

Oplopanphesides A—C, Three New Phenolic Glycosides from the Root Barks of *Oplopanax horridus*

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Three new phenolic glycosides, named oplopanphesides A—C (1—3), have been isolated from the root barks of *Oplopanax horridus*. Their structures were elucidated by a combination of spectroscopic analyses, including 1D- and 2D-NMR techniques. These phenolic glycosides possess a novel feature in their sugar moieties that a 3-hydroxy-3-methylglutaryl moiety was connected with C-6 of the β -D-glucopyranosyl group. Those compounds showed no cytotoxic effects against human cancer cell lines (MDA-231 and MCF-7) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

Key words *Oplopanax horridus*; phenolic glycoside; oplopanpheside; 3-hydroxy-3-methylglutaryl

Oplopanax horridus belongs to the *Oplopanax* genus classified to the Araliaceae family.^{1–3} Traditionally, the inner bark and roots of *O. horridus* are commonly used to treat such as diabetes, tuberculosis, headaches, lung hemorrhages and cancer by many indigenous peoples of Alaska and the Pacific Northwest.⁴ In order to discover new natural compounds with interesting biological activities, phytochemical investigations were carried out on the root barks of *O. horridus* collected in America. Previous phytochemical studies of the title plant have led to the identification of some polyynes, polyenes and volatile oil from the hydrophobic fraction.^{5–7} However, the chemical constituents from the hydrophilic parts of this medicinal plant were rarely known. An investigation of bioactive molecules from its hydrophilic fraction had led to the isolation and identification of three new phenolic glycosides, named oplopanphesides A—C (1—3). Phenolic glycosides have not been reported from *O. horridus* but only from *O. elatus* of this genus.⁸ Phenolic glycosides had been demonstrated to have antioxidant, anticancer, anticancerogenic, immunomodulatory, antimicrobial and antiparasitic activities.^{9–11} The present paper describes the isolation and structural elucidation of the new compounds on the basis of their spectroscopic data and the bioassay of their cytotoxicity against two kinds of human cancer cell lines MDA-231 and MCF-7).

Results and Discussion

The filtrate of an 85% aqueous ethanol extract of air-dried root barks of *O. horridus* afforded three new phenolic glycosides named oplopanphesides A—C (1—3) (Fig. 1). The phenolic glycosides were isolated by successive column chromatography on macroporous resins (D-101), subsequent silica gel, reversed-phase C₁₈ silica gel and preparative reversed-phase HPLC.

Oplopanpheside A (**1**) was obtained as a colorless gum with negative optical rotation [α]_D²⁰ –13.6 (c =0.7, MeOH). The molecular formula was determined to be C₂₁H₂₈O₁₃ by high resolution-electrospray ionization-mass spectra (HR-ESI-MS), which showed a [M+Na]⁺ ion at m/z 511.1420 (Calcd for C₂₁H₂₈O₁₃Na⁺: 511.1422). The IR spectrum of **1** showed the presence of hydroxyl (3420 cm^{–1}), carbonyl

