Food Chemistry 231 (2017) 324–331

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Extraction, identification, and antioxidant and anticancer tests of seven dihydrochalcones from *Malus* 'Red Splendor' fruit

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ARTICLE INFO

Article history:
Received 4 January 2017
Received in revised form 17 March 2017
Accepted 21 March 2017
Available online 23 March 2017

Keywords:
Antioxidant capacity
Cytotoxicity
Dihydrochalcone
Extraction
Malus

ABSTRACT

Five dihydrochalcone compounds, including phlorizin, trilobatin, 3-hydroxyphlorizin, sieboldin and phloretin 2′-xyloglucoside, were isolated from ornamental *Malus* 'Red Splendor' fruit. The chemical structures of these compounds were elucidated by LC-ESI-MS and NMR. Phloretin and 3-hydroxyphloretin were produced by hydrolysis. The antioxidant capacities of these seven compounds were examined by DPPH and ABTS assays, while their cytotoxicity to five cancer cell lines were evaluated by the MTT assay. The results showed that the DPPH assay mainly reflected the antioxidant capacity of the B ring, whereas the ABTS assay was mostly related to the A ring of the dihydrochalcone molecule. Moreover, 3-hydroxyphloretin was the best antioxidant among the seven compounds. Both glycosylation of the A ring and the ortho phenolic hydroxyl groups of the B ring were important for the cytotoxicity of dihydrochalcone molecules. Sieboldin and 3-hydroxyphlorizin exhibited better cytotoxicity than other dihydrochalcone compounds. Dihydrochalcones from *Malus* may benefit human health.

1. Introduction

Dihydrochalcone is a class of the flavonoid family, but it has only two aromatic rings and a saturated C_3 chain in the basic C_6-C_3-C_6 skeleton structure. Dihydrochalcone compounds play an important role in plants against either biotic or abiotic stresses (Gaucher et al., 2013; Gosch, Halbwirth, & Stich, 2010; Popovici et al., 2010), but they also have some specific biological activities that are beneficial to human health. For example, phlorizin (phloretin 2′-glucoside) may inhibit sodium glucose co-transporter 2 (Ehrenkranz, Lewis, Kahn, & Roth, 2005; White, 2010), whereas trilobatin (phloretin 4′-glucoside), an isomer of phlorizin, has inhibitory potential against α-glucosidase and α-amylase (Dong, Li, Zhu, Liu, & Huang, 2012). These bioactivities are believed to have great significance for the treatment of type 2 diabetes. By hydrogenating neohesperidin extracted from bitter orange, an artificial sweetener, neohesperidin dihydrochalcone, can be produced and used as food additive E959 in Europe (Janvier, Goscinny, Donne, & Loco, 2015).

To date, more than 200 kinds of dihydrochalcone were identified (Rozmer & Perjési, 2016). Approximately 30 plant families, including Rosaceae (Petersen, 1835), Fabaceae (Koeppen & Roux, 1966), Fagaceae (Rui-Lin, Tanaka, Zhou, & Tanaka, 1982) and Asteraceae (Altunkaya & Gökmen, 2009), have been found to contain natural dihydrochalcone compounds, mostly in the form of glycosylated products but not aglycones. Among these plants, *Malus* is unique with its very high dihydrochalcone content. It has been reported that dihydrochalcone accounts for 66%, 70–80% or 80–90% of the total phenolic compounds in seeds, bark or leaves, respectively, in *Malus domestica* (Guyot, Marnet, Laraba, Sanoner, & Drilleau, 1998; Mayr, Michalek, Treutter, & Feucht, 1997; Mornau, 2004; Pontais, Treutter, Paulin, & Brisset, 2008). However, the dihydrochalcone compounds are mainly phlorizin in cultivated apples (Li, Ma, & Cheng, 2013; Bi et al., 2014; Kalinowska, Bielawska, Lewandowska-Siwkiewicz, Priebe, & Lewandowski, 2014), whereas 3-hydroxyphlorizin and phloretin 2′-xyloglucoside were also detected in some apple or crabapple cultivars (Fromm, Loos, Bayha, Carle, & Kammerer, 2013; Górnaś et al., 2015). Other dihydrochalcone compounds such as trilobatin and sieboldin have been found in certain wild *Malus* species (Gosch et al., 2010; Williams, 1961). However, these five compounds have never been reported together in a *Malus* species. Moreover, although some studies have examined the bioactivities of phenolic

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http://dx.doi.org/10.1016/j.foodchem.2017.03.111
0308-8146/

compounds from *Malus* plants (Bi et al., 2014; Li, Shi, & Wang, 2014; Rana et al., 2016), information is still lacking regarding the bioactivities of the above five dihydrochalcone compounds. For example, whether the isomers (phlorizin versus trilobatin; 3-hydroxyphlorizin versus sieboldin) have the same bioactivities or whether hydroxyl group at the 3-position affects the bioactivities of 3-hydroxyphlorizin and sieboldin in comparison to phlorizin and trilobatin remain unknown. In addition, although metabolic engineering of *Saccharomyces cerevisiae* for the production of dihydrochalcone has been reported (Eichenberger et al., 2017), the extraction of dihydrochalcone from natural plant materials is still important for industrial production. Plant material containing all target compounds may lower the economic cost during processing.

In this study, five major dihydrochalcone compounds were isolated and identified from an ornamental *Malus* cultivar—'Red Splendor' fruit. Two kinds of aglycones were also produced from the purified natural dihydrochalcones. Subsequently, the antioxidant ability and the cytotoxicity of five cancer cell lines of these compounds were evaluated to obtain deeper insight into the bioactivities of the dihydrochalcone compounds from *Malus* plants.

2. Materials and methods

2.1. Chemicals and regents

Dimethyl sulfoxide-d6 (DMSO-d_6), 2,2-azino-bis-(3-ethylbenzo thiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), potassium persulphate ($K_2S_2O_8$), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-car boxylic acid (trolox) and L(+)-ascorbic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Paclitaxel and 10-hydroxycamptothecin were obtained from J&K Scientific (Beijing, China). Sephadex LH-20 was obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). SiliaSphere PC18 was purchased from SiliCycle (Quebec, Canada). Polyamide powder was obtained from Taizhou City Luqiao Sijia Biochemical Plastic Factory (Zhejiang, China). Deionized water was prepared using a ULUPURE pure water system (Sichuan, China). Ultra-pure water was prepared using a Millipore Milli-Q system (Darmstadt, Germany). HPLC grade methanol, acetonitrile and formic acid were purchased from Guanghua Sci-Tech Co.,Ltd (Guangdong, China). All reagents of cell culture process were purchased from Gibco Invitrogen (Carlsbad, CA, USA).

