

Three Isoflavonoid Glycosides from the Rhizomes of *Achyranthes bidentata* and their Protective Effects on H₂O₂ Induced H9c2 Cardiomyocytes Injury

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Abstract: A new isoflavonoid glycoside, achyranthoside C (**1**), along with two known compounds (**2** and **3**) were isolated from the rhizomes of *Achyranthes bidentata*. Compounds **1-2** were two rare natural isoflavonoid glycosides with C8 hydroxymethylene derivatization. Their structures were elucidated through comprehensive 1D and 2D NMR, UV, IR, and HRMS analyses. Bioassay results showed all the three compounds had obviously cell-protective effects against H₂O₂-induced H9c2 cardiomyocytes injury in a concentration -dependent manner.

Keywords: *Achyranthes bidentata*; isoflavonoid glycosides; antioxidant activity. © 2021 ACG Publications. All rights reserved.

1. Introduction

Achyranthes bidentata Blume is a perennial herbaceous plant of Amaranthaceae, which is widely planted in Henan province of China and commonly called as “Huai-Niu-Xi” [1]. Huai-Niu-Xi is also known as one of “Four Famous Huai Medicines” and has been widely used in the clinical

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applications for thousands of years due to its medical value for tonifying liver and kidney, strengthening muscles and bones [2]. For the medicinal parts of *A. bidentata*, there are two main records in the ancient herbal literatures, including removing the rhizomes (Lutou) from the roots, and not removing the rhizomes [3]. The Chinese Pharmacopoeia defined the dried roots without rhizomes as the medicine part. However, the scientific basis for removing the rhizomes of *A. bidentata* is still unclear. Besides that, the removal of rhizomes is time-consuming and power-wasting, and wasteful of plant resources. Accordingly, the systematic study on the phytochemistry and pharmacological activities of both the rhizomes and roots of *A. bidentata* to explore whether to remove or not is necessary and urgent.

At present, there are large number of studies focusing on the roots of *A. bidentata*, which were revealed to have a variety of natural substances, including phytosteroids [4-5], triterpenoid saponins [6], flavonoids [7-8] and lignans [9], and show many activities including anti-inflammatory [8], antioxidant [10] and anti-tumor effects [11-13]. However, the research on the rhizomes of *A. bidentata* is much fewer. In this study, the phytochemical and pharmacological investigation of the rhizomes of *A. bidentata* was carried out firstly, which resulted in the isolation of one new isoflavonoid glycosides, namely achyranthoside C (**1**), and two known isoflavonoid glycosides (**2** and **3**) (Figure 1). The protective effect of **1-3** against hydrogen peroxide (H_2O_2) induced H9c2 cardiomyocyte injury were evaluated. The data showed they all had obviously cell-protective effects, and 25 and 50 μM of **1** and **2** displayed activity equivalent to or higher than positive control vitamin C.

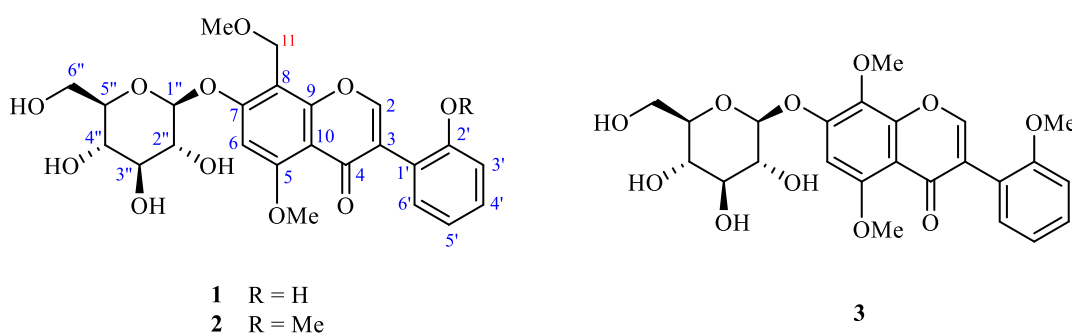


Figure 1. Chemical structures of compounds **1-3**

2. Materials and Methods

2.1. Apparatus and Reagents

UV spectrum was measured on a Shimadzu double-beam 210A spectrophotometer. IR spectrum was recorded on a Thermo Nicolet iS5 spectrometer. HRESIMS data was carried out on a Thermo UPLC-LTQ orbitrap XL Spectrometer. 1D and 2D NMR spectra were recorded on Bruker Avance III 500-NMR instruments with TMS as the internal standard. Silica gel (100–200 and 200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), ODS- C_{18} (50 μM , YMC, Kyoto, Japan), Waters e2695 series HPLC, semi-prep HPLC (QBH LC-52, Beijing Qingbohua Technology Co., Ltd., Beijing, China) with a YMC (250 mm \times 10 mm, I.D. 5 μM) column were used for chromatographic separations.

