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Two new cytotoxic glycosides isolated from the green walnut husks of *Juglans mandshurica* Maxim.

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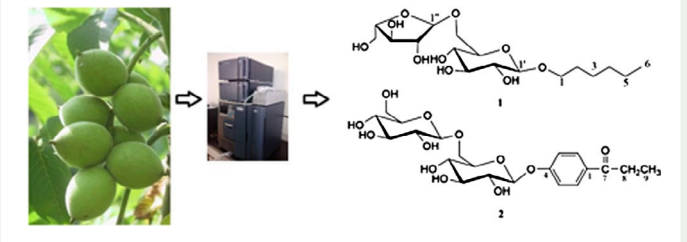
ABSTRACT

Two new glycosides including an alcohol glycoside and a phenolic glycoside: hexyl-1-*O*-*α*-D-arabinofuranosyl-(1→6)-*β*-D-glucopyranoside (**1**), 4-hydroxypropiophenone-4-*O*-*β*-D-glucopyranosyl(1 → 6)-*β*-D-glucopyranoside(**2**), along with six known naphthalenyl glucosides (**3**–**8**) were isolated from green walnut husks of *Juglans mandshurica*, and their structures were elucidated on the basis of spectroscopic studies. All compounds were evaluated for their inhibitory effects on tumour cells (BGC-823, HepG-2, MCF-7). The results showed that new compounds **1** and **2** had superior inhibitory activity in comparison with other naphthalenyl glucosides.



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KEYWORDS
Juglans mandshurica; alcohol glycoside; phenolic glycoside; cytotoxic activity

1. Introduction

Juglans mandshurica Maxim. (Juglandaceae) is one of the rare species of trees for pharmacy resources and distributed throughout urban and rural areas in north-east of Asia (Wu & Raven 1999). Its leaves, roots, fruit and barks have been used as medicinal parts in use or development (Li et al. 2003; Liu et al. 2004; Lin et al. 2014; Yao et al. 2015a). Recent pharmacological studies of medicinal parts from *J. mandshurica* have reported they had many biological properties, including antitumour, of green husks, roots, barks and leaves (Lee et al. 2002; Li et al. 2008; Liu et al. 2010; Guo et al. 2015; Zhou et al. 2015a; Gao et al. 2016), anti-inflammatory, of barks (Ju et al. 2009), anticomplement activity, of stem-barks (Min et al. 2003), antioxidant, of stem-barks (Ngoc et al. 2008), as well as protection of skin fibroblasts from

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damage by regulating the oxidative defence system of leaves (Park et al. 2012), anti-human immunodeficiency virus-type 1 of stem-barks (Min et al. 2002). In addition, the types of components isolated from different parts of *J. mandshurica* were multiple and different. Naphthoquinones and their derivatives widely existed in the roots, stem barks, leaves and green husks (Lee et al. 2000; Min et al. 2002; Li et al. 2008; Lin et al. 2014; Wang et al. 2014; Yao et al. 2015b; Zhou et al. 2015a). Diarylheptanoids were distributed among leaves, green husks and roots (Li et al. 2003; Chen et al. 2015; Jin et al. 2015; Yao et al. 2015b), also including flavonoids from stem-barks (Min et al. 2003), phenolic glycoside from barks and stem-barks (Machida et al. 2009; Yao et al. 2014), fatty acid from walnuts, barks and stem-barks (Bouabdallah et al. 2014; Yao et al. 2015a; Gao et al. 2016) and so on. According to the reports, the types of components isolated from green walnut husks of this plant mainly focused on naphthoquinones, *α*-tetralones and their glycosides, triterpenes and diarylheptanoids (Liu et al. 2004, 2010; Chen et al. 2015; Zhou et al. 2015b). As part of our continuing search for biologically active compounds from green walnut husks of *J. mandshurica* Maxim, we investigated the BuOH fraction of the EtOH extract which was barely reported, since this polarity fraction exhibited potential cytotoxic activity in our screening procedures. Indeed, we isolated two new glycosides including an alcohol glycoside (**1**) and a phenolic glycoside (**2**), together with six known naphthalenyl glucosides (**3**–**8**). The isolated compounds were tested for their cytotoxic activities against three human cancer cell lines *in vitro*. The results showed that new compounds **1, 2** showed superior inhibitory activity and the other naphthalenyl glucosides showed almost no activity.