2.2. Plant materials and dihydrochalcone compound isolation

Five kilograms of *Malus* 'Red Splendor' fruits were collected on April 26, 2016 from the Crabapple Garden of Northwest A&F University, Yangling, Shaanxi province, China. The red fruits (diameter, 1.2–1.5 cm) including seeds and pedicels were frozen in liquid nitrogen, and then lyophilized for two weeks at room temperature using a vacuum freeze drier (ScanVac Coolsafe 110-4, LaboGene, Solrød Strand, Denmark) with the cold trap temperature being set at −100 °C. The vacuum was maintained using a Rotary Vane Vacuum Pump (Vacuubrand RZ 2.5, Wertheim, Germany).

The lyophilized fruits were ground into a powder using a disintegrator (Hangta LS-O2A, Fangyan Yeniu Hardware Machinery Factory, Zhejiang, China), and then extracted three times with 80% ethanol (15 L) at room temperature for 24 h. The extraction solution was stirred by an electric stirrer (JJ-1, Chengdong Xinrui Instrument Factory, Sichuan, China). After vacuum filtration using a sintered glass funnel with two layers of gauze to remove the crude sediment, the extraction solution was centrifuged for 10 min at 8000*g* (Avanti J-25, Beckman Coulter, Brea, CA, USA) to further remove the fine sediment. The supernatant was evaporated using a rotary evaporator to obtain a viscous substance. One liter of deionized water was added to one eighth of the viscous substance (totally, 8 L water were used), followed by ultrasonic vibration for 2 h until the viscous substance was dispersed into turbid liquid. One liter of the turbid liquid was extracted three times with 0.4 L petroleum ether, followed by the extraction with 0.6 L ethyl acetate three times. The ethyl acetate fractions were combined and dried by evaporation to obtain the crude phenolic fraction, F_{EA}. The water phase was freeze-dried to obtain the crude phenolic fraction, F_W. The two parts were subjected to a custom-made open polyamide column chromatography (200–400 mesh, ∅ 9 × 40 cm), respectively. Different concentrations of methanol solution were used for elution in the sequences of 0% (1 L), 30% (2 L), 50% (2 L) and 80% (2 L) for F_{EA} and 0% (8 L), 30% (2 L) and 50% (2 L) for F_W, respectively. The fraction collected with the 80% or 50% methanol eluent was used for F_{EA} or F_W, separately. Each concentrated part was subjected to a custom-made open Sephadex LH-20 column chromatography (∅ 3 × 120 cm) and eluted with methanol to obtain four fractions (F_{EA1}–F_{EA4}) or three fractions (F_{W1}–F_{W3}), respectively, based on HPLC analysis as described by Bi et al. (2014). Phlorizin (**D1**) was obtained by crystallization from F_{EA2}. F_{EA3} was dried and re-dissolved in 30% methanol solution and then loaded onto a custom-made open SiliaSphere PC18 column (50 μm, ∅ 2.6 × 30 cm). After washing with 100 mL 30% methanol, the F_{EA3} was eluted with step gradients of 40% (150 mL), 50% (150 mL) and 60% (150 mL) methanol to obtain three sub-fractions (F_{EA3-1}—F_{EA3-3}). The flow rate was approximately 3 mL/min. F_{EA3-1}, F_{EA3-2}, F_{EA3-3} and F_{W2} were further purified by LC-20A liquid chromatography equipped with a FRC-10A automatic fraction collector and a photo-diode array detector (Shimadzu Corporation, Tokyo, Japan) to produce sieboldin (**D4**), 3-hydroxyphlorizin (**D3**), trilobatin (**D2**) and phloretin 2′-xyloglucoside (**D5**), respectively. A YMC-Pack ODS-A column (5 μm, 10 mm × 250 mm, YMC CO., Ltd. Kyoto, Japan) was used with a mobile phase of 50% methanol. These five compounds were re-crystallized to increase their purities. Briefly, the compound was re-dissolved into methanol with almost saturated concentration, and then slowly evaporated with nitrogen gas. After the compound crystal precipitated, the solution with precipitated crystal was moved to −20 °C for overnight to further enhance the crystallization. After centrifuged at 8000*g* for 20 min at 4 °C, the sediment was the compound with higher purity.

2.3. LC-ESI-MS analysis

For LC-ESI-MS analysis, a 20A HPLC system coupled to an API2000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Waltham, Massachusetts, USA) via a turbo spray electrospray ionization (ESI) interface was used for mass analysis and detection. Each sample was filtered through a 0.22-μm filter before injection. An Inertsil ODS-3 column (5.0 μm particle size, 4.6 mm × 250 mm, GL Sciences Inc., Tokyo, Japan) was used for the analysis, preceded by an Inertsil ODS-3 Guard Column (5.0 μm, 4.0 mm × 10 mm). Mobile phase A consisted of 10% formic acid dissolved in water, and mobile phase B was 10% formic acid and 1.36% water in acetonitrile. The gradient consisted of 95% A (0 min), 85% A (25 min), 78% A (42 min), 64% A (60 min) and 95% A (65 min), sequentially. The post-run time was 5 min. The flow rate was 1 ml min^{-1} at 35 °C. Positive ion mass spectra were recorded in the range from 180–800 *m/z*. The instrument was operated with an ion spray voltage of 3800 V, curtain gas of 20 psi, nebulizer gas of 50 psi, heater gas of 50 psi, and heater gas temperature of 450 °C. All of the gases used were nitrogen. The data were collected and analyzed by Analyst 1.5.1 data

acquisition and processing software (Applied Biosystems/MDS Sciex, Waltham, Massachusetts, USA).

2.4. Hydrolysis of dihydrochalcone glycoside derivatives

The hydrolysis was performed as described by Baranowska, Hejniak, and Magiera (2016) with some modifications. The purified phlorizin (0.2 g) and sieboldin (0.2 g) were dissolved in 25 mL and 2 mL 4 M HCl solution and incubated in a water bath at 90 °C for 12 h. The hydrolysate was allowed to cool by standing at 4 °C for 4 h for crystallization, followed by centrifugation at 8000 g. The insoluble compound was collected and washed three times in cold water and then re-dissolved in methanol. The compounds were further purified by loading onto a Sephadex LH-20 column (∅ 1.6 × 120 cm) and eluted with methanol. The fraction containing phloretin or 3-hydroxyphloretin was collected based on the HPLC analysis, and then evaporated with nitrogen gas.

2.5. NMR analysis

NMR experiments were performed on a Bruker-500 (Bruker Corporation, Fällanden, Switzerland) at 500 MHz for ^{1}H NMR spectra and 125 MHz for ^{13}C spectra. The reference compound tetramethylsilane (TMS) was used as the internal standard. All samples were dissolved in DMSO-d_6.

