Antioxidative Phenols and Phenolic Glycosides from *Curculigo orchioides*

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A new orcinol glucoside, orcinol-1-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (3), was isolated from the rhizomes of *Curculigo orchioides* GAERTN., together with seven known compounds: orcinol glucoside (1), orcinol-1-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside (2), curculigoside (4), curculigoside B (5), curculigoside C (6), 2,6-dimethoxyl benzoic acid (7), and syringic acid (8). The structures of these compounds were elucidated using spectroscopic methods. The antioxidant activities of these isolated compounds were evaluated by colorimetric methods based on their scavenging effects on hydroxyl radicals and superoxide anion radicals, respectively. All the compounds showed potent antioxidative activities and the structure–activity relationship is discussed.

Key words *Curculigo orchioides*; Hypoxidaceae; phenol; phenolic glycoside; antioxidative activity

Curculigo orchioides GAERTN. is a tiny herbal plant widely distributed in China, India, Malaya, Japan and Australia. Its rhizome, known as "Xianmao" in China, is a common kidney-Yang-reinforcing and anti-aging traditional Chinese medicine. It has the properties of warming kidney, invigorating Yang, expelling cold and eliminating dampness, and is used to treat impotence, enuresis, cold sperm, cold pain of back and knee, and numbness of the limb.[1,2] In India, the tuberous root of this plant is also considered to be tonic, alterative, demulcent, diuretic, and restorative, and is used as a poultice for itch and skin disease.[3] Previous phytochemical investigations on the rhizomes of this species revealed the presence of curculigoside,[4,5] curculigoside B,[5,6] curculigoside C,[5] orcinol glycosides,[4] 2,6-dimethoxyl benzoic acid, curculigines A—C,[7] curculigol,[8] curculigosaponins A—M,[9] 1,3,7-trimethylxanthine, daucosterol,[4] and aliphatic long-chain ketones.[10] Apart from curculigoside, which showed high phagocytic activity,[4] the bioactivities of these compounds from this plant were not clear. Venukumar and Latha reported that the methanol extract of *C. orchioides* served as an effective hepatoprotection agent with significant antioxidative activity, but the responsible antioxidative components are still unknown.[11]

In our screening program of searching for antioxidative natural substances from traditional Chinese medicine, the ethanol extract of *C. orchioides* showed some antioxidative activities. Fractionation of this extract with a D-101 resin column chromatography confined the antioxidative activities to the 20% and 40% ethanol eluates. The concentrations exhibited 50% inhibition (IC_{50}) on hydroxyl radicals and superoxide anion radicals were 3.42 and 4.17 mg/ml for the 20% eluate, and 1.56 and 2.48 mg/ml for the 40% eluate, respectively. Further bioassay-guided analysis led to the isolation of 8 phenols and phenolic glycosides from these two active eluates of the *C. orchioides* extract. In this paper, we report the isolation and structural elucidation of a new orcinol glycosides, orcinol-1-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**3**), along with seven known compounds obtained from this plant. The antioxidative activities of these 8 compounds were evaluated based on their scavenging effects on hydroxyl radicals produced by H_2O_2/Fe^{2+}, and superoxide anion radicals produced by xathine/xanthine oxidase systems, and compared with epigallocatechin gallate (EGCG), a known antioxidant.[16]

Results and Dicussion

The ethanol extract of the plant was re-suspended in water and partitioned with petroleum ether (60—90 °C). The water phase was filtered and the filtrate was loaded onto a D-101 resin column and then eluted sequentially with H_2O followed by 20%, 40%, 60%, 80% and 95% aqueous EtOH. The fractions eluted from 20% and 40% EtOH were separately chromatographed on silica gel and Sephadex LH-20 to afford compounds **1**—**3** and compounds **4**—**8**, respectively. Of the 8 compounds elucidated, the seven known compounds were subsequently identified as orcinol glucoside (**1**), orcinol-1-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside (**2**),[4] curculigoside (**4**), curculigoside B (**5**), curculigoside C (**6**),[5] 2,6-dimethoxyl benzoic acid (**7**),[7] and syringic acid (**8**),[12,13] by comparison of spectral data with the data reported in the literature. The purity of these compounds was confirmed by TLC and HPLC (purity >95% for all compounds).