(1717 cm^{–1}), methyl (2935 cm^{–1}), and aromatic ring (1602, 1512 cm^{–1}). The UV absorption maxima occurred at 233 and 280 nm, implying the presence of conjugated double bond system in this compound. The ¹H-NMR spectrum of **1** displayed signals due to three aromatic protons at δ_H 7.64 (1H, dd, J =2.0, 8.5 Hz), 7.60 (1H, d, J =2.0 Hz), and 7.18 (1H, d, J =8.5 Hz), two methoxyl groups at δ_H 3.90 (3H, s) and 3.88 (3H, s), as well as an anomeric proton at δ_H 5.03 (1H, d, J =7.5 Hz) (Table 1). Analysis of the ¹³C-NMR, distortionless enhancement by polarization transfer (DEPT) and heteronuclear multiple quantum correlation (HMQC) spectra revealed the presence of twenty one carbons (Table 1), including six sp^2 carbons (δ_C 152.0, 150.6, 125.7, 124.5, 116.8, 114.3) due to a 1,3,4-trisubstituted aromatic ring; six oxygen-bearing sp^3 carbons (δ_C 101.8, 78.0, 75.5, 74.8, 71.5, 64.5) assignable to a glucose unit; two methoxy carbon signals at δ_C 56.8 and 52.7; and seven additional carbon signals which were assigned to a methyl group (δ_C 27.8), two methylene groups (δ_C 46.4, 45.9), a quaternary carbon (δ_C 70.7), and three carboxyl carbons (δ_C 175.0, 172.4, 168.3), respectively. Acid hydrolysis of **1** afforded D-glucose. The coupling constant (J =7.5 Hz) of the anomeric H-atom revealed β -configuration of the D-glucopyranose.¹² With the aid of ¹H–¹H correlation spectroscopy (COSY), HMQC and heteronuclear multiple bond correlation (HMBC) experiments, the NMR data of **1** were assigned as shown in Table 1. The HMBC correlations of H-2'' [δ_H 2.74 (d, J =14.7 Hz) and 2.64 (d, J =14.7 Hz)] with C-1'' (δ_C 172.4), H-4'' [δ_H 2.64 (d, J =15.2 Hz) and 2.62 (d, J =15.2 Hz)] with C-5'' (175.0), and CH₃-3'' [δ_H 1.26 (s)] with C-3'' (70.7) revealed the existence of a 3-hydroxy-3-methylglutaryl unit (HMG) as a substructure.^{13,14} (Fig. 2) Furthermore, the HMBC correlations between H-6' (δ_H 4.47, 4.21) and C-1'' (δ_C 172.4) indicated that the HMG unit was connected to the C-6' position of glucopyranosyl. The HMBC cross peak between anomeric proton (δ_H 5.03) of glucose unit and C-4 (δ_C 152.0) of aromatic ring assigned the glycosidic linkage site. Thus, the structure of **1** was identified as (–)-methyl benzoate-3-methoxy-4-O-[6'-O-3''-hydroxy-3''-methylglutaryl]- β -D-glucopyranoside.

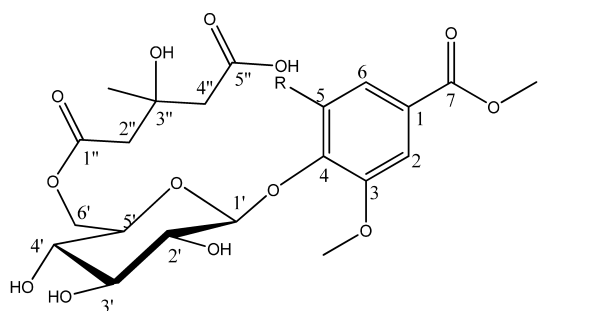
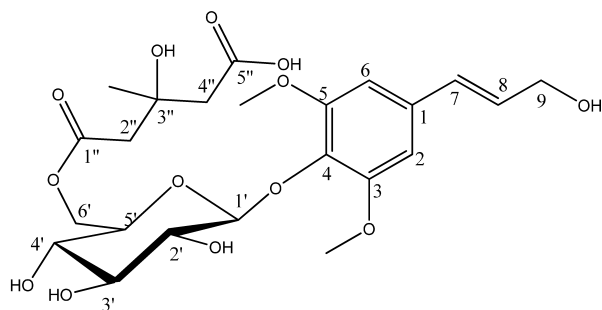
Oplopanpheside B (**2**) was prepared as a colorless gum with negative optical rotation [α]_D²⁰ –15 (c =0.4, MeOH). Its

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Table 1. ^1H - (500 MHz) and ^{13}C -NMR (125 MHz) Data of **1**–**3** in $\text{CD}_3\text{OD}^{a,b}$ (δ in ppm, J in Hz)