2.6. Antioxidant capacity

Antioxidant capacities were evaluated using the ABTS or DPPH assay. The DPPH assay was performed as described by Sousa et al. (2016) with some modifications. The reaction mixture contained 60 μM DPPH and 10 μM dihydrochalcone compound or standard dissolved in methanol. A sample containing only DPPH was used as a control. After incubation for 30 min in the dark, the absorbance was measured at 517 nm using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan).

The ABTS assay was carried out according to the methods of Li, Du, and Ma (2011) with minor modifications. Briefly, 7 mM ABTS methanol solution and 2.5 mM potassium persulphate water solution were mixed to produce an ABTS radical cation ($ABTS^{\cdot+}$). This reaction mixture was kept in the dark for 14 h at room temperature before use. The $ABTS^{\cdot+}$ solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 734 nm. After the addition of 100 μL of 100 μM dihydrochalcone compound or standard to 0.9 mL of the diluted $ABTS^{\cdot+}$ solution, the absorbance was measured at 734 nm. One hundred microliters of methanol mixed with 0.9 mL of the diluted $ABTS^{\cdot+}$ solution was used as a control.

The results are expressed as the standard compound equivalent antioxidant capacity. Trolox and L(+)-ascorbic acid were used as standard compounds.

2.7. Cell culture

MG-63, Hela, Hep G2 and SK-OV-3 cell lines were purchased from the American Type Culture Collection (ATCC, respectively CRL-1427, CCL-2, HB-8065, HTB-77). The MDA-MB-231 cell line was kindly provided by M.M. Yanhui Zhang (Institute of Cerebrovascular Disease, The Affiliated Hospital of Qingdao University, Qingdao, China). MDA-MB-231 cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640), and all other cells were cultured in Dulbecco's Modified Eagle's Medium (high glucose) with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 units mL^{-1} penicillin and 100 mg mL^{-1} streptomycin at 37 °C in an atmosphere of 5% CO_2/air. When the cells grew to approximately 80% confluency, they were sub-cultured or treated with compounds.

2.8. MTT assay

Survival was determined by the 2, 5-diphenyl tetrazolium bromide (MTT) assay at 550 nm with a microplate reader (Bio-Tek, Vermont, USA). After determining the cell number, the cells were seeded in 96-well plates in a 100-μL volume (7×10^3 per well) and allowed to grow for 24 h before treatment with 1% FBS medium containing the compounds (five doses of each compound) for 24 h. At the end of the experiments, 20 μL of 5 μg mL^{-1} MTT was added to each well. The cells were then incubated at 37 °C for 4 h. Formazan was solubilized in 150 μL of DMSO and measured at 550 nm. The 50% inhibitory concentration (IC_{50}) was calculated by using Graphpad prism software 6.0 (Hearne Scientific Software Pty Ltd., Victoria, Australia).

2.9. Statistical analysis

All data are presented as means ± SE (n = 5) and analyzed statistically by the *t*-test using SPSS 16.0 software (IBM, New York, USA) with $P < 0.05$.

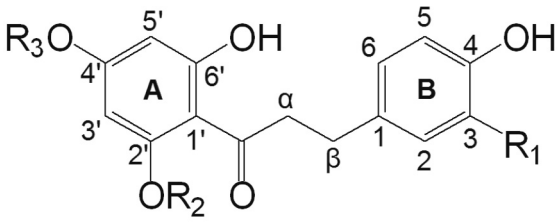
3. Results and discussion

3.1. Identification of dihydrochalcone compounds

Five dihydrochalcone compounds (Fig. 1) were extracted and purified from the fruit of *Malus* 'Red Splendor'. ESI-MS, ^{1}H NMR and ^{13}C NMR were used to identify these compounds (Fig. 2, Table 1, Supplementary Figs. S1–S12).

Compound 1 (**D1**) was a white powder with a purity of 99.17% and retention time (Rt) at 41.0 min. The ESI-MS analysis showed that **D1** had a quasi-molecule ion at *m*/*z* 437.4 $[M + H]^+$. The fragment at *m*/*z* 275.2 indicates a loss of the glucoside moiety, consistent with the molecular weight of phloretin in positive mode. The ^{1}H NMR analysis (500 MHz, DMSO-d_6) yielded the following data: δ_H 13.52 (1H, s, OH-6′), 10.60 (1H, s, OH-4′), 9.11 (1H, s, OH-4), 7.04 (2H, d, *J* = 8.3 Hz, H-2, 6), 6.65 (2H, d, *J* = 8.3 Hz, H-3, 5), 6.13 (1H, d, *J* = 2.2 Hz, H-3′), 5.94 (1H, d, *J* = 2.1 Hz, H-5′), 5.31 (1H, d, *J* = 5.0 Hz, Glu OH-4″), 5.17 (1H, d, *J* = 4.4 Hz, Glu OH-2″), 5.07 (1H, d, *J* = 5.3 Hz, Glu OH-3″), 4.94 (1H, d, *J* = 7.1 Hz, Glu H-1″), 4.62 (1H, t, *J* = 5.7 Hz, Glu OH-6″), 3.71 (1H, ddd, *J* = 2.1, 5.3, 12.1 Hz, Glu H-6β″), 3.51 (1H, dt, *J* = 5.7, 11.6 Hz, Glu H-6α″), 3.24 – 3.38 (5H, m, overlapped, H-α, Glu H-2″, 3″, 5″), 3.19 (1H, td, *J* = 5.1, 8.9 Hz, Glu H-4″) and 2.79 (2H, t, *J* = 7.4 Hz, H-β). The data of ^{13}C NMR analysis were: δc 204.63 (C═O), 165.56 (C-4′), 163.89 (C-6′), 160.33 (C-2′), 154.40 (C-4), 131.94 (C-1), 128.42 (C-1, 6), 114.15 (C-3, 5), 104.92 (C-1′), 100.15 (Glu C-1″), 96.42 (C-5′), 93.52 (C-3′), 76.55 (Glu C-3″), 76.46 (Glu C-5″), 72.77 (Glu C-2″), 69.18 (Glu C-4″), 60.52 (Glu C-6″), 44.98 (C-α), and 28.89 (C-β). **D1** was identified as phlorizin, consistent with previous studies (Hilt et al., 2003).