Compound **3**, a colorless amorphous powder, had a molecular formula of $C_{18}H_{26}O_{11}$ as established by its ESI-MS (*m/z* 441.6 $[M+Na]^+$) and NMR data. It gave positive reactions with 3% $FeCl_3$ and Molish reagents. Its UV spectrum (MeOH) revealed characteristic absorptions of orcinol derivatives at 271 and 277 nm. The IR spectrum (KBr) showed absorptions for a hydroxyl moiety (3404 cm^{-1}) and a benzene ring (1601, 1466 cm^{-1}). The ^{1}H-NMR spectrum of **3** showed signals for a hydroxyl group at δ 11.35, three aromatic broad singlets at δ 7.15, 6.91, and 6.78 (each 1H, br s), and a methyl group at δ 2.31 (3H, s), corresponding to an aglycone moiety of orcinol.[3] These proton signals were in agreement with the relevant signals at δ_C 160.3 (s), 160.0 (s), 140.6 (s), 111.4 (d), 109.3 (d), 102.7 (d), and 21.8 (q) in the ^{13}C-NMR and DEPT spectra (Table 1). Moreover, the ^{1}H-, ^{13}C-NMR and DEPT spectral data suggested the presence of a D-glucosyl and a D-apiosyl moieties. Two anomeric signals at δ_H 5.60 (1H, d, J=7.2 Hz) and δ_C 102.9 (d); and δ_H 5.81 (1H, d,

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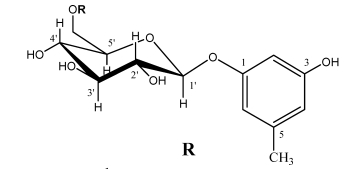
Table 1. ^{1}H- and ^{13}C-NMR Data for Compound **3** (400 and 100 MHz, Pyridine-d_5, ppm)

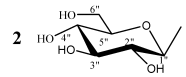
Position	δ_H	δ_C
Aglycone		
1		160.3 s
2	7.15 br s	102.7 d
3	11.35 br s (OH)	160.0 s
4	6.91 br s	109.3 d
5		140.6 s
6	6.78 br s	111.4 d
7	2.31 s	21.8 q
Glucosyl moiety		
1′	5.60 d (7.2)	102.9 d
2′	4.35 m	75.1 d
3′	4.35 m	78.7 d
4′	4.19 m	71.7 d
5′	4.23 m	77.4 d
6′	4.78 br d (10.0)	68.9 t
	4.21 (overlap)	
Apiosyl moiety		
1″	5.81 d (2.3)	111.2 d
2″	4.84 d (2.3)	77.9 d
3″		80.6 s
4″	4.65 d (9.3)	75.2 t
	4.41 d (9.3)	
5″	4.26 s	65.8 t

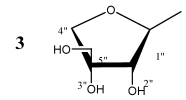
J=2.3 Hz) and δ_C 111.2 (d) showed both sugars to be β-oriented.[3,14]

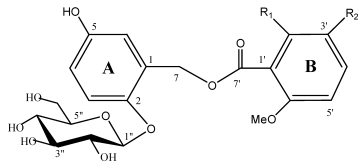
On the basis of ^{1}H–^{1}H COSY, HMBC and HMQC spectral analysis, the structure of **3** was further determined, and all the proton and carbon signals were fully assigned. In the HMBC spectrum (Fig. 2), the proton at δ_H 5.60 (1H, d, J=7.2 Hz, H-1′) correlated with C-1 (δ_C 160.3), indicating that the β-D-glucosyl moiety was located at C-1. The β-D-apiosyl moiety was attached to C-6′ of the glucose as evidenced by the HMBC cross-peaks between the proton at δ_H 5.81 (1H, d, J=2.3 Hz, H-1″) and C-6′ (δ_C 68.9). On the other hand, the following long-range correlation were observed: the proton at δ_H 7.15 (1H, br s, H-2) with C-4 (δ_C 109.3) and C-6 (δ_C 111.4); the signal at δ_H 6.91 (1H, br s, H-4) with C-3 (δ_C 160.0) and C-7 (δ_C 21.8); and the broad singlet at δ_H 6.78 (1H, br s, H-6) with C-7. The structure of the aglycone was thus confirmed. Consequently, compound **3** was determined as orcinol-1-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside.