2.2. Plant Material

Achyranthes bidentata was collected in September 2019 from Henan province of China. The species was identified by Professor Liping Dai of Henan University of Chinese Medicine. The voucher specimen (no. 2019-0916) was deposited in the Key Laboratory of Traditional Chinese Medicine Chemistry and Resources of Henan province.

2.3. Extraction and Isolation

The dried materials (40.0 kg) of *A. bidentata* rhizomes were cut into small pieces, and were refluxed with 70% EtOH ($2 \times 120 \text{ L} \times 2 \text{ h}$). The extract was evaporated under reduced pressure using a rotary evaporator. Non-alcoholic smell residue (2401 g) was dissolved in water and then partitioned with EtOAc (223.0 g) and *n*-butanol (639.0 g), successively. *n*-Butanol fraction was chromatographed on silica gel column (100–200 mesh), eluted with dichloromethane-methanol system (1:0~0:1) to afford six fractions (Fr. 1 ~ 6), monitored by TLC. Fr. 3 (19.0 g) was subjected to separation with an ODS column eluted with MeOH-H₂O (15% ~ 100%), to afford Fr. 3A~3D. Fr.3B (31.5 mg) was purified by semi-prep HPLC with 17% ACN-H₂O (3 mL/min) to afford compound **1** (4.8 mg) at 36 min. Fr. 3C (200.0 mg) was further submitted to semi-prep HPLC with 20% ACN-H₂O, and compounds **2** (8.0 mg), **3** (16.0 mg) were respectively obtained at 35 and 42 min.

2.4. Spectral Data

Achyranthoside C (1): Yellow amorphous powder; $[\alpha]_{\text{D}}^{25} - 42.262$ (*c* 0.02, CH₃OH); IR (KBr) λ_{max} : 3303, 1606, 1453, 1216, 1076 cm⁻¹; UV (MeOH) λ_{max} (log ϵ): 203 (3.54), 246 (3.33) and 281 (2.99) nm; HR-ESI-MS at *m/z* 491.1555 [M+H]⁺ (calcd for C₂₄H₂₇O₁₁, 491.1547); ¹H and ¹³C NMR (500 MHz, methanol-*d*₄) data (Table 1).

Table 1. ¹H and ¹³C NMR spectral data (500/125 MHz) for compound **1** in methanol-*d*₄

Position	δ_{H}	δ_{C}
2	8.11 (1H, s)	155.1
3		125.5
4		177.4
5		161.5
6	7.20 (1H, s)	101.0
7		162.3
8		120.3
9		160.7
10		114.6
11	4.69 (2H, s)	64.1
1'		120.6
2'		156.9
3'	6.92 (1H, m)	117.1
4'	7.25 (1H, m)	130.8
5'	6.92 (1H, m)	120.7
6'	7.23 (1H, m)	132.6
1''	5.13 (1H, d, <i>J</i> = 7.5 Hz)	102.3
2''	3.59 (1H, m)	74.8
3''	3.58 (1H, m)	78.5
4''	3.44 (1H, brd, <i>J</i> = 9.9 Hz)	71.2
5''	3.54 (1H, m)	77.9
6''	3.94 (1H, d, <i>J</i> = 12.0 Hz)	62.5
	3.73 (1H, dd, <i>J</i> = 12.0, 5.8 Hz)	
5-OMe	3.90 (3H, s)	63.6
11-OMe	3.42 (3H, s)	58.4

Achyranthoside A (2): Yellow amorphous powder; ¹H NMR (500 MHz, methanol-*d*₄) δ_{H} : 8.02 (1H, s, H-2), 7.40 (1H, td, *J* = 7.9, 1.8 Hz, H-4'), 7.25 (1H, dd, *J* = 7.4, 1.8 Hz, H-6'), 7.17 (1H, s, H-