Compound 2 (**D2**) was a white powder with a purity of 99.15%. **D2** showed similar MS data to **D1** but a different Rt at 47.2 min. The ^{1}H NMR analysis (500 MHz, DMSO-d_6) data were as follows: δ_H 12.27 (2H, s, OH-2′, 6′), 9.12 (1H, s, OH-4), 7.02(2H, d, *J* = 8.4 Hz, H-2, 6), 6.66(2H, d, *J* = 8.5 Hz, H-3, 5), 6.04 (2H, s, H-3′, 5′), 5.31 (1H, d, *J* = 5.3 Hz, Glu OH-4″), 5.07 (1H, d, *J* = 4.9 Hz, Glu OH-2″), 5.00 (1H, d, *J* = 5.3 Hz, Glu OH-3″), 4.87 (1H, d, *J* = 7.7 Hz, H-1″), 4.54 (1H, t, *J* = 5.7 Hz, Glu OH-6″), 3.68 (1H, dq, *J* = 2.1, 11.9 Hz, Glu H-6β″), 3.49 (1H, dt, *J* = 5.4, 11.4 Hz, Glu H-6α″), 3.13–3.35 (6H, m, overlapped, H-α, Glu H-2″, 3″, 4″, 5″) 2.78 (2H, t, *J* = 7.7 Hz, H-β). The ^{13}C NMR data were: δc 205.09 (C═O), 163.79 (C-2′, 6′), 163.43 (C-4′), 155.45 (C-4), 133.90 (C-1), 129.19 (C-2, 6), 1115.13 (C-3, 5), 105.32 (C-1′), 99.60 (Glu C-1″), 95.12 (C-3′, 5′), 77.19 (Glu C-3″), 76.47 (Glu C-5″), 73.10 (Glu C-2″), 69.49



Phlorizin (**D1**) : R1 = H, R2 = glucoside, R3 = H

Trilobatin (**D2**) : R1 = H, R2 = H, R3 = glucoside

3-Hydroxyphlorizin (**D3**) : R1 = OH, R2 = glucoside, R3 = H

Sieboldin (**D4**) : R1 = OH, R2 = H, R3 = glucoside

Phloretin 2'-xyloglucoside (**D5**) : R1 = H, R2 = xyloglucoside, R3 = H

Phloretin (**D6**): R1 = H, R2 = H, R3 = H

3-Hydroxyphloretin (**D7**): R1 = OH, R2 = H, R3 = H

Fig. 1. Structures of phlorizin (**D1**), trilobatin (**D2**), 3-hydroxyphlorizin (**D3**), sieboldin (**D4**), phloretin 2′-xyloglucoside (**D5**), phloretin (**D6**) and 3-hydroxyphloretin (**D7**).

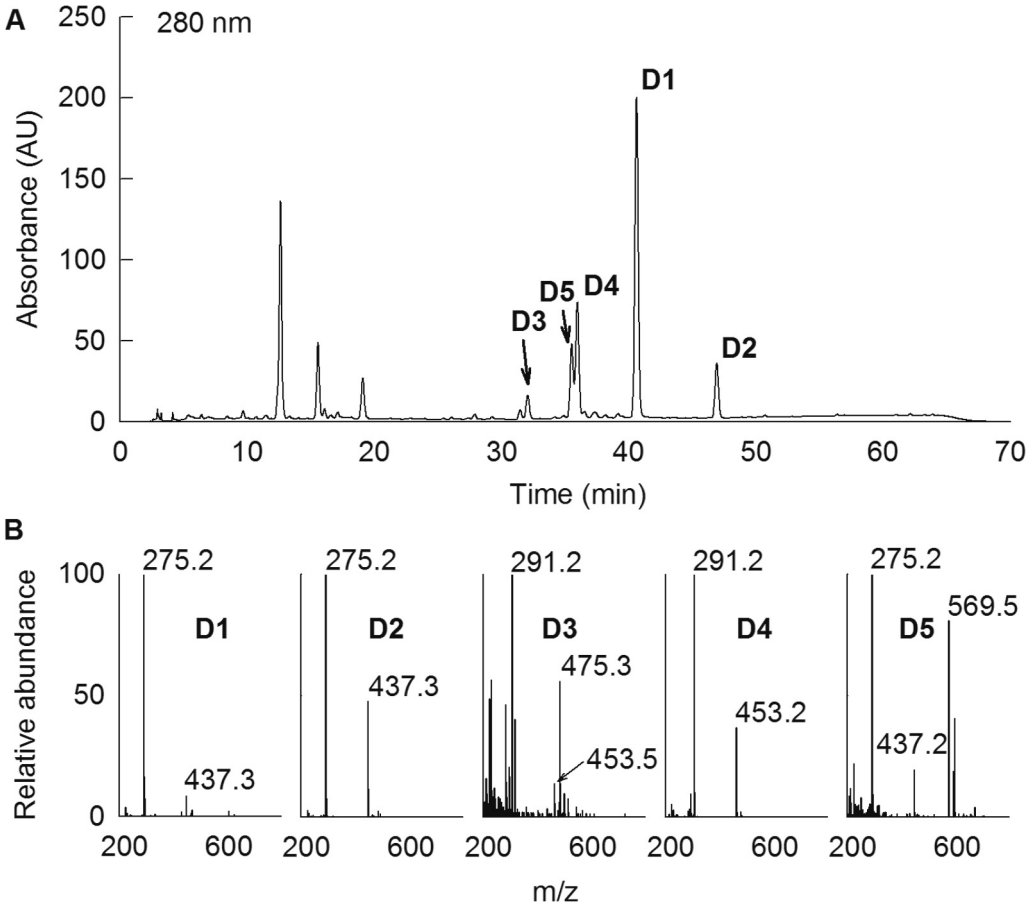


Fig. 2. HPLC chromatogram and ESI-MS spectra in positive mode of dihydrochalcone compounds in *Malus* 'Red Splendor' fruit.

(Glu C-4″),60.55 (Glu C-6″), 45.74 (C-α) and 29.37 (C-β). **D2** was identified as trilobatin, consistent with previous results (Qin & Liu, 2004).

Compound 3 (**D3**) was a slightly orange powder with a purity of 97.99%. **D3** (Rt = 32.4 min) showed two quasi-molecule ion fragments, $[M + Na]^+$ at *m*/*z* 475.3 and $[M + H]^+$ at 453.5. The fragment at *m*/*z* 291.2 indicated a loss of the glucoside moiety, and it was 16 larger than the molecular weight of phloretin. Thus, it is very possible that the aglycone was a hydroxyl group added by phloretin. The NMR analysis yielded the following data: ^{1}H NMR: δ_H 13.54 (1H, s, OH-6′), 10.60 (1H, s, OH-4′), 8.67, 8.60 (2H, s, exchanged, OH-3, 4), 6.66 – 6.60 (2H, m, H-2, 5), 6.51 (1H, dd, *J* = 2.1, 8.0 Hz, H-6), 6.15 (1H, d, *J* = 2.4 Hz, H-3′), 5.95 (1H, d, *J* = 2.2 Hz, H-5′), 5.31 (1H, d, *J* = 4.2 Hz, Glu OH-4″), 5.18 (1H, d, *J* = 3.8 Hz, Glu OH-2″), 5.08 (1H, d, *J* = 5.3 Hz, Glu OH-3″), 4.95 (1H, d, *J* = 7.4 Hz, Glu H-1″), 4.64 (1H, t, *J* = 5.6 Hz, Glu OH-6″), 3.74 (1H, m,

Table 1
NMR-data of five dihydrochalcone compounds in DMSO-d_6.