Position	1		2		3	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1		125.7		127.3		135.3
2	7.60 (d, 2.0)	114.3	7.33 (s)	108.4	6.72 (s)	105.4
3		150.6		154.5		154.5
4		152.0		140.0		135.6
5	7.18 (d, 8.5)	116.8		154.5		154.5
6	7.64 (dd, 2.0, 8.5)	124.5	7.33 (s)	108.4	6.72 (s)	105.4
7		168.3		168.1	6.54 (d, 15.9)	131.3
8					6.34 (dt, 5.5, 15.9)	130.0
9					4.23 (d, 5.5)	63.6
1'	5.03 (d, 7.5)	101.8	5.04 (d, 7.6)	104.0	4.85 (d, 7.6)	104.9
2'	3.54 (t, 7.8)	74.8	3.52 (t, 7.8)	75.6	3.50 (t, 8.0)	75.5
3'	3.48 (t, 8.1)	78.0	3.43 (m)	77.8	3.42 (m)	77.7
4'	3.40 (t, 9.0)	71.5	3.36 (m)	71.7	3.38 (m)	71.6
5'	3.67 (m)	75.5	3.40 (m)	75.7	3.40 (m)	75.6
6'a	4.21 (dd, 6.3, 12.0)	64.5	4.33 (dd, 2.0, 11.9)	64.5	4.33 (dd, 5.5, 10.9)	64.6
6'b	4.47 (dd, 2.2, 12.0)		4.19 (dd, 5.9, 11.9)		4.21 (dd, 2.0, 10.9)	
1''		172.4		172.3		172.4
2''a	2.74 (d, 14.7)	46.4	2.61 (d, 14.7)	46.4	2.62 (d, 14.5)	45.9
2''b	2.64 (d, 14.7)		2.54 (d, 14.7)		2.56 (d, 14.5)	
3''		70.7		70.6		70.7
4''a	2.62 (d, 15.2)	45.9	2.52 (d, 15.3)	46.0	2.54 (s)	46.3
4''b	2.64 (d, 15.2)		2.50 (d, 15.3)			
5''		175.0		175.1		175.0
3-OCH ₃	3.90 (s)	56.8	3.88 (s)	57.1	3.84 (s)	57.0
5-OCH ₃			3.88 (s)	57.1	3.84 (s)	57.0
7-OCH ₃	3.88 (s)	52.7	3.90 (s)	52.8		
3''-CH ₃	1.26 (s)	27.8	1.24 (s)	27.6	1.26 (s)	27.6

a) TMS was used as an internal standard in spectra experiments. b) Assignments based on HMQC and HMBC experiments.

Oplopanpheside A (**1**) R = HOplopanpheside B (**2**) R = OCH₃Oplopanpheside C (**3**)Fig. 1. Structures of Oplopanpheside A–C (**1**–**3**)

molecular formula, $\text{C}_{22}\text{H}_{30}\text{O}_{14}$, was determined from the $[\text{M}+\text{Na}]^+$ ion peak at 541.1548 (Calcd for $\text{C}_{22}\text{H}_{30}\text{O}_{14}\text{Na}^+$: 541.1528) in the HR-ESI-MS. The UV absorption maxima (358, 258, 220, 217 nm) and IR absorptions (3430, 2929, 1720, 1597, 1510 cm^{-1}) suggested that compound **2** had the same structural skeleton as compound **1**. Compared with compound **1**, only two aromatic protons at δ_{H} 7.33 were recorded on the ^1H -NMR spectrum but one more methoxy group (δ_{H} 3.88) was observed. A 1,3,4,5-tetrasubstituted aromatic ring could be deduced according to the ^1H -, ^{13}C -NMR, DEPT and HMQC spectra analysis, which confirmed that the aromatic H-5 of **1** was substituted by a methoxy group to afford the new compound **2**. The NMR spectra of **2** revealed the presence of a glucose unit (δ_{C} 104.0, 77.8, 75.7, 75.6, 71.7, 64.5) and an HMG moiety (δ_{C} 175.1, 172.3, 70.6, 46.4, 46.0, 27.6). Acid hydrolysis of **1** afforded D-glucose. Compared to the known compound methyl benzoate-3,5-dimethoxy-4-O- β -D-glucopyranoside,¹⁵ the downfield shift of C-6' resonance of the glucosyl moiety from δ_{C} 62.6 to 64.5 and the resonance of H-6' (δ_{H} 4.19, 4.33) suggested that the HMG moiety was attached to C-6' of the glucopyranosyl group, which could be further confirmed by the HMBC correlation between H-6' (δ_{H} 4.33, 4.19) and C-1'' (δ_{C} 172.3). Based on the above results, the structure of **2** was elucidated as (–)-methyl benzoate-3,5-dimethoxy-4-O-[6'-O-3''-hydroxy-3''-methylglutaryl]- β -D-glucopyranoside.