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6), 7.07 (1H, d, $J = 8.3$ Hz, H-3'), 7.01 (1H, td, $J = 7.5, 1.0$ Hz, H-5'), 5.13 (1H, d, $J = 7.5$ Hz, H-1''), 4.68 (2H, s, H-11), 3.94 (1H, dd, $J = 12.2, 2.3$ Hz, H-6''), 3.88 (3H, s, 5-OCH₃), 3.79 (3H, s, 2'-OCH₃), 3.73 (1H, dd, $J = 12.1, 5.9$ Hz, H-6''), 3.61–3.54 (2H, m, H-3'', 5''), 3.52 (1H, d, $J = 9.1$ Hz, H-2''), 3.44 (1H, d, $J = 9.3$ Hz, H-4''), 3.41 (3H, s, 11-OCH₃). ¹³C NMR (125 MHz, methanol-*d*₄) δ_C : 176.7 (C-4), 162.3 (C-7), 161.4 (C-5), 160.7 (C-9), 159.2 (C-2'), 154.3 (C-2), 132.5 (C-6'), 131.1 (C-4'), 125.6 (C-3), 122.3 (C-1'), 121.5 (C-5'), 120.2 (C-8), 114.6 (C-10), 112.2 (C-3'), 102.3 (C-1''), 101.0 (C-6), 78.6 (C-3''), 77.9 (C-5''), 74.8 (C-2''), 71.2 (C-4''), 64.0 (C-11), 63.6 (5-OCH₃), 62.5 (C-6''), 58.4 (11-OCH₃), 56.1 (2'-OCH₃).

Achyranthoside B (**3**): Yellow amorphous powder; ¹H NMR (500 MHz, methanol-*d*₄) δ_H : 8.00 (1H, s, H-2), 7.39 (1H, t, $J = 8.0$ Hz, H-4'), 7.24 (1H, dd, $J = 7.4, 1.8$ Hz, H-6'), 7.17 (1H, s, H-6), 7.07 (1H, d, $J = 8.3$ Hz, H-3'), 7.01 (1H, t, $J = 7.5$ Hz, H-5'), 5.15 (1H, d, $J = 7.6$ Hz, H-1''), 3.93 (3H, s, 8-OCH₃), 3.91 (3H, s, 5-OCH₃), 3.79 (3H, s, 2'-OCH₃), 3.73 (dd, $J = 12.1, 5.8$ Hz, 1H, H-6''), 3.56 (3H, dq, $J = 22.0, 9.0$ Hz, H-2'', 3'', 5''), 3.44 (1H, t, $J = 9.3$ Hz, H-4''). ¹³C NMR (125 MHz, methanol-*d*₄) δ_C : 177.0 (C-4), 159.3 (C-2'), 157.2 (C-9), 155.9 (C-7), 154.5 (C-2), 154.0 (C-5), 142.2 (C-8), 132.6 (C-6'), 131.0 (C-4'), 124.8 (C-3), 122.4 (C-1'), 121.5 (C-5'), 115.1 (C-10), 112.2 (C-3'), 101.9 (C-1''), 101.5 (C-6), 78.5 (C-3''), 78.1 (C-5''), 74.8 (C-2''), 71.22 (C-4''), 62.7 (5-OCH₃), 62.45 (C-6''), 62.3 (8-OCH₃), 56.07 (2'-OCH₃).

2.5. Bioassay for Antioxidant Activity

2.5.1. H2c9 Cell Viability

H9c2 cells were seeded in 96-well plates (8×10^4 cells/well) and incubated in a humidified 5% CO₂ atmosphere at 37°C. After 24 h, the cells were treated with 0, 6.25, 12.5, 25, 50, 100 μ M of isolated compounds. After 24 h, cell viability was measured by CCK-8 method [14].

2.5.2. SOD Activity

To investigate the antioxidant effects of the isolated compounds on H₂O₂-treated H9c2 cells, SOD activity was measured. H9c2 cells were seeded in 6-well plates (8×10^4 cells/well) and incubated in 37°C, 5% CO₂ incubator. After 24 h, the plates were treated with 12.5, 25, 50 μ M of compounds **1-3** and then added H₂O₂ (200 μ M) for 2 h. After modeling, cells were collected and broken. According to the SOD detection kit, the SOD activity was calculated.