The antioxidative activities of compounds **1**—**8** were estimated based on their scavenging effects on hydroxyl radicals produced by H_2O_2/Fe^{2+}, and superoxide anion radicals produced by xathine/xanthine oxidase systems. The results of these assays are given in Table 2. Compound **3** showed scavenging effects on hydroxyl radical and superoxide anion radical with IC_{50} values of 1.17 mM and 1.84 mM, slightly weaker than those of the positive control EGCG (0.43 mM, 0.53 mM, respectively). In the scavenging assay of hydroxyl radicals, compounds **6**, **4**, and **2** showed significant scavenging effects with IC_{50} values of 0.25, 0.54 and 0.87 mM, respectively, comparable with that of EGCG. In the scavenging assay of superoxide anion radicals, only compound **6**, with an IC_{50} value of 0.88 mM, exhibited comparable scavenging effect with that of EGCG. The antioxidative activities of compounds **1**—**3**, the orcinol glycosides, seemed related to the number and type of sugar chains in the molecular structures.

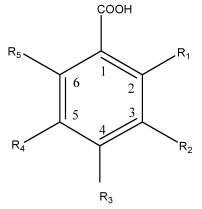








	R_1	R_2
4	OMe	H
5	OH	H
6	OMe	OH



	R1	R2	R3	R4	R5
7	OMe	H	H	H	OMe
8	H	OMe	OH	OMe	H

Fig. 1. The Structures of Compounds **1**—**8**

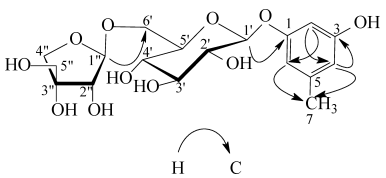


Fig. 2. The Key HMBC Correlations of **3**

Table 2. Hydroxyl Radical and Superoxide Anion Radical Scavenging Activities of Compounds **1**—**8**

Compound	IC_{50} (mM)	
	Hydroxyl radical	Superoxide anion
1	1.39	2.49
2	0.87	1.56
3	1.17	1.84
4	0.54	1.35
5	1.11	1.48
6	0.25	0.88
7	1.51	3.21
8	2.61	3.46
EGCG	0.43	0.53

Compounds **2** and **3**, containing two sugar moieties, exhibited better antioxidative activities than compound **1**, which contains only one sugar moiety. And it was also found that the glucopyranosyl group substituted at C-6′ position of

compound **2** showed better antioxidative activities than the apiofuranosyl group substituted at the same position of compound **3**. As for compounds **4**—**8**, the benzoic acid derivatives, their antioxidative activities were influenced by the 5-hydroxyl-2-*O*-*β*-D-glucopyranosyl benzyl moiety as well as the number and position of the hydroxyl and methoxyl substitutions in the molecular structures. Compounds **4**—**6** showed stronger scavenging effects on both hydroxyl radical and superoxide anion radical than did compounds **7** and **8**, suggested that the 5-hydroxyl-2-*O*-*β*-D-glucopyranosyl benzyl moiety played a key role in their antioxidative activities. The influence on their antioxidative activities of C-2′ methoxyl substitution in Compound **4** and **6** was greater than that of the hydroxyl substitution at the same position as in **5**, and the introduction of a hydroxyl group at C-3′ in compound **6** further increased its antioxidative activities. Moreover, it could be concluded from compounds **7** and **8** that the 2,6-dimethoxyl substitutions (**7**) showed more potent effects than did the 3,5-dimethoxyl derivative (**8**). These results also suggest that the antioxidative activities of *C. orchioides* are partly attributed to these phenols and phenolic glycosides.