Oplopanpheside C (**3**) was isolated as amorphous powder, with specific rotation $[\alpha]_{\text{D}}^{20} -10.7$ ($c=0.5$, MeOH). The HR-ESI-MS showed the $[\text{M}+\text{Na}]^+$ ion at m/z 539.1741 (Calcd for $\text{C}_{23}\text{H}_{32}\text{O}_{13}\text{Na}^+$: 539.1735), indicating the molecular formula $\text{C}_{23}\text{H}_{32}\text{O}_{13}$. The UV spectrum of **3** showed absorption

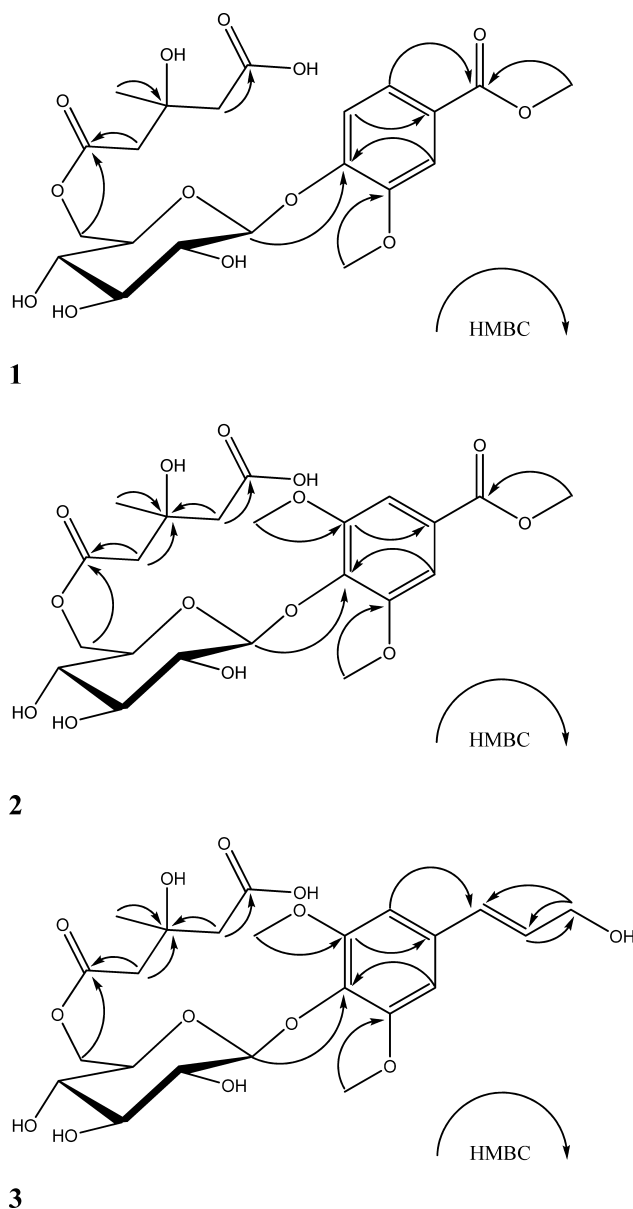


Fig. 2. Key HMBC for Determination of Oplopanpheside A—C (1—3)

maxima at 219 and 261 nm. The IR spectrum indicated the presence of hydroxyl group (3400 cm^{-1}), conjugated carbonyl (1714 cm^{-1}), and aromatic ring (1587 , 1507 cm^{-1}). The ^1H - and ^{13}C -NMR signals of **3** were assigned on the basis of ^1H - ^1H COSY, nuclear Overhauser effect spectroscopy (NOESY), HMQC and HMBC experiments. The ^1H -NMR spectrum of **3** displayed signals for two aromatic protons [δ_{H} 6.72 (2H, s)] of a symmetrical 1,3,4,5-tetrasubstituted aromatic ring, two *trans*-olefinic protons [δ_{H} 6.54 (d, $J=15.9\text{ Hz}$), and 6.34 (dt, $J=15.9$, 5.5 Hz)] and two methoxy group at δ_{H} 3.84. The ^{13}C -NMR spectra of **3** revealed the presence of a syringin moiety,¹⁶ (Table 1) and an HMG moiety (δ_{C} 175.0, 172.4, 70.7, 46.3, 45.9, 27.6). In the HMBC spectrum, correlations between H-6' (δ_{H} 4.33, 4.21) and C-1'' (δ_{C} 172.4), between H-1' [δ_{H} 4.85 (d, $J=7.6\text{ Hz}$)] and C-4 (δ_{C} 135.3) were clearly observed, revealing the HMG group was connected to C-6' of glucopyranosyl. From the above results, the structure of **3** was characterized as (–)-6'-*O*-3''-hy-

droxy-3''-methylglutaryl-syringin.

