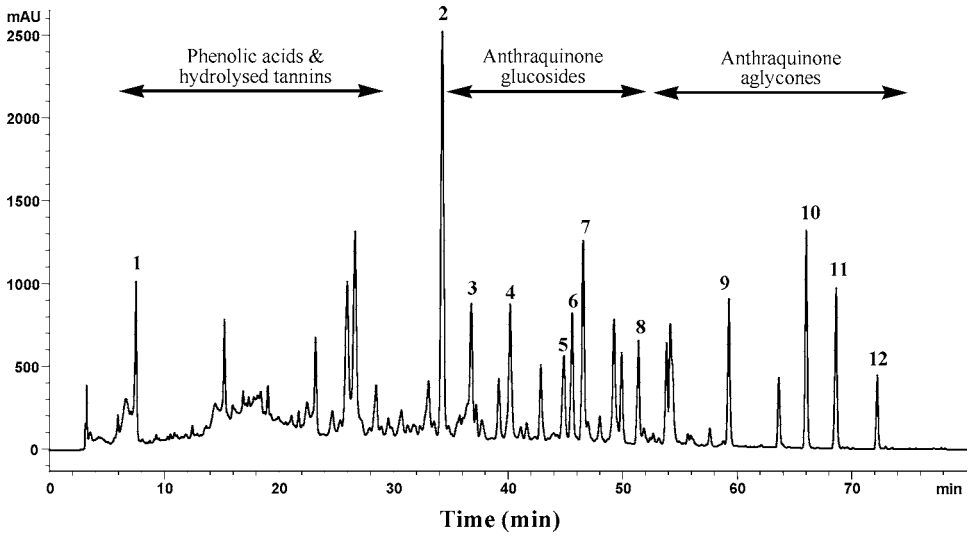


Figure 1. Preparative HPLC elution profile of methanolic extract of *R. officinale* roots. Elution monitored at 280 nm. Peaks: 1, gallic acid; 2–4, unelucidated anthraquinone-glucosides; 5, chrysophanol-1-*O*-β-D-glucopyranoside; 6, chrysophanol-8-*O*-β-D-glucopyranoside; 7, emodin-1-*O*-β-D-glucopyranoside; 8, aloe-emodin; 9, rhein; 10, emodin; 11, chrysophanol; and 12, physcion. Peak numbers correspond to the numbers in **Table 1**.