No.	Phlorizin		Trilobatin		3-Hydroxyphlorizin		Sieboldin		Phloretin 2′-xyloglucoside	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1		131.94		133.90		132.40		132.28		131.59
2	7.04 (d, *J* = 8.3 Hz)	128.42	7.02(d, *J* = 8.4 Hz)	129.19	6.66–6.60 (m)	115.91	6.61 (m)	115.78	7.06 (d, *J* = 8.4 Hz)	129.26
3	6.65 (d, *J* = 8.3 Hz)	114.15	6.66(d, *J* = 8.5 Hz)	115.13	8.60, 8.67 (s)[b]	144.95	8.60, 8.68 (s)[b]	145.05	6.67 (d, *J* = 8.5 Hz)	115.02
4	9.11 (s)	154.40	9.12 (s)	155.45	8.60, 8.67 (s)[b]	143.21	8.60, 8.68 (s)[b]	143.33	9.12 (s)	155.29
5	6.65 (d, *J* = 8.3 Hz)	114.15	6.66(d, *J* = 8.5 Hz)	115.13	6.66–6.60 (m)	115.47	6.61 (m)	115.51	6.67 (d, *J* = 8.5 Hz)	115.02
6	7.04 (d, *J* = 8.3 Hz)	128.42	7.02(d, *J* = 8.4 Hz)	129.19	6.51 (dd, *J* = 8.0, 2.1 Hz)	118.93	6.47 (dd, *J* = 8.0, 2.2 Hz)	118.90	7.06 (d, *J* = 8.4 Hz)	129.26
C=O		204.63		205.09		204.76		205.12		204.64
α	3.24–3.38 (m)[a]	44.98	3.13–3.35 (m)[a]	45.74	3.27–3.36 (m)[a]	44.96	3.18–3.35 (m)[a]	45.66	3.19–3.51 (m)[a]	44.95
β	2.79 (t, *J* = 7.4 Hz)	28.89	2.78 (t, *J* = 7.7 Hz)	29.37	2.75 (td, *J* = 7.1, 3.6 Hz)	29.20	2.72 (t, *J* = 7.7 Hz)	29.53	2.81 (t, *J* = 7.4 Hz)	28.98
1′		104.92		105.32		105.16		105.32		105.05
2′		160.33	12.27 (s)	163.79		160.91	12.27 (s)	163.79		160.83
3′	6.13 (d, *J* = 2.2 Hz)	93.52	6.04 (s)	95.12	6.15 (d, *J* = 2.4 Hz)	94.40	6.04 (s)	95.14	6.22 (d, *J* = 2.3 Hz)	94.63
4′	10.60 (s)	165.56		163.43	10.60 (s)	165.43		163.42	10.51(s)	165.40
5′	5.94 (d, *J* = 2.1 Hz)	96.42	6.04 (s)	95.12	5.95 (d, *J* = 2.2 Hz)	96.88	6.04 (s)	95.14	5.95 (d, *J* = 1.5 Hz)	97.02
6′	13.52 (s)	163.89	12.27 (s)	163.79	13.54 (s)	164.64	12.27 (s)	163.79	13.52 (s)	164.78
1″	4.94 (d, *J* = 7.1 Hz)	100.15	4.87 (d, *J* = 7.7 Hz)	99.60	4.95 (d, *J* = 7.4 Hz)	100.88	4.87 (d, *J* = 7.7 Hz)	99.60	4.94 (m)[a]	101.00
2″	3.24–3.38 (m)[a]	72.77	3.13–3.35 (m)[a]	73.10	3.27–3.36 (m)[a]	73.22	3.18–3.35 (m)[a]	73.10	3.19–3.51 (m)[a]	73.20
3″	3.24–3.38 (m)[a]	76.55	3.13–3.35 (m)[a]	77.19	3.27–3.36 (m)[a]	77.36	3.18–3.35 (m)[a]	77.19	3.19–3.51 (m)[a]	76.36
4″	3.19 (td, *J* = 8.9, 5.1 Hz)	69.18	3.13–3.35 (m)[a]	69.49	3.23 (dt, *J* = 9.3, 4.6 Hz)	69.48	3.18–3.35 (m)[a]	69.49	3.19–3.51 (m)[a]	69.30
5″	3.24–3.38 (m)[a]	76.46	3.13–3.35 (m)[a]	76.47	3.27–3.36 (m)[a]	76.80	3.18– .35 (m)[a]	76.48	3.56 (dd, *J* = 9.8, 6.0 Hz)	76.00
6″	3.71 (ddd, *J* = 12.1, 5.3, 2.1 Hz)		3.68 (dq, *J* = 11.9, 2.1 Hz)		3.74 (m)		3.68 (ddd, *J* = 12.0, 5.2, 2.1 Hz) 3.49 (dt, *J* = 11.5, 5.6 Hz)	60.56		
OH-2″	3.51 (dt, *J* = 11.6, 5.7 Hz)	60.52	3.49 (dt, *J* = 11.4, 5.4 Hz)	60.55	3.54 (dt, *J* = 11.3, 5.2 Hz)	60.63	5.07 (d, *J* = 4.9 Hz)		3.65 (dd, *J* = 11.6, 6.0 Hz)	68.05
OH-3″	5.17 (d, *J* = 4.4 Hz)		5.07 (d, *J* = 4.9 Hz)		5.18 (d, *J* = 3.8 Hz)		5.00 (d, *J* = 5.3 Hz)		5.25 (d, *J* = 3.9 Hz)	
OH-4″	5.07 (d, *J* = 5.3 Hz)		5.00 (d, *J* = 5.3 Hz)		5.08 (d, *J* = 5.3 Hz)		5.31 (d, *J* = 5.3 Hz)		5.18 (d, *J* = 5.3 Hz)	
OH-6″	5.31 (d, *J* = 5.0 Hz)		5.31 (d, *J* = 5.3 Hz)		5.31 (d, *J* = 4.2 Hz)		4.54 (t, *J* = 5.7 Hz)		5.35 (d, *J* = 4.5 Hz)	
1‴										
2‴									4.20 (d, *J* = 7.4 Hz)	103.94
3‴									3.02 (m)	73.46
4‴									3.11 (t, *J* = 8.8 Hz)	76.61
5‴									3.19–3.51 (m)[a]	69.61

[a] Peaks were overlapped.
[b] Peaks were exchanged.