Since compounds **1**—**3** were obtained at a very low yield, the usual approach to absolute configuration of the HMG moiety based on reductive hydrolysis could not be applied.¹⁷ The absolute configuration of the HMG moiety in compounds **1**—**3** was not elucidated yet.

Oplopanphesides A—C (**1**—**3**) had been evaluated for their α -glucosidase inhibition activity, they were inactive ($\text{IC}_{50}>50\text{ }\mu\text{M}$, respectively). In addition, the cytotoxic activities of these new glycosides against the human cancer cell lines (MDA-231 and MCF-7) were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. As a result, the activities of these compounds were inactive ($\text{IC}_{50}>100\text{ }\mu\text{M}$, respectively).

Experimental

General Procedures Optical rotations were measured on a PerkinElmer Model 341 polarimeter. UV spectra were recorded on a Beckman Coulter DU 640 spectrophotometer. IR spectra were obtained with a PerkinElmer Spectrum 100 FT-IR spectrometer with KBr pallets. The ^1H -, ^{13}C -, and 2D-NMR spectra were recorded on a Bruker AV-500 spectrometer (δ in ppm, J in Hz) with tetramethylsilane (TMS) as an internal standard (Bruker, Germany). ESI-MS and HR-ESI-MS measurements were carried out on an Agilent 1100 series LC/MSD Trap VL mass spectrometer and an Agilent time-of-flight (TOF) mass spectrometer respectively (Agilent, U.S.A.). Macroporous resin (pre-treated type, D-101) was purchased from Haiguang Chemical Industrial Company (Tianjin, China). Silica gel (100—200 and 200—300 mesh) (Qingdao Haiyang Chemical Co., Ltd., China) and Alltech Reversed-phase C_{18} (RP- C_{18}) silica gel (40—63 μm) (Alltech, U.S.A.) were used for column chromatography (CC). Precoated silica gel GF₂₅₄ plates (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) were used for TLC. Supercritical fluid extraction was manipulated on a supercritical fluid extractor (SFT-250, Supercritical Fluid Technologies, Inc., U.S.A.). Analytical HPLC was performed on an Agilent 1100 liquid chromatograph with an Agilent Zorbax SB RP- C_{18} column (250 mm $\times$ 4.6 mm inside diameter (i.d.), 5 μm , Alltech, U.S.A.). Preparative HPLC was carried out with an Agilent 1100 liquid chromatograph with a Phenomenex Luna RP- C_{18} column (250 mm $\times$ 22 mm i.d., 5 μm). HPLC-grade methanol and Acetonitrile were the products of Merck (Merck, Germany). The deionized water used for HPLC was purified by a Milli-Q purification system (Millipore, U.S.A.). α -Glucosidase type I (EC 3.2.1.20) from *Saccharomyces cerevisiae*, *p*-nitrophenyl α -D-glucopyranoside (PNPG), as the substrate of α -glucosidase, and acarbose were purchased from Sigma (St. Louis, MO, U.S.A.).

Plant Collection The air-dried roots bark of *O. horridus* was collected and identified by one of the authors (C.-Z. Wang) from Chicago, IL of U.S.A. in March, 2009. A voucher specimen has been deposited in the Laboratory of Quality Control, Institute of Chinese Medicine Sciences, University of Macau, Macao, China.