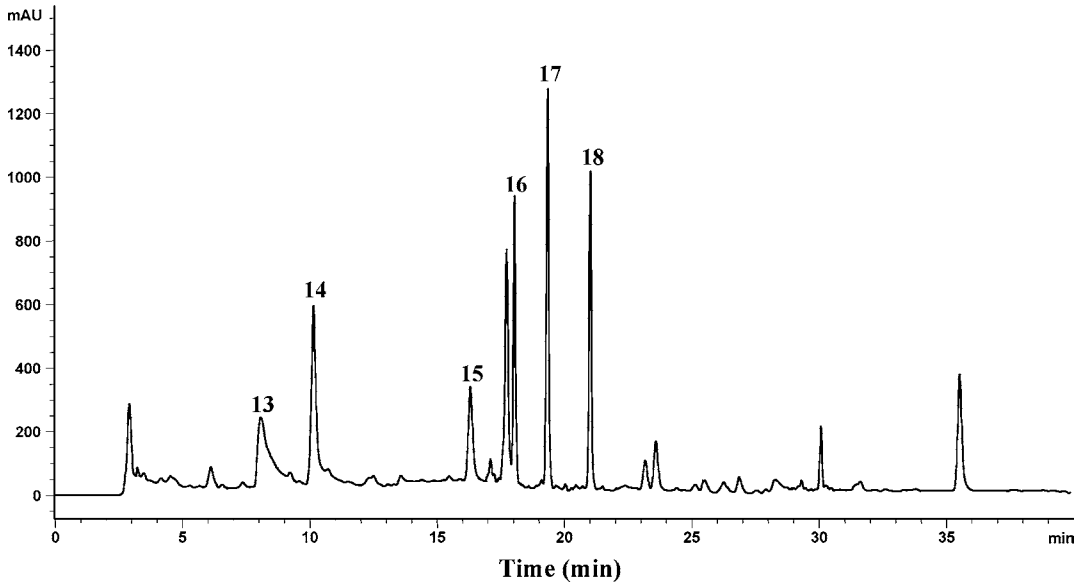


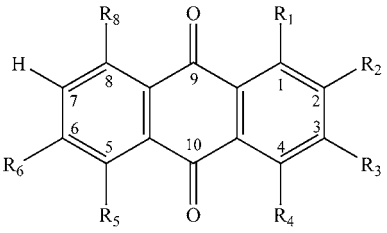
Figure 2. Preparative HPLC elution profile of methanolic extract of *R. cordifolia* roots. Elution monitored at 280 nm. Peaks: 13, ruberythric acid; 14, alizarin-2-*O*-β-D-glucopyranoside; 15, pseudopurpurin; 16, munjistin; 17, alizarin; and 18, purpurin. Peak numbers correspond to the numbers in **Table 1**.

(16), alizarin (17), and purpurin (18), which are typical hydroxyanthraquinone aglycones in the plants of the family Rubiaceae. RP-HPLC elution order (retention time R_t, **Table 1** and **Figures 1** and **2**) suggested that hydroxyanthraquinone aglycones were normally retained significantly longer than their glucosides because the polarity of their glucosides was much higher than that of their respective aglycones. The peaks 2−7 (**Figure 1**) and peaks 13 and 14 (**Figure 2**) were identified as hydroxyanthraquinone glucosides, such as chrysophanol-1-*O*-β-D-glucopyranoside (5), chrysophanol-8-*O*-β-D-glucopyranoside (6), emodin-1-*O*-β-D-glucopyranoside (7), ruberythric acid (13), and alizarin-2-*O*-β-D-glucopyranoside (14). The peaks 2−4 were also tentatively identified as hydroxyanthraquinone glucosides based on their UV/vis spectra, typical fragments (MS data), retention times, and comparison with literature data (**Table 1**), but their structures could not be completely elucidated yet.

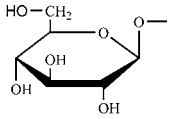
Interestingly, there were obvious differences in hydroxyanthraquinone structures between *R. officinale* and *R. cordifolia*. As seen **Tables 1** and **2**, in *R. officinale*, hydroxy groups and other substituents were usually located on both two benzene rings of the basic structure of anthraquinones, but in *R. cordifolia*, hydroxy groups and other substituents were generally located on a single benzene ring of the anthraquinone structure. The structural differences of hydroxyanthraquinones from *R. officinale* and *R. cordifolia* would significantly affect the radical scavenging activities of the respective plant extracts.

Other phenolic constituents were also detected in the tested roots of *R. officinale*, such as tannins and gallic acid (**Figure 1**). Peak 1 was identified as gallic acid according to the corresponding standard. Tannins, complex polyphenol compounds, were dominant constituents in the roots of *R. officinale*, and their total content reached 8.7% (**Table 1**). It was reported that the tannins from *R. officinale* are complex mixtures of hydrolyzable tannins and condensed tannins (*8*). Hydrolyzable tannins usually contain a central core of polyhydric alcohol (e.g., glucose), which is esterified by gallic acid or hexahydroxydiphenic acid (*11*). The peaks from 14 to 27 min (R_t) in **Figure 1** were not more closely identified in this study but might be

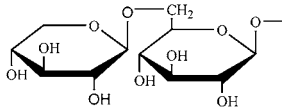
Table 1. Identification and Chemical Structures of Hydroxyanthraquinones and Other Phenolic Constituents from Roots of *R. officinale* and *R. cordifolia*



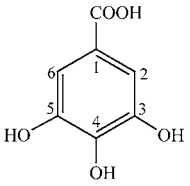
Anthraquinone structure



β-D-glucopyranosyl (Glc)



β-D-primeverosyl (Prv)



