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Characterization of tyrosinase inhibitors in the twigs of *Cudrania tricuspidata* and their structure–activity relationship study

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ABSTRACT

The twigs of *Cudrania tricuspidata* were found to show strong tyrosinase inhibitory activity, and further detailed component analysis resulted in the isolation of a new flavanol glucoside, (2S,3S)-2,3-*trans*-dihydromorin-7-*O*-*β*-D-glucoside (**1**), plus twenty-seven known compounds (**2**–**28**). Their structures were elucidated on the basis of ESI-MS and NMR spectral data. Among the isolated compounds, *trans*-dihydromorin (**8**), oxyresveratrol (**9**), and steppogenin (**12**) were found to exhibit significant tyrosinase inhibition activities. Moreover, the structure–activity relationship of these isolated compounds was also discussed.

1. Introduction

Enzymatic browning occurs widely in plant-derived food products [1], which usually results in color changes and loss of nutritional quality of these products [2]. In the process of enzymatic browning, tyrosinase (EC 1.14.18.1) plays an important role as a catalyzing enzyme. On the other hand, tyrosinase also catalyzes the tyrosine to form melanin pigments in mammals [3]. The inhibition of tyrosinase activity is of great promise approach in preventing the undesirable browning of vegetables and fruits and the accumulation of an excessive level of melanin pigments in mammals. So far, numerous compounds from natural and synthetic sources have been tested to inhibit the activity of tyrosinase [4,5]. However, few of them were applied in food and cosmetic as additives because of their low tyrosinase inhibition activities, source limitation, and safety consideration. Therefore, the search of safe tyrosinase inhibitors with rich resources and strong inhibition activity are highly demanded by the food and cosmetics industry.

Cudrania tricuspidata is a deciduous tree growing in East Asia, including China, Japan and Korea. Its root has been used as a traditional medicine for the treatment of digestive apparatus tumor [6]. This plant is rich in isoprenylated xanthones and flavonoids [7,8]. Biological studies showed that its constituents demonstrate many biological activities, such as anti-inflammatory [7], α-glucosidase inhibitory [8], antifungal [9], anti-lipid peroxidative [10], antioxidative and cytotoxic activity [11,12]. However, previous phytochemical studies mainly focused on the root and stem, no detailed phytochemical study on the twigs of *C. tricuspidata* has been conducted up to now. In our screening process of natural tyrosinase inhibitors, we found that the twig extracts showed potent inhibitory activity against mushroom tyrosinase (IC_{50} = 113.5 μg/mL). Column chromatographic separation technology and spectral analysis therefore were applied to characterize the tyrosinase inhibition components of the twigs. Another aim of the present research was to study the structure–activity relationship of these tyrosinase inhibitors.

2. Experimental

2.1. General

Ethyl acetate, hexane, methanol (MeOH), 95% ethanol (EtOH), dichloromethane (CH_2Cl_2), sodium dihydrogen

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orthophosphate ($NaH_2PO_4.2H_2O$), dimethyl sulfoxide (DMSO), and HPLC grade solvent acetonitrile (ACN) were purchased from BDH (Poole, UK). Mushroom tyrosinase (5370 units/mg), L-tyrosine, formic acid, methanol-d_6 (CD_3OD), DMSO-d_6, acetone-d_6, chloroform-d ($CDCl_3$) and kojic acid were purchased from Sigma Chemical Co (St. Louis, USA). Silica gel (200–300 mesh) for column chromatography was from Qingdao Marine Chemical Company (Qingdao, PR China). Sephadex LH-20 was from GE Healthcare Bio-Sciences AB (Uppsala Sweden). Anhydrous di-sodium hydrogen phosphate (Na_2HPO_4) and TLC plates (25 DC-platten Kieselgel 60 F_{254}) were from Merck (Darmstadt, Germany). Preparative HPLC were carried out on a Waters 600 system equipped with a 2487 dual-wavelength detector and the Empower Pro software. A Phenomenex Luna C18 (2) column (250×21.2 mm, 5 μm) was used for preparative HPLC. ^{1}H NMR and ^{13}C NMR were acquired on a Bruker 300 DRX NMR spectrometer. Mass spectrometry was carried out on an Applied Biosystems Q-trap LC-MS mass spectrometer. Spectrophotometric measurements for the tyrosinase inhibition assay were taken on a UV-1206 Spectro-photometer (Shimadzu Corporation, Japan). Minigrinder (Model: DF-15) for grinding was purchased from Shenzhen Laitong Company, Shenzhen, PR China.