Experimental

General Melting points were measured on an XT-4 micro-melting point apparatus and are uncorrected. IR spectra were recorded on a Nicolet Impact-410 instrument with KBr pellets. ESI-MS data were obtained on a FINNIGAN-MAT-95 mass spectrometer. 1D-NMR spectra were recorded on a DM500 instrument, and 2D-NMR spectra were obtained on a Bruker DRX-400 instrument. Column chromatography was performed on silica gel H (10—40 μm, Qingdao Marine Chemical Factory, Qingdao, Shandong, China), D-101 resin (Tianjin Farm Chemical Factory, Tianjing, China), and Sephadex LH-20 (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Materials The rhizomes of *Curculigo orchioides* GAERTN. were collected in Sichuan Province, People's Republic of China, in September 2000, and authenticated by Dr. Tong-Shui Zhou. A voucher specimen was deposited at the Herbarium of School of Life Sciences, Fudan University. Xanthine (X), xanthine oxidase (XO) and (−)-epigallocatechin gallate (EGCG) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). All other reagents were local products of analytical grade and were used without further purification.

Extraction and Isolation The air-dried and powdered rhizomes of *C. orchioides* (5.0 kg) were extracted twice each with 35 l of 90% EtOH under reflux for 2 h. The extracts were combined, evaporated and the aqueous residue (380 g) was re-suspended in 15 l of H_2O and partitioned with petroleum ether (60—90 °C, 3 l×5). The H_2O phase was filtered and the filtrate was loaded onto a column containing D-101 resin (1 kg), and then eluted sequentially with H_2O followed by 20%, 40%, 60%, 80% and 95% aqueous EtOH. The fraction eluted from 40% EtOH (19 g) was applied to a silica gel column (1.2 kg) which was eluted with $CHCl_3$–MeOH (10 : 1, 9 : 1, 8 : 1, 7 : 1, 6 : 1, 5 : 1 and 4 : 1) to give seven fractions (G_1—G_7). Each fraction was repeatedly separated and purified on a Sephadex LH-20 (100 g) column. Compounds **7** (800 mg) and **8** (440 mg) were obtained from fraction G_1. Compounds **4** (1.2 g) and **5** (40 mg) were obtained from fraction G_5. And compound **6** (20 mg) was isolated from fraction G_6. The fraction eluted from 20% EtOH (40 g) was subjected to another silica gel column (1.2 kg) and eluted with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5) to give five fractions (A—E). Fraction B was separated and purified repeatedly on a Sephadex LH-20 (100 g) column to yield compounds **1** (350 mg), **2** (440 mg), and **3** (50 mg).

Hydroxyl Radical Scavenging Assay The hydroxyl radical scavenging assay was carried out by measuring the clearance of test compounds for hydroxyl radicals generated from the H_2O_2/Fe^{2+} system. The reaction mixture contained 10 mmol salicylic acid, 4 mmol $FeSO_4$ dissolved in 2 mmol EDTA, 0.4 mol phosphate buffered saline (pH 7.4). The samples at various concentrations were added. Then 20 mmol H_2O_2 was added to activate the reaction. After co-incubation at 37 °C for 2 h, the absorption value at 510 nm was measured with a Shimadzu UV-260 spectrophotometer. EGCG was used as a positive control and the solvent dissolving samples was used as blank. Reactions were carried out in triplicates. The relative activity was calculated from the ratio of sample treated with all compounds *versus* blank at the same concentration and time. And the effective activity of each sample was expressed in terms of the median inhibitory concentration or IC_{50}.

Superoxide Anion Radical Scavenging Assay The scavenging assay of superoxide anion radicals was evaluated as previously described by Beauchamp.[15] The reaction mixture contained 1 mmol xanthine (X), 10 μmol·min^{-1}·l^{-1} xanthine oxidase (XO), 25 μmol nitroblue tetrazolium (NBT) and 0.1 mmol EDTA, dissolved in 50 mmol sodium carbonate buffer (pH 10.2) to the final volume of 3.0 ml. The various concentrations of the test compounds were added into this reaction system. EGCG was used as positive control and the solvent dissolving samples was used as blank. The mixtures were incubated for 30 min at 25 °C. And the absorbance of blue product was measured at 560 nm. The relative activity was calculated from the ratio of sample treated with all compounds *versus* blank at the same concentration and time. And the effective activity of each sample was expressed in terms of IC_{50}.

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