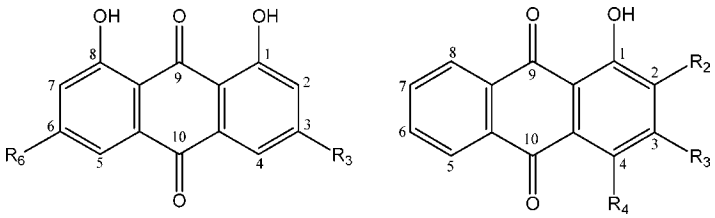
Gallic acid

constituent[a]			substituent position								UV/visible
number	name	R_t (min)[b]	R_1	R_2	R_3	R_4	R_5	R_6	R_8	M_w [c]	λ_{max} (nm)
			hydroxyanthraquinones from roots of *R. officinale*								
1	gallic acid	7.53	(1-COOH; 3,4,5-OH)							170	270
2	anthraquinone glucoside	34.28	(unelucidated)							462	225, 258, 286sh, 418
3	anthraquinone glucoside	36.80	(unelucidated)							614	
4	anthraquinone glucoside	40.19	(unelucidated)							418	222, 261, 282sh, 416
5	chrysophanol-1-*O*-Glc	44.88	Glc	H	CH_3	H	H	H	OH	416	220, 260, 284sh, 411
6	chrysophanol-8-*O*-Glc	45.61	OH	H	CH_3	H	H	H	Glc	416	221, 260, 284sh, 410
7	emodin-1-*O*-Glc	46.56	Glc	H	CH_3	H	H	H	OH	432	222, 257, 282sh, 410
8	aloe-emodin	51.38	OH	H	CH_2OH	H	H	H	OH	270	226, 256, 272, 288, 433
9	rhein	59.28	OH	H	COOH	H	H	H	OH	284	228, 258, 428
10	emodin	66.04	OH	H	CH_3	H	H	OH	OH	270	221sh, 254, 266, 286, 435
11	chrysophanol	68.65	OH	H	CH_3	H	H	H	OH	254	258, 284, 431
12	physcion	72.23	OH	H	CH_3	H	H	OCH_3	OH	284	263, 288, 432
			hydroxyanthraquinones from roots of *R. cordifolia*								
13	ruberythric acid	8.31	OH	Prv	H	H	H	H	H	534	227, 258, 276sh, 332, 417
14	alizarin-2-*O*-Glc	10.20	OH	Glc	H	H	H	H	H	402	228, 260, 281sh, 421
15	pseudopurpurin	16.38	OH	OH	COOH	OH	H	H	H	300	257, 286, 488, 518sh
16	munjistin	18.09	OH	COOH	OH	H	H	H	H	284	249, 289, 422
17	alizarin	19.27	OH	OH	H	H	H	H	H	240	248, 278, 330sh, 429
18	purpurin	21.06	OH	OH	H	OH	H	H	H	256	256, 290, 478, 518sh
			other hydroxyanthraquinone standards								
19	anthraquinone (non hydroxy)		H	H	H	H	H	H	H		
20	1,4-dihydroxyanthraquinone		OH	H	H	OH	H	H	H		
21	2,3-dihydroxyanthraquinone		H	OH	OH	H	H	H	H		
22	2,6-dihydroxyanthraquinone		H	OH	H	H	H	OH	H		
23	1,5-dihydroxyanthraquinone		OH	H	H	H	OH	H	H		

[a] The numbers in the table coincide with the numbers in the text and the peak numbers in the figures and **Table 2**. Glc, β-D-glucopyranosyl; Prv, β-D-primeverosyl. [b] R_t, retention time of phenolic constituents isolated by HPLC. [c] Measured by LC-MS. Major MS spectra/fragment data (*m*/*z*) at positive mode and negative mode of parts of peaks. Peak 2: 293.0, 417.1, 485.1 $[M + Na]^+$, 461.1 $[M - H]^-$. Peak 3: 445.0, 637.0 $[M + Na]^+$, 613.0 $[M - H]^-$. Peak 5 (chrysophanol-1-*O*-glucoside): 439.1 $[M + Na]^+$, 414.9 $[M - H]^-$, 252.9, 483.0 $[M + HCOONa - H]^-$. Peak 6 (chrysophanol-8-*O*-glucoside): 439.1 $[M + Na]^+$, 415.1 $[M - H]^-$, 252.9, 483.1 $[M + HCOONa - H]^-$. Peak 7 (emodin-1-*O*-glucosied): 455.1 $[M + Na]^+$, 431.1 $[M - H]^-$. Peak 8 (aloe-emodin): 271.1 $[M + H]^+$, 268.9 $[M - H]^-$. Peak 9 (rhein): 285.0 $[M + H]^+$, 282.9 $[M - H]^-$. Peak 10 (emodin): 271.0 $[M + H]^+$, 268.9 $[M - H]^-$. Peak 13 (ruberythric acid): 241.2, 535.1 $[M + H]^+$, 239.3, 533.1 $[M - H]^-$. Peak 17 (alizarin): 241.1 $[M + H]^+$, 239.3 $[M - H]^-$.