2.2. Plant material

The fresh twigs of *C. tricuspidata* were collected from Anhui Province, P.R. China, in August 2011. Voucher specimen (accession number 20110801) was deposited at the School of Biological Sciences, The University of Hong Kong.

2.3. Extraction and isolation of C. tricuspidata twig phytochemicals

The twigs of *C.tricuspidata* (3.5 kg) were ground by a minigrinder and packed into a paper extraction bag. Extraction was performed with 95% ethanol (3×10 L) at room temperature using a traditional Chinese medicine extractor. The extraction solutions were combined and concentrated in vacuo at 50 °C. The crude part (130.0 g) was subjected to an Amberlite XAD16 column and was eluted with 20%, 40%, 60%, 80%, and 95% ethanol, which offered eleven fractions (Fr.1-11).

Fr.1 (20% ethanol fraction) was purified by Sephadex LH-20 [eluted by MeOH/H_2O (1:1)] to give two sub-fractions (Fr.A1-A2). Fr.A1 was purified by preparative HPLC and eluted by 10% ACN to offer compounds **1** (27.0 mg) and **2** (289.5 mg). Fr.A2 was separated by preparative HPLC and eluted by 10% ACN to give compound **3** (3.8 mg). Fr.2 (40% ethanol fraction) was separated by silica gel [ethyl acetate–MeOH (20:1)] and then was purified by preparative HPLC (gradient eluted by ACN from 5% to 35%) to give two compounds **5** (5.4 mg) and **6** (444.5 mg). Fr.3 (40% ethanol fraction) was firstly separated by silica gel [ethyl acetate-hexane (1:1)] and then was purified by preparative HPLC (eluted by 18% ACN) to offer three compounds **7** (1070.5 mg), **8** (275 mg), and **9** (47.8 mg). Fr.4 (40% ethanol fraction) was firstly separated by silica gel [ethyl acetate–hexane (1:1)] and then purified by preparative HPLC (eluted by 18% ACN) to offer compounds **4** (6.3 mg). Fr.5 (40% ethanol fraction) was subjected to silica gel [ethyl acetate–hexane (1:2)] and then separated by Sephadex LH-20 [MeOH–H_2O (1:1)] to give **10** (100.6 mg), **11** (9.1 mg), **12** (6.6 mg), and **13** (3.3 mg). Fr.6 (60% ethanol fraction) was firstly separated by silica gel [ethyl acetate–hexane (1:2)] and then purified by Sephadex LH-20 [MeOH–H_2O (1:1)] and further separated by silica gel [CH_2Cl_2–MeOH (30:1)] to give compound **14** (80.3 mg). Fr.7 (60% ethanol fraction) was firstly separated by silica gel [ethyl acetate–hexane (1:2)] and further purified by preparative HPLC (eluted by 30% ACN) to offer compound **15** (20.5 mg) and **16** (31.1 mg). Fr.8 (60% ethanol fraction) was firstly separated by silica gel and further separated by preparative HPLC (eluted by 30% ACN) to give compound **17** (2.9 mg). Fr.9 (80% ethanol fraction) was separated by silica gel [MeOH–CH_2Cl_2, (1:50)] into two fractions (Fr.C1–C2). Fr.C1 was purified by preparative HPLC (eluted by 50% ACN) to offer compound **18** (8.5 mg) and **19** (88.8 mg). Fr.C2 was purified by preparative HPLC (eluted by 56% ACN) to give compound **20** (309.5 mg). Fr.10 (80% ethanol fraction) was separated by silica gel (eluted by MeOH–CH_2Cl_2, 1:50) into two fractions (Fr.D1–D2). Fr.D1 was further purified by preparative HPLC (eluted by 56% ACN) to give compound **21** (2.4 mg). Fr.D2 was further separated by preparative HPLC (gradient eluted by ACN from 45% to 75%) to offer compound **22** (4.6 mg). Fr.10 (80% ethanol fraction) was separated by silica gel and was eluted by hexane–CH_2Cl_2 (2:1) to offer two fractions (Fr.E1–E2). Fr.E1 [hexane–CH_2Cl_2 (2:1)] was further separated by Sephadex LH-20 (eluted by MeOH) to get compound **27** (2.8 mg). Fr.E2 was further purified by preparative HPLC (gradient eluted by ACN from 55% to 95%) to offer compound **23** (3.2 mg), **24** (2.4 mg), **25** (36.2 mg), and **26** (2.9 mg). Fr.11 (95% ethanol fraction) was firstly separated by silica gel (eluted by CH_2Cl_2) and separated by preparative HPLC (gradient eluted by ACN from 55% to 100%) to offer compound **28** (3.3 mg).