3. Results and Discussion

3.1. Structure Elucidation

Achyranthosides C (**1**) was obtained as a yellow amorphous powder and its UV spectrum showed three absorption peaks at 203, 246 and 281 nm, indicating it might be an isoflavonoid derivative. Its molecular formula C₂₄H₂₆O₁₁ was determined by positive HR-ESI-MS of the pseudomolecular ion peak at m/z 491.1555 [M+H]⁺ (calcd for C₂₄H₂₇O₁₁, 491.1547). The IR spectrum revealed the presence of hydroxyl (3354 cm⁻¹) and carbonyl (1639 cm⁻¹) groups, and benzene ring (1606 cm⁻¹). The ¹H-NMR spectral data (Table 1) compound **1** showed six aromatic protons appearing as two singlets and two overlapped multiplets. The typical downfield proton at δ_H 8.11 (1H, s) was readily diagnosed as H-2 in isoflavonoid [15]. The proton at δ_H 7.20 (1H, s, H-6) suggested the tri-substituted aromatic ring A. The aromatic resonances of δ_H 7.25 (1H, d, $J = 8.4$ Hz, H-4'), 7.23 (1H, m, H-6'), 6.92 (2H, d, $J = 7.1$ Hz, H-3', 5') were diagnosed with a 2-substituted aromatic ring B. The coupling constant ($J = 7.5$ Hz) of the anomeric proton at δ_H 5.13 indicated that the glucopyranosyl moiety was in β configuration. Additionally, an isolated methylene group at δ_H 4.69 (2H, s) and two methoxy groups at δ_H 3.90 (3H, s), 3.42 (3H, s) were also observed in ¹H NMR spectrum. The ¹³C-NMR and HSQC data of compound **1** revealed 24 resonances, attributed to one ester carbonyl at δ_C 177.4 (C-4), seven double bond carbons at δ_C 162.3, 161.5, 160.7, 156.9, 155.1, 132.6, 130.8, 125.5, 120.7, 120.6,

120.3, 117.1, 114.6, 101.0, a glucosyl unit at δ_C 102.3 (C-1''), 78.5, 77.9, 74.8, 71.2, 62.5, a methylene group at δ_C 64.1, and two methoxy groups at δ_C 63.6, 58.4.

Two spin–spin coupling systems of H-3'/H-4'/H-5'/H-6' and H-1''/H-2''/H-3''/H-4''/H-5''/H-6'' in the ^1H - ^1H COSY spectrum of compound **1** (Figure 2), together with the detailed analysis of HMBC correlations, allowed the assignments of chemical shifts of aromatic ring B and β -glucose group. The HMBC correlations (Figure 2) from H-2 (δ_H 8.11) to C-3 (δ_C 125.5), C-4 (δ_C 177.4), C-9 (δ_C 160.7), C-1' (δ_C 120.6) confirmed the isoflavonoid skeleton of the compound. The key correlations from -OMe (δ_H 3.42) to C-11 (δ_C 64.1), and from H₂-11 (δ_H 4.69) to C-7 (δ_C 162.3), C-8 (δ_C 120.3) and C-9, together with correlations from H-6 (δ_H 7.20) to C-5 (δ_C 161.5), C-7, C-8, and C-10 (δ_C 114.6), and from -OMe (δ_H 3.90) to C-5 (δ_C 161.5), demonstrated that the isolated methylene of C-11 was located at C-8, as well as two methoxy groups were respectively linked to C-5 and C-11. The anomeric proton H-1'' (δ_H 5.14) showed obvious HMBC correlation to C-7 and revealed that the glucose was linked to C-7. Finally, a free hydroxyl group linked to C-2'' was identified by considering its molecular formula. Therefore, the structure of compound **1** was determined as illustrated in Figure 1, named achyranthosides C.

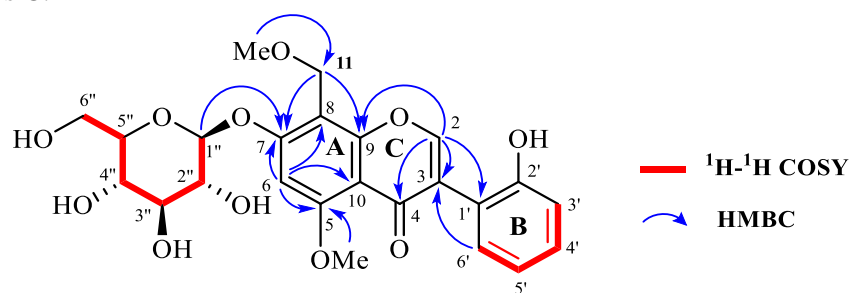


Figure 2. Key ^1H - ^1H COSY and HMBC correlations of compound **1**

Two known compounds **2** and **3** were identified as achyranthoside A and B by analyzing of their NMR data [8]. Structurally, compound **2** was determined as a methoxylated derivative of compound **1** that was supported by the remained methoxy signal at δ_H 3.41 (3H, s) and δ_C 58.4. Compound **3** was a common isoflavonoid glycoside, which was different from compound **2** in that one methoxy group was directly connected to C-8.