the hydrolyzed components of tannins in the roots of *R. officinale*. Therefore, these peaks (14−27 min) were collected together and freeze-dried. The collected samples could be further hydrolyzed into gallic acid and glucose, which were easily identified by HPLC and a common chemical method. Through determination of total phenolic content, the collected samples contained surprisingly high levels of phenolics, indirectly confirming that they were tannin components.

Table 2. Radical Scavenging Activity of Hydroxyanthraquinones and Other Phenolic Constituents Isolated from Roots of *R. officinale* and *R. cordifolia*[a]



Hydroxyanthraquinones from Roots of *R. officinale*			
constituent		content	radical scavenging
number[b]	name	(%)[c]	activity (mM)[d]
2	anthraquinone glucoside	6.8	0.172 ± 0.003
5	chrysophanol-1-O-β-D-glucopyranoside	1.7	ND
6	chrysophanol-8-O-β-D-glucopyranoside	2.2	ND
7	emodin-1-O-β-D-glucopyranoside	3.0	0.171 ± 0.002
8	aloe-emodin (R_3 = CH_2OH; R_6 = H)	1.5	0.173 ± 0.001
9	rhein (R_3 = COOH; R_6 = H)	1.9	0.174 ± 0.001
10	emodin (R_3 = CH_3; R_6 = OH)	2.6	0.172 ± 0.002
11	chrysophanol (R_3 = CH_3; R_6 = H)	1.9	0.170 ± 0.001
12	physcion (R_3 = CH_3; R_6 = OCH_3)	0.8	0.171 ± 0.002
	other phenolics from roots of *R. officinale*		
1	gallic acid (1-COOH; 3,4,5-OH)	2.2	3.613 ± 0.043
	tannins	8.7[e]	233.6 ± 1.92[f]
	crude extracts from *R. officinale*	NA	88.6 ± 6.4[f]

Hydroxyanthraquinones from Roots of *R. cordifolia*			
constituent		content	radical scavenging
number[b]	name	(%)[c]	activity (mM)[d]
13	ruberythric acid (R_2 = primeverosyl)	9.7	0.219 ± 0.007
14	alizarin-2-O-β-D-glucopyranoside	8.2	0.238 ± 0.010
15	pseudopurpurin (R_2, R_4 = OH; R_3 = COOH)	6.8	1.216 ± 0.011
16	munjistin (R_2 = COOH; R_3 = OH; R_4 = H)	7.7	0.575 ± 0.007
17	alizarin (R_2 = OH; R_3, R_4 = H)	9.5	1.019 ± 0.008
18	purpurin (R_2, R_4 = OH; R_3 = H)	8.4	1.680 ± 0.009
	crude extracts from *R. cordifolia*	NA	24.9 ± 0.036[f]
	other hydroxyanthraquinone standards		
19	anthraquinone (without hydroxyl groups)		0.171 ± 0.001
20	1,4-dihydroxyanthraquinone (quinizarin)		0.516 ± 0.003
21	2,3-dihydroxyanthraquinone		0.309 ± 0.001
22	2,6-dihydroxyanthraquinone		0.182 ± 0.002
23	1,5-dihydroxyanthraquinone		0.184 ± 0.001
	$LSD_{0.05}$[g]		0.229

[a] The numbers in the table coincide with the numbers in the text and the peak numbers in the figures and **Table 1**. [b] Results are means ± SD of triplicate measurements. ND, not determined; NA, not applicable. [c] Individual hydroxyanthraquinone content was expressed as a percentage of peak area determined by HPLC. [d] Radical scavenging activity was assayed by $ABTS^{\bullet+}$ method and expressed as total equivalent trolox equivalent (TEAC) (mM). [e] Tannin content was expressed as percentage of dry weight. [f] Total antioxidant activity of tannins and crude extracts was also measured by $ABTS^{\bullet+}$ method but expressed as mM trolox/100 g dry weight. [g] LSD ($p < 0.05$) was used for difference comparison among means of various hydroxyanthraquinones and gallic acid.