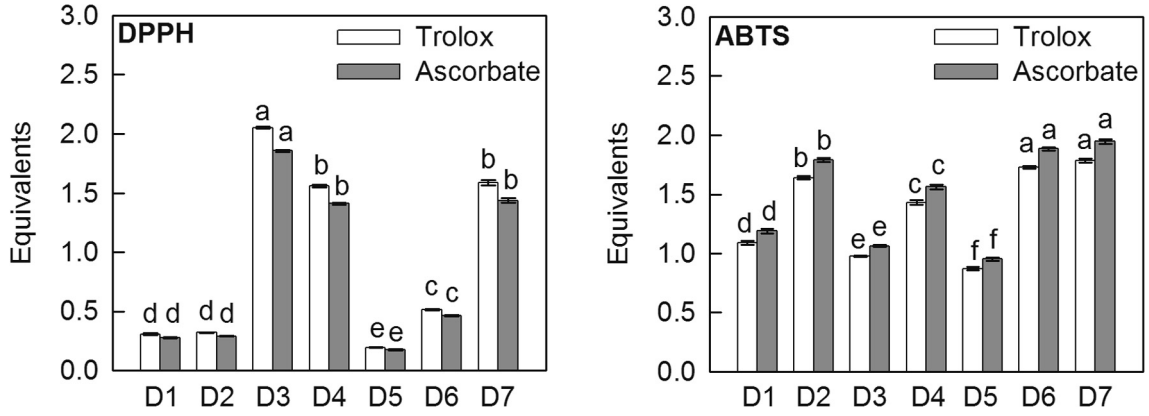


Fig. 3. Antioxidant abilities of dihydrochalcone compounds evaluated by DPPH and ABTS assays. All data are means ± SE (n = 5).

Glu-6β″), 3.54 (dt, *J* = 1H, 11.3, 5.2 Hz, Glu-6α″), 3.18–3.49 (5H, m, overlapped, H-α, Glu H-2″, 3″, 5″), 3.23 (1H, dt, *J* = 9.3, 4.6 Hz, Glu H-4″) and 2.75 (2H, td, *J* = 7.1, 3.6 Hz, H-β); ^{13}C NMR: δ_c 204.76 (C=O), 165.43 (C-4′), 164.64 (C-6′), 160.91 (C-2′), 144.95 (C-3), 143.21 (C-4), 132.40 (C-1), 118.93 (C-6), 115.91 (C-2), 115.47 (C-5), 105.16 (C-1′), 100.88 (Glu C-1″), 96.88 (C-5′), 94.40 (C-3′), 77.36 (Glu C-3″), 76.80 (Glu C-5″), 73.22 (Glu C-2″), 69.48 (Glu C-4″), 60.63 (Glu C-6″), 44.96 (C-α) and 29.20 (C-β). **D3** was identified as 3-hydroxyphlorizin.

Compound 4 (**D4**) was a white powder with a purity of 99.07%. **D4** (Rt = 36.2 min) exhibited a quasi-molecule ion at *m*/*z* 453.2 $[M + H]^+$ and an aglycone fragment at *m*/*z* 291.2, similarly to **D3**. The ^{1}H NMR analysis (500 MHz, DMSO-d_6) yielded the following data: δ_H 12.27 (2H, s, OH-2′, 6′), 8.68, 8.60 (2H, s, exchanged, OH-3, 4), 6.61 (2H, m, H-2, 5), 6.47 (1H, dd, *J* = 2.2, 8.0 Hz, H-6), 6.04 (2H, s, H-3′, 5′), 5.31 (1H, d, *J* = 5.3 Hz, Glu OH-4″), 5.07 (1H, d, *J* = 4.9 Hz, Glu OH-2″), 5.00 (1H, d, *J* = 5.3 Hz, Glu OH-3″), 4.87 (1H, d, *J* = 7.7 Hz, Glu-1″), 4.54 (1H, t, *J* = 5.7 Hz, Glu OH-6″), 3.68 (1H, ddd, *J* = 12.0, 5.2, 2.1 Hz, Glu-6β″), 3.49 (1H, dt, *J* = 11.5, 5.6 Hz, Glu-6α″), 3.18–3.35 (6H, m, overlapped, H-α, Glu H-2″, 3″, 4″, 5″) and 2.72 (2H, t, *J* = 7.7 Hz, H-β). The ^{13}C NMR data were: δ_c 205.12 (C=O), 163.79 (C-2′, 6′), 163.42 (C-4′), 145.05 (C-3), 143.33 (C-4), 132.28 (C-1), 118.90 (C-6), 115.78 (C-2), 115.51 (C-5), 105.32 (C-1′), 99.60 (Glu C-1″), 95.14 (C-3′, 5′), 77.19 (Glu C-3″), 76.48 (Glu C-5″), 73.10 (Glu C-2″), 69.49 (Glu C-4″), 60.56 (Glu C-6″), 45.66 (C-α) and 29.53 (C-β). **D4** was identified as sieboldin. Notably, in the ^{1}H spectrum, two hydrogen atoms on the B ring were indistinguishable from 3-hydroxyphlorizin and sieboldin, respectively. Therefore, they are labeled as 'exchanged'.