3.2. Antioxidant Activity

The *in vitro* cytotoxicity of isolated compounds (**1-3**) on rat cardiomyocytes (H9c2) were evaluated by CCK-8 assay. The results showed they all had no cytotoxicity and could protect H9c2 cells in a concentration-dependent manner at the concentration of 6.25-100 μM (Figure 3). Further, their antioxidant capacities were investigated in H9c2 cells submitted to hydrogen peroxide (H_2O_2). It was found that the activities of SOD in H9c2 cells pretreated with compounds **1-3** (12.5, 25, 50 μM) were all obviously decreased than control groups ($P < 0.05$) (Figure 4). SOD activities were improved in a concentration-dependent manner relative to the H_2O_2 group ($P < 0.05$), indicating that the isoflavonoid glycosides (**1-3**) could enhance the oxidation resistance in H9c2 cells. Significantly, 25 and 50 μM of compounds **1** and **2** displayed activity equivalent to or higher than positive control vitamin C.

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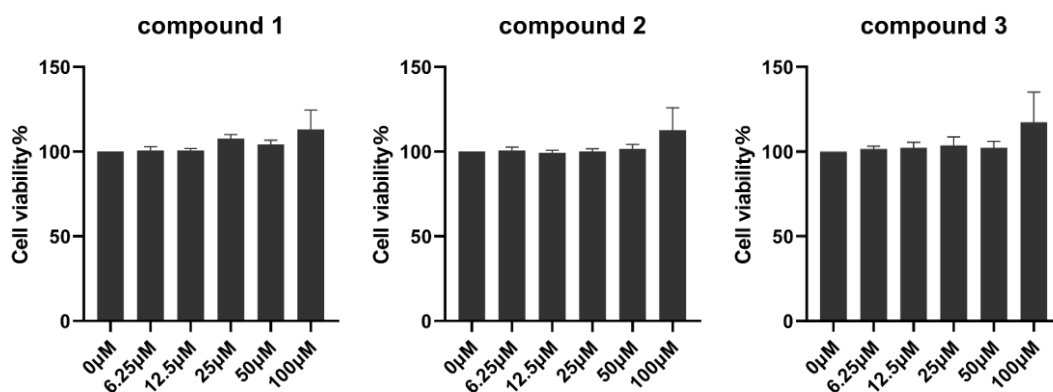


Figure 3. Cell viabilities of H9c2 cells in the presence of compounds 1–3

* $P < 0.05$, ** $P < 0.001$, # $P < 0.05$, compared with the control group

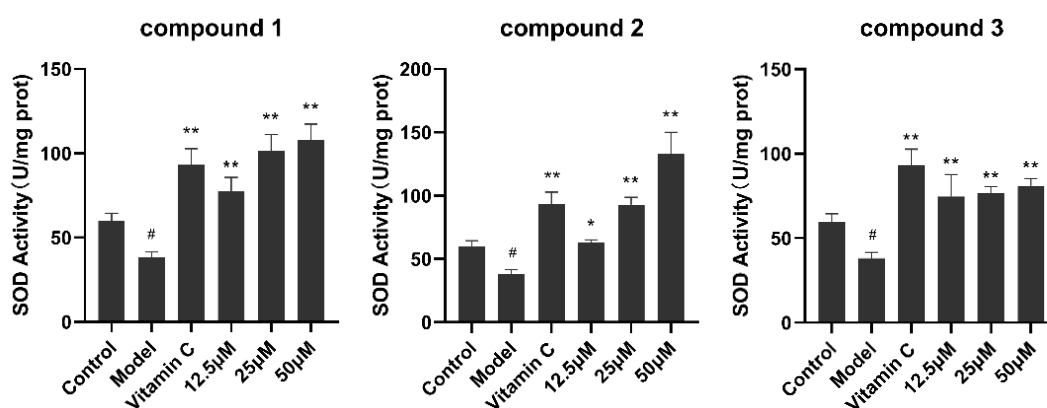


Figure 4. Effect of compounds 1–3 on SOD activities in H9c2 cells treated with H_2O_2 . Vitamin C was used as a positive control (20 $\mu\text{g/L}$ approximately equal to 0.11 μM)

* $P < 0.05$, ** $P < 0.001$, # $P < 0.05$, compared with the H_2O_2 group

4. Conclusion

This study reports the isolation, structural elucidation, and the antioxidant activity of three isoflavonoid glycosides from the rhizomes of *A. bidentata*. Compounds 1–2 are the rare type of C8-hydroxymethylated isoflavonoid glycosides in nature. Bioassay results indicated that all the three monomers had certain antioxidant activity, and 25 and 50 μM of compounds 1 and 2 displayed activity equivalent to or higher than positive control vitamin C. This study can provide effective information for further understanding of the rhizomes of *A. bidentata*.

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Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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