Radical ScavengingActivity of Phenolic Constituents in the Tested Roots. Crude extracts from the roots of *R. officinale* contained much more phenolic constituents (total phenolic content, 8.37 g gallic acid equivalent/100 g DW) than those of *R. cordifolia* (2.11 g/100 g DW). Crude extracts from *R. officinale* (**Table 2**) had a much higher total antioxidant activity (88.6 mM Trolox equivalent/100 g dry weight) than those from *R. cordifolia* (24.9 mM Trolox/100 g DW). Hydroxyanthraquinones were the only major kind of phenolic constituent in the roots of *R. cordifolia*, but in the roots of *R. officinale*, there were three major kinds of phenolic constituents, i.e., tannins, hydroxyanthraquinones, and phenolic acids (gallic acid). Six tested hydroxyanthraquinones in the roots of *R. cordifolia* possessed radical scavenging activity (0.22−1.68 TEAC units) (**Table 2**), predominantly contributing to total antioxidant capacity of the crude extracts. However, in the roots of *R. officinale*, tannins (233.6 mM Trolox/100 g DW) and gallic acid (3.6 TEAC units) exhibited significantly stronger radical scavenging activities than seven tested hydroxyanthraquinones (0.170−0.174 TEAC units) (**Table 2**). The predominant contribution to total antioxidant activity would therefore be expected from tannins and gallic acid in the roots of *R. officinale*. It was estimated that approximately 85−90% of total antioxidant capacity of the crude extracts from *R. officinale* would be due to tannins.

Structure−Radical Scavenging Activity Relationships of Hydroxyanthraquinones. Generally, the number and positions of hydroxy groups or other hydrogen-donating groups in the phenolic molecular structures and the glycosylation of the phenolic aglycones influenced their antioxidant activity. The structure−antioxidant activity relationships of flavonoids and phenolic acids are well-known (*12*, *13*), but the structure−

activity relationships of hydroxyanthraquinones have not been studied so far. As described above, hydroxyanthraquinones were major phenolic constituents in the roots of *R. officinale* and *R. cordifolia*. To clarify their structure−activity relationships, the relevant pure hydroxyanthraquinones were required. The majority of hydroxyanthraquinone aglycones tested in the study could be obtained from commercial companies, but the hydroxyanthraquinone glucosides were not available. Samples of pure hydroxyanthraquinone glucosides were obtained through separation and purification by preparative HPLC. Also, to confirm the structure−activity more completely, further hydroxyanthraquinone standards were included (**Table 2**), which were not found in the roots investigated.

TEAC values (mM) of different hydroxyanthraquinones from *R. officinale* and *R. cordifolia* and other hydroxyanthraquinone standards are listed to compare their radical scavenging activity (**Table 2**). It was found that, surprisingly, all hydroxyanthraquinones (e.g., emodin, aloe-emondin, rhein, chrysophanol, etc.) from *R. officinale* had low TEAC values (0.170−0.174 mM), similar to the TEAC value (0.171 mM) of anthraquinone, which, without any hydroxy groups, does not have a radical scavenging activity, indicating a very poor antioxidant activity. In contrast, all of the hydroxyanthraquinones (e.g., purpurin, pseudopurpurin, alizarin, etc.) from *R. cordifolia* had higher TEAC values (0.22−1.68 mM), representing an antioxidant activity or even potent activity. Their structural differences (**Table 2**) show that for the former, the hydroxy groups and other substituents were located on both benzene rings of the anthraquinone structure, but for the latter, the hydroxy groups and other substituents were located on a single benzene ring of the anthraquinone structure. Our findings suggested that the positions of hydroxy groups and other substituents on one or both sides of the anthraquinone structure significantly affected the radical scavenging activity of the hydroxyanthraquinones. Hydroxy groups and other substituents on one side of the anthraquinone structure resulted in a markedly increasing level of radical scavenging activity. Our results may explain that while some authors reported that hydroxyanthraquinones exhibited antioxidant activity, others reported that they had no antioxidant activity or only exhibit poor activity (*3*, *14*, *15*). In contrast, Yen et al. (*14*) reported that there was a significant scavenging effect of alizarin, emodin, and aloe-emodin on hydroxy radicals, but the present findings do not support that emodin and aloe-emodin have a radical scavenging activity. Hydroxy groups and other substituents of emodin and aloe-emodin molecules are on both rings of the anthraquinone structure and are also not *ortho*-dihydroxy structure. Such kinds of hydroxyanthraquinone structures showed poor activity in our $ABTS^{\bullet+}$ assay and also in the DPPH assay reported by Mastsuda et al. (*3*) and Ömür Demirezer et al. (*15*).