Compound 5 (**D5**) was a loose white powder with a purity of 98.88%. **D5** (Rt = 35.8 min) exhibited a quasi-molecule ion at *m*/*z* 569.5 $[M + H]^+$ and two fragments at *m*/*z* 437.2 and *m*/*z* 275.2. The fragment at *m*/*z* 437.2 indicated a loss of the xyloside moiety, while that at *m*/*z* 275.2 was an aglycone fragment with an additional loss of a glucoside moiety. The ^{1}H NMR analysis (500 MHz, DMSO-d_6) yielded the following data: δ_H 13.52 (1H, s, OH-6′), 10.51 (1H, s, OH-4′), 9.12 (1H, s, OH-4), 7.06 (2H, d, *J* = 8.4 Hz, H-2, 6), 6.67 (2H, d, *J* = 8.5 Hz, H-3, 5), 6.22 (1H, d, *J* = 2.3 Hz, H-3′), 5.95 (1H, d, *J* = 1.5 Hz, H-5′), 5.35 (1H, d, *J* = 4.5 Hz, Glu OH-4″), 5.25 (1H, d, *J* = 3.9 Hz, Glu OH-2″), 5.18 (1H, d, *J* = 5.3 Hz, Glu OH-3″), 4.94 (1H, m, overlapped, Glu H-1″), 4.20 (1H, d, *J* = 7.4 Hz, xylo-1‴), 3.99 (1H, *J* = 11.7 Hz, d, Glu H-6″), 3.71 (1H, dd, *J* = 11.3, 5.3 Hz, xylo-5β‴), 3.65 (1H, dd, *J* = 11.6, 6.0 Hz, Glu H-6″), 3.56 (1H, dd, *J* = 9.8, 6.0 Hz, Glu H-5″), 3.19 – 3.51 (6H, m, overlapped, H-α, Glu H-2″, 3″, 4″, xylo-4H‴), 3.11 (1H, t, *J* = 8.8 Hz, xylo-3H‴), 3.02 (2H, m, xylo-2H‴, 5‴) and 2.81 (2H, t, *J* = 7.4 Hz, H-β). The hydrogen atoms on the hydroxyl group belonging to xylopyranosyl were not detected. ^{13}C NMR data were: δc 204.64 (C=O), 165.40 (C-4′), 164.78 (C-6′), 160.83 (C-2′), 155.29 (C-4), 131.59 (C-1), 129.26 (C-2,6), 115.02 (C-3,5), 105.05 (C-1′), 103.94 (Xylo C-1‴), 101.00 (Glu C-1″), 97.02 (C-5′), 94.63 (C-3′), 76.61 (Xylo C-3‴), 76.36 (Glu C-3″), 76.00 (Glu C-5″), 73.46 (Xylo C-2‴), 73.20 (Glu C-2″), 69.61 (Xylo C-4‴), 69.30 (Glu C-4″), 68.05 (Glu C-6″), 65.66 (Xylo C-5‴), 44.95 (C-α) and 28.98 (C-β). **D5** was identified as phloretin 2′-xyloglucoside, consistent with previous studies (Will, Zessner, Becker, & Dietrich, 2007).

Phlorizin and sieboldin were used to produce aglycones via hydrolysis. Phloretin (**D6** Rt = 60.0 min) and 3-hydroxyphloretin (**D7** Rt = 49.6 min) were a slightly pink powder with a purity of 99.69% and 99.84%, respectively.

3.2. Antioxidant capacity of dihydrochalcone compounds

In the DPPH assay, the antioxidant capacities of the seven compounds demonstrated the following order: **D3** > **D4** = **D7** > **D6** > **D1** = **D2** > **D5** (Fig. 3). Moreover, the capacities of **D3**, **D4,** and **D7** were higher and the other four compounds were lower in comparison to trolox and ascorbate. The antioxidant capacity of **D3** was almost double but that of **D5** was only one fifth of the capacity of trolox or ascorbate. Clearly, the compounds with *ortho*-dihydroxyl conformation in the B ring exhibited stronger antioxidant capacities. The *ortho*-dihydroxyl groups in the B ring of **D3**, **D4** and **D7** may form a hydrogen bond, which lower the bond dissociation enthalpies and make hydrogen atom donation become easier (Galano et al., 2016; Leopoldini, Russo, & Toscano, 2011). Therefore, hydrogen atom transfer (HAT) mechanism might dominantly occur during the DPPH radical scavenging by **D3**, **D4** and **D7**. As a result, **D3**, **D4** and **D7** showed remarkably higher antioxidant capacities than the other four compounds.

Interestingly, it was found that **D3** showed significantly stronger DPPH scavenging capacity than **D7** did. By ^{1}H NMR analysis, we noticed that the glycosylation at the 2′-position in the A ring of these dihydrochalcones may enhance the ionization of the phenolic hydroxyl group at the 4′-position (Supplemental Figs. S13 & S14). This is favorable to the occurrence of the sequential proton loss electron transfer (SPLET) mechanism (Foti, Daquino, Dilabio, & Ingold, 2011). Because the A ring and B ring of dihydrochalcone molecule are two discontinuously conjugated systems, these two rings may be independent when reacting with free radicals. So, both the HAT and SPLET mechanisms might occur when **D3** scavenging DPPH radical.

For **D1**, **D2**, **D5** and **D6**, which have only one hydroxyl group in the B ring, they are unlikely to react with DPPH radical by HAT mechanism. The different DPPH scavenging capacities among them could not be simply explained by SPLET mechanism either. In the present experiment, the reactions with DPPH radical were carried

Table 2
Cytotoxicity (IC_{50}) of dihydrochalcone compounds on human cancer cell lines. All data are mean ± SE (n = 5).

Compounds	IC_{50} (μM)				
	MG-63	Hela	Hep G2	MDA-MB-231	SK-OV-3
D1	>150[a]	>150[a]	>150[a]	>150[a]	>150[a]
D2	>150[a]	>150[a]	>150[a]	>150[a]	>150[a]
D3	51.10 ± 1.39	66.90 ± 2.09	34.13 ± 2.87	55.48 ± 2.06	80.80 ± 5.04
D4	32.64 ± 2.65	73.77 ± 2.78	40.93 ± 0.28	79.95 ± 2.21	68.31 ± 3.00
D5	>150[a]	>150[a]	>150[a]	>150[a]	>150[a]
D6	142.54 ± 4.11	118.79 ± 4.46	68.67 ± 2.25	66.154 ± 1.05	121.279 ± 1.16
D7	60.25 ± 3.61	59.37 ± 4.46	50.92 ± 1.82	79.87 ± 1.40	101.07 ± 3.00
Paclitaxel[b]	0.023 ± 0.003	0.187 ± 0.014	0.095 ± 0.001	4.502 ± 0.316	0.011 ± 0.000
10-Hydroxycamptothecin[b]	0.592 ± 0.078	18.593 ± 0.764	0.919 ± 0.116	12.286 ± 0.777	0.099 ± 0.006

MG-63, bone cancer cell line; Hep G2, liver cancer cell line; Hela, cervix cancer cell line; MDA-MB-231, breast adenocarcinoma cell line; SK-OV-3, ovarian cancer cell line.
[a] No cytotoxicity was detected at 150 μM.
[b] Paclitaxel and 10-Hydroxycamptothecin were used as positive controls.

out in methanol, a kind of partially ionize solvent, allowing both electron and hydrogen transfer (Xie & Schaich, 2014). Therefore, the process of DPPH radical scavenging by these four dihydrochalcone compounds might involve other mechanisms.

Using the ABTS assay, the following antioxidant capacity was found: **D6** = **D7** > **D2** > **D4** > **D1** > **D3** > **D5** (Fig. 3). Excluding **D3** and **D5**, all of the compounds showed higher antioxidant capacities than trolox and ascorbate. Among the seven compounds, **D2**, **D4**, **D6** and **D7** have free hydroxyl groups at both 2′- and 6′-position of the A ring and relatively higher $ABTS^{\cdot+}$ radical scavenging capacity than the other three which have glycosyl at the 2′-position. This result indicates the $ABTS^{\cdot+}$ radical scavenging capacity mainly depends on the A ring of dihydrochalcone molecules.