Compound **1**: yellow powder; ^{1}H NMR (300 MHz, CD_3OD) δ 7.22 (1H, d, J=9.0 Hz, H-5′), 6.34 (2H,overlapped, H-3′, 6′), 6.20 (1H, d, J=2.1 Hz, H-8), 6.17 (1H, d, J=2.1 Hz, H-6), 5.41 (1H, d, J=11.7 Hz, H-2), 4.96 (1H, d, J=7.2 Hz, H-1″), 4.81 (1H, d, J=11.7 Hz, H-3). 3.46~3.89 (6H, m, H-2″, 3″, 4″, 5″, 6″); ^{13}C NMR (75 MHz, CD_3OD) δ 199.8 (C=O, C-4), 167.2 (C, C-7), 164.7 (C, C-5), 164.7 (C, C-9), 160.2 (C, C-4′), 158.6 (C, C-2′), 131.0 (CH, C-6′), 115.3 (C, C-1′), 107.8 (CH, C-3′), 103.6 (CH, C-5′), 103.5 (C, C-10), 101.2 (CH, C-1″), 98.1 (CH, C-6), 96.9 (CH, C-8), 80.2 (CH, C-2), 72.6 (CH, C-3), 78.2 (CH, C-5″), 77.7 (CH, C-3″), 74.6 (CH, C-2″), 71.1 (CH, C-4″), 62.3 (CH_2, C-6″); Negative HRESIMS m/z 465.1038 $[M-H]^-$ (Calcd for $C_{21}H_{21}O_{12}$, 465.1039).

Compound **21**: yellow powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.01 (1H, s, OH-1), 9.65 (1H, br s, OH-5), 7.64 (1H, d, J=8.8 Hz, H-8), 7.19 (1H, d, J=8.8 Hz, H-7), 6.61 (1H, d, J=2.0 Hz, H-8), 6.37 (1H, d, J=2.0 Hz, H-6), 3.96 (3H, s, OCH_3-6), 3.89 (3H, s, OCH_3-3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.99 (C=O), 166.2 (C, C-3), 162.6 (C, C-1), 157.3 (C, C-4a), 152.9 (C, C-7), 145.2 (C, C-4b), 134.0 (C, C-5), 115.5 (CH, C-8), 114.3 (C, C-8b), 109.0 (CH, C-7), 102.4 (C, C-8a), 56.4 (CH_3, OCH_3-3), 56.0 (CH_3, OCH_3-6); Negative ESI-MS m/z 287 $[M-H]^-$.

Compound **27**: gum; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (1H, s, H-2), 7.96 (2H, m, H-2′, 6′), 7.55 (2H, m, H-2″, 3″), 7.47 (6H, m, H-3′, 4′, 5′, 4″, 5″, 6″), 3.33 (3H, br s, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.0 (C=O, C-1), 147.5 (CH, C-2), 136.3 (C, C-1″), 131.3 (C, C-1′), 129.8 (CH, C-4′), 129.4 (CH, C-4″), 128.7 (CH, C-3″, 5″), 128.6 (CH, C-3′, 5′), 128.5 (C, C-3), 127.3 (CH, C-2′, 6′), 125.8 (CH, C-2″, 6″), 106.6 (C, C-4), 51.5 (CH_3, OCH_3-4); Negative ESI-MS m/z 265 $[M-H]^-$.