Extraction and Isolation After the volatile oil was removed from the air-dried, powdered roots bark of *O. horridus* (10.5 kg) by supercritical fluid extraction (SFE), the residue (10.2 kg) was extracted by 85% EtOH under refluxing, and the crude extract (1900 g) was suspended in water and then extracted successively with petroleum ether (60—90°C), EtOAc, and *n*-BuOH to give the corresponding fractions P (124 g), E (570 g) and B (610 g), respectively. The *n*-BuOH-soluble fraction B (560 g) was separated by Macroporous resins (D-101) column chromatography (CC), eluted with a gradient of EtOH-H₂O (0:1 to 1:0) to give four fractions (B1—B4). Fraction B2 (105 g) was then subjected to CC of silica gel (100—200 mesh), eluting with CHCl_3 -MeOH (10:1 to 0:1), to give nine subfractions (B2a—B2i). Subfraction B2c (13 g) was chromatographed on RP- C_{18} silica gel CC (MeOH-H₂O, 40:60), then prepared on Prep-HPLC (acetonitrile-H₂O, 24:76) to afford **1** (8 mg) and **2** (7 mg). Fraction B2e (18 g) was subjected to silica gel (200—300 mesh) CC, eluting with CHCl_3 -MeOH (5:1), to afford five subfractions (B2e1—B2e5). Subfraction B2e2 (2 g) was chromatographed on RP- C_{18} silica gel CC (MeOH-H₂O, 40:60), then by prep-HPLC (acetonitrile-H₂O, 20:80) to afford **3** (4.7 mg).

Oplopanpheside A (**1**): Colorless gum, [α]_D²⁰ –13.6 ($c=0.7$, MeOH). UV λ_{max} (MeOH) nm (log ϵ): 233 (3.19), 280 (1.48), 358 (0.08). IR (KBr) cm^{-1} : 3420, 2935, 1717, 1601, 1512, 1436, 1417, 1337, 1298, 1275, 1073, 897, 765. ^1H - and ^{13}C -NMR spectrometric data are given in Table 1. HR-ESI-MS m/z : 511.1420 [$\text{M}+\text{Na}$]⁺ (Calcd for $\text{C}_{21}\text{H}_{28}\text{O}_{13}\text{Na}$: 511.1422).

Oplopanpheside B (**2**): Colorless gum, $[\alpha]_D^{20} -15$ ($c=0.4$, MeOH). UV $\lambda_{\max}$ (MeOH) nm (log ϵ): 217 (2.71), 220 (2.77), 358 (0.03). IR (KBr) cm^{-1} : 3440, 2935, 1720, 1602, 1515, 1465, 1422, 1384, 1271, 1224, 1074, 818, 746. ^1H - and ^{13}C -NMR spectrometric data are given in Table 1. HR-ESI-MS m/z : 541.1548 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{22}\text{H}_{30}\text{O}_{14}\text{Na}$: 541.1528).

Oplopanpheside C (**3**): Colorless amorphous powder, $[\alpha]_D^{20} -10.7$ ($c=0.5$, MeOH). UV $\lambda_{\max}$ (MeOH) nm (log ϵ): 198 (0.60), 215 (2.84), 219 (2.87), 261 (1.55). IR (KBr) cm^{-1} : 3400, 2924, 1714, 1587, 1507, 1455, 1420, 1382, 1356, 1333, 1244, 1195, 1127, 1080, 1019, 909, 843, 702. ^1H - and ^{13}C -NMR spectrometric data are given in Table 1. HR-ESI-MS m/z : 539.1741 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{23}\text{H}_{32}\text{O}_{13}\text{Na}$: 539.1735).

Acid Hydrolysis Compounds **1** (2 mg) and **2** (2 mg) were hydrolyzed with $2\text{ mol}\cdot\text{L}^{-1}$ TFA (1 ml) in a sealed glass tube with screw cap which were filled with N_2 at 100°C for 2 h, respectively. The hydrolyzed solution was evaporated to dryness under 50°C and then methanol (2 ml) was added for further evaporation and complete removal of trifluoroacetic acid (TFA). The hydrolysate was used for derivatization.