The present study also revealed the importance and influence of the *ortho*-diphenolic structure in the hydroxyanthraquinone molecules on their radical scavenging activity. Purpurin, pseudopurpurin, and alizarin had high TEAC values, i.e., 1.68, 1.22, and 1.02 mM, respectively, showing potent radical scavenging activity. According to their structures (**Tables 1** and **2**), all of them possessed the same structural features, i.e., an *ortho*-dihydroxy structure at C_1/R_1 and C_2/R_2. Other similar hydroxyanthraquinones, e.g., munjistin, 1,4-dihydroxyanthraquinone, and 2,3-dihydroxyanthraquinone, did not exhibit the *ortho*-dihydroxy structure, and their TEAC values ranged from 0.31 to 0.58 mM, obviously lower ($p < 0.05$) than those of purpurin, pseudopurpurin, and alizarin (1.02−1.68 mM). The results indicated that the *ortho*-diphenolic structure in the hydroxyanthraquinone molecules could markedly enhance their radical scavenging effects. This was similar to the results of Rice-Evans et al. (*12*) and Cao et al. (*16*) who reported that the *ortho*-dihydroxy structure in the B ring of flavonoid molecules caused potent antioxidant activity of flavonoids. Additionally, a hydroxy group at the C_4/R_4 position seemed to improve the radical scavenging activity of the hydroxyanthraquinones. Both purpurin and 1,4-dihydroxyanthraquinone, with a 4-hydroxy group, had higher TEAC values than other any hydroxyanthraquinones without a 4-hydroxy (munjistin and 2,3-dihydroxyanthraquinone).

The glycosylation of flavonoids reduced their activity when compared to the corresponding aglycones (*12*). In this study, it was also observed that the glycosylation of hydroxyanthraquinones diminished their radical scavenging activity. By comparison of hydroxyanthraquinone glucosides and their aglycones in the roots of *R. cordifolia* **(Table 2)**, the radical scavenging activity of ruberythric acid with primeverosyl (two sugar units) (0.22 mM) and alizarin-2-*O*-*β*-D-glucopyranoside (with one sugar unit) (0.24 mM) was obviously lower ($p < 0.05$) than those of their aglycones (pseudopurpurin, munjistin, alizarin, and purpurin, 0.58−1.68 mM). However, there were no obvious differences in radical scavenging activity between the hydroxyanthraquinone glucosides and their aglycones in the roots of *R. officinale*, possibly because all kinds of such hydroxyanthraquinones from *R. officinale* did not have a strong radical scavenging activity (∼0.17 mM); therefore, the expected differences were too small to be discerned.

In addition, tannins and gallic acid isolated from the roots of *R. officinale* showed a maximum radical scavenging activity in this study. Tannin molecules contain a range of phenolic hydroxy groups (*11*) and are excellent radical scavengers. Gallic acid (3,4,5-trihydroxybenzoic acid) is also a good radical scavenger. High concentrations of tannins and gallic acid with potent activity in the roots of *R. officinale* could explain the reason its crude extracts demonstrated such stronger antioxidant activities as compared to crude extracts from *R. cordifolia*.

In conclusion, 17 kinds of hydroxyanthraquinones, gallic acid, and tannins were separated and identified from the roots of *R. officinale* and *R. cordifolia*. They were the major phenolic constituents in the tested roots. This study is the first report to systematically reveal the structure−radical scavenging activity relationships of the tested hydroxyanthraquinones: Hydroxy groups on one benzene ring of anthraquinone structure were essential for hydroxyanthraquinones to show activity, the *ortho*-dihydroxy structure in the hydroxyanthraquinone molecules could greatly enhance their radical scavenging effect, and glycosylation of the hydroxyanthraquinones reduced activity.

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