The phenolic hydroxyl groups near the carbonyl group of the A ring of flavonoid compounds can form intramolecular hydrogen bonds (Musialik, Kuzmicz, Pawłowski, & Litwinienko, 2009). For **D1**, **D3** and **D5**, as the hydroxyl group at the 2′-position of the A ring is occupied by glycosyl, the hydroxyl group at the 6′-position could form a hydrogen bond with the carbonyl group. Meanwhile, for **D2**, **D4**, **D6** and **D7**, the hydroxyl groups at the 2′- and 6′-position of the A ring do not differ greatly in their chemical structure under our experimental conditions, as supported by the NMR data. The hydroxyl hydrogen atoms at the 2′- and 6′- positions of **D2** and **D4** displayed the same chemical shift and were shown as one peak in the ^1H NMR spectrum (Table 1, Supplementary Figs. S4 & S8). Therefore, even the carbonyl group forms a hydrogen bond with one of the hydroxyls at 2′- and 6′-position of the A ring, there is still a free hydroxyl group of **D2**, **D4**, **D6** and **D7**. Differed from DPPH radical, $ABTS^{\cdot+}$ radical is more reactive and likely react with antioxidants by single electron transfer (SET) mechanism (Ak & Gülçin, 2008). Clearly, the free hydroxyl group of the A ring of **D2**, **D4**, **D6** and **D7** might play an important role in the process of electron transfer. However, other mechanisms might also be involved in the reaction between $ABTS^{\cdot+}$ radical with the dihydrochalcone compounds (Tian & Schaich, 2013). The steric accessibility may also affect the reaction (Schaich, Tian, & Xie, 2015). For instance, the glycosylation of **D1**, **D3** and **D5** at the 2′-position and of **D2** and **D4** at the 4′-position of the A ring lowered their antioxidant capacities in comparison to aglycones. Meanwhile, the hydroxyl group at the 3-position of the B ring also exhibited a slight effect on the antioxidant capacity in the ABTS assay, but this effect depended on the glycosylation of the A ring. Further studies are needed to clarify the mechanisms of reaction between $ABTS^{\cdot+}$ radical with the dihydrochalcone compounds.

Clearly, the DPPH assay mainly reflected the antioxidant capacity of the B ring, whereas the ABTS assay was mostly related to the A ring of the dihydrochalcone molecule. Antioxidants may scavenge free radicals with kinds of mechanisms, which depend on the molecular structures of antioxidants, the properties of free radicals and the reacting conditions (Schaich et al., 2015; Tian & Schaich, 2013; Xie & Schaich, 2014). Unlike most other flavonoids, the basic chemical structure of dihydrochalcone molecule contains two independent conjugated systems of the A ring and B ring, with the connection between these two conjugated systems being flexible. Consequently, these two rings may independently react with free radicals, which lead to the reaction between dihydrochalcones with free radicals occurring by different mechanisms, and result in different scavenging capacities.

3.3. Cytotoxicity of dihydrochalcone compounds in vitro

Five cancer cell lines, including MG-63 (bone cancer), Hep G2 (liver cancer), Hela (cervix cancer), MDA-MB-231 (breast adenocarcinoma) and SK-OV-3 (ovarian cancer), were used to evaluate the cytotoxicity of the dihydrochalcone compounds (Table 2). For MG-63, the cytotoxicity of **D4** was strongest, whereas for Hela the cytotoxicities of **D3**, **D4** and **D7** were relatively stronger than those of the other compounds. For Hep G2 and SK-OV-3, **D3** and **D4** exhibited relatively higher levels of cytotoxicity. For MDA-MB-231, **D3** showed the best cytotoxicity.

It was very interesting to note that among these dihydrochalcone compounds, **D3**, **D4**, **D6** and **D7** displayed cytotoxicity against all of the cancer cell lines, while **D1**, **D2** and **D5** did not show any cytotoxicity even when their concentrations increased to 150 μM. Clearly, the hydroxyl at the 3-position contributed to the increase in cytotoxicity, but only after the glycosylation of dihydrochalcones. While it did not decrease the cytotoxicity of the dihydrochalcone molecule with the hydroxyl at 3-position, the glycosylation significantly decreased the cytotoxicity of the dihydrochalcone molecule without the hydroxyl at the 3-position. These results suggest that both glycosylation and the ortho phenolic hydroxyl groups were important for the cytotoxicity of dihydrochalcone molecules. It should be noted that although **D3** and **D4** exhibited better cytotoxicity than other dihydrochalcone compounds, their IC_{50} values ranged from 30 to 80 μM for all cancer cell lines tested, which were significantly higher than those of paclitaxel or 10-hydroxycamptothecin. However, the dihydrochalcone compounds were more easily available in comparison to paclitaxel and 10-hydroxycamptothecin and may have the potential to prevent cancer as a food resource. Moreover, previous studies have shown that phloretin can potentiate the anticancer actions of Paclitaxel on Hep G2 cells (Yang et al., 2009), potentially representing an effective route for the treatment of cancer with dihydrochalcone compounds.

4. Conclusion

In general, seven dihydrochalcone compounds were obtained from *Malus* 'Red Splendor'. Among the seven dihydrochalcone

compounds, **D7**, 3-hydroxyphloretin was the best antioxidant, as evidenced by both the DPPH and ABST assays. The free radical quenching by dihydrochalcones is not a single reaction but embraces multiple mechanisms. It is very important to use a variety of methods to evaluate the antioxidant capacity of dihydrochalcone compounds or crude extracts which contain dihydrochalcones. To evaluate the cytotoxicity of dihydrochalcone molecules, **D3** and **D4**, namely 3-hydroxyphlorizin and sieboldin, exhibited higher cytotoxicity in cancer cell lines. Dihydrochalcones from *Malus* may have great potential for human health. For most of apple cultivars (*Malus domestica*), their fruits mainly contain **D1** and **D5**, which showed relatively lower antioxidant capacities and cytotoxicities. It is of significance to increase the concentrations of other dihydrochalcone compounds in apple fruits by breeding in future.

Acknowledgements

This study was supported by ‘‘the Fundamental Research Funds for the Central Universities‘‘, China. We are grateful to Dr. Qiang Zhang (College of Science, Northwest A&F University, Yangling), Prof. Hongli Zhang (State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling) and Dr. Jiangjiang Tang (College of Science, Northwest A&F University, Yangling) for their helps on compounds structural identification and discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.03.111.

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