Sugar Analysis The stock solution of standard monosaccharides (1 ml) was treated with hydroxylamine hydrochloride-pyridine solution (1 ml) in a sealed glass tube at 90°C for 30 min. Acetic anhydride (1 ml) was added and continuing heated for another 30 min after the solution cooling to room temperature. The cooled solution was evaporated to dryness under diminished pressure at 50°C . The residue was dissolved in dry methanol (2 ml) was added. The mixture was filtered through $0.45\text{ }\mu\text{m}$ syringe filter (Agilent Technologies) prior to injection into GC-MS system. The hydrolysate was reacted with hydroxylamine hydrochloride and acetic anhydride to form the derivatives directly as in the procedures mentioned above for sugar determination. GC-MS was performed on an Agilent 6890 gas chromatography instrument coupled with an Agilent 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA, U.S.A.). A HP-5MS capillary column ($30\text{ m}\times 0.25\text{ mm}$, i.d.) coated with $0.25\text{ }\mu\text{m}$ film 5% phenyl methyl siloxane was used for separation. The column temperature was set at 175°C and held for 7 min, then programmed at $5^\circ\text{C}\cdot\text{min}^{-1}$ to 185°C and held for 5 min, then at $4^\circ\text{C}\cdot\text{min}^{-1}$ to 230°C . Split injection ($2\text{ }\mu\text{l}$) with a split ratio of 1:50 was applied. High purity helium was used as carrier gas with flow rate of $1.0\text{ ml}\cdot\text{min}^{-1}$. The mass spectrometer was operated in electron-impact (EI) mode, the scan range was $40\text{--}550\text{ amu}$, the ionization energy was 70 eV and the scan rate was 2.89 s per scan . The inlet, ionization source temperature were 250 and 280°C , respectively.

α -Glucosidase Inhibition Assay The inhibitory activity of the phenolic glycosides on α -glucosidase was measured spectrophotometrically on 96-well microplate reader. The absorbance (A_S) was recorded at 405 nm . Briefly, $20\text{ }\mu\text{l}$ of 100 mM phosphate buffer (pH 6.8), $20\text{ }\mu\text{l}$ of 2.5 mM PNPg, and $20\text{ }\mu\text{l}$ of investigated compounds in the buffer were mixture to a total of $60\text{ }\mu\text{l}$ reaction system in the wells, which was added $20\text{ }\mu\text{l}$ of 0.2 U/ml α -glucosidase in phosphate buffer and mixed well. After incubation for 15 min at 37°C , the reaction was stopped by adding $80\text{ }\mu\text{l}$ of 0.2 M sodium carbonate solution. The control sample was the mixture of the test sample with solvent instead. The sample and control blanks were the mixtures of sample and control, respectively, except α -glucosidase was instead with buffer, respectively. The inhibition (%) of test sample on α -glucosidase could be calculated as inhibition $[\text{inhibition} (\%) = 100 \times (A_S - A_{SB}) / (A_C - A_{CB})]$, where A_S , A_{SB} , A_C , and A_{CB} are the absorbance of sample, sample blank, control, and control blank, respectively. The measurement was performed in triplicates.

Cytotoxicity Assay MDA-231 (human breast cancer cell line) and MCF-7 (human breast cancer cell line) were utilized. The cells were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium (DMEM) (Hyclone,

Logan, UT, U.S.A.), added with 10% (v/v) fetal bovine serum (Hyclone, U.S.A.) in a humidified atmosphere with 5% CO_2 at 37°C . Cell viability was determined by recording colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of MTT (Sigma, St. Louis, MO, U.S.A.). In brief, before drug addition, $100\text{ }\mu\text{l}$ adherent cells were seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h, while suspended cells were seeded each with initial density of 1×10^5 cells/ml in $100\text{ }\mu\text{l}$ medium. Each tumor cell line was exposed to the tested compound at various concentrations (0.1, 1.0, 10, $100\text{ }\mu\text{M/l}$) in triplicates for 48 h, with *cis*-platin (Sigma, U.S.A.) as positive control. After the incubation, MTT ($100\text{ }\mu\text{g}$) was added to each well, and the incubation was stopped after 4 h at 37°C . The cells were lysed with $100\text{ }\mu\text{l}$ 20% sodium dodecyl sulfate (SDS)–50% *N,N*-dimethylformamide (DMF) after removal of $100\text{ }\mu\text{l}$ medium. The optical densities (A) of 570 nm were measured using a Bio-Rad 580 enzyme-linked immunosorbent assay microplate reader. The cytotoxicity was calculated as cytotoxicity (%) = $[(A_{570}$ of untreated cells – A_{570} of treated cells) / A_{570} of untreated cells] $\times 100\%$.

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