2. Results and discussion

Compound **1** was obtained as white amorphous powder. The positive-mode-HR-ESI-MS data showed a quasi-molecular ion peak $[M + Na]^+$ at *m/z* 419.4137, suggesting a molecular mass of 396.2011 and thus a molecular formula of **1** as $C_{17}H_{32}O_{10}$. The ^{1}H NMR data of **1** (Table S1 and Figure S2) showed the methyl group at δ_H 0.90 (3H, t, J = 6.8 Hz), and four methylene proton signals at δ_H 1.61 (2H, dt, J = 14.9, 6.8 Hz) and 1.28–1.35 (6H, *m*) indicating the presence of saturated straight-chain paraffin. Further, the ^{1}H NMR data revealed two anomeric proton signals [δ_H 4.24 (d, J = 7.8 Hz, Glc H-1), δ_H 4.95 (d, J = 1.1 Hz, Ara H-1)] implying the occurrence of two sugar units: one *β*-glucopyranose and one *α*-arabinofuranose which were determined by the coupling constant of the anomeric proton. The proton signals for the disaccharide sugar unit of **1** were identical to the published compound Bumaldoside A (Hideaki et al. 2010) The ^{13}C NMR data (Table S1 and Figure S3) and distortionless enhanced polarisation transfer spectra (DEPT, Table S1 and Figure S4) of **1** showed 17 carbons including seven methylene carbons, nine methine carbons and one methyl group. The ^{13}C NMR data also revealed two anomeric carbon signals at δ_C 104.4 (Glc C-1) and 110.0 (Ara C-1) in accordance with the two anomeric signals in the ^{1}H NMR spectrum. In the HMBC spectrum (Figure S6), a suggestive correlation was observed between the anomeric proton signal of glucopyranose and an oxymethylene carbon signal at δ_C 71.1 (C-1) of the aglycone, indicating that the sugar moieties were linked at the C-1 position. Moreover, the connectivity of the arabinofuranose with the glucopyranose was indicted by the cross-peak between the anomeric proton H-1 of arabinofuranose and C-6 of glucopyranose. The sugar moieties of **1** linked at 1-*O*- were assigned as 1-*O*-*α*-L-arabinofuranosyl (1 → 6)-*β*-D-glucopyranoside, combined with the result of acid hydrolysis of **1**. The aqueous layer was separated by HPLC to give D-glucose

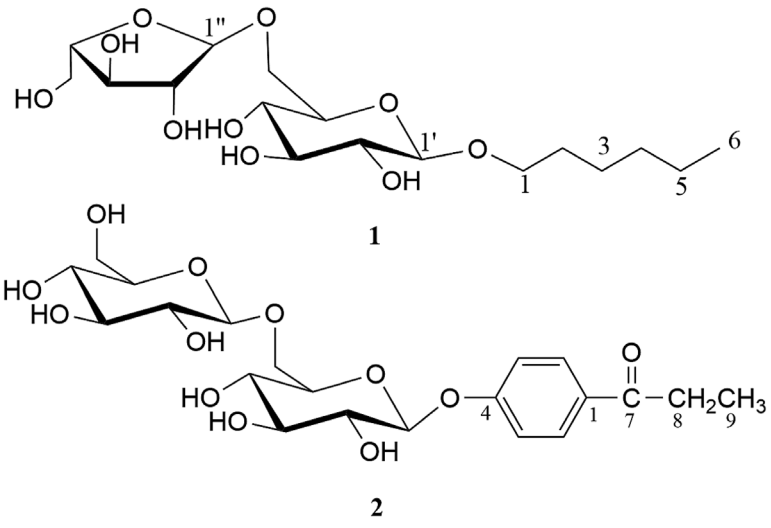

Figure 1. Structures of new compounds **1** and **2**.

and L-arabinose. On the basis of the above evidence, the structure of **1** was established as the new compound hexyl-1-*O*-*α*-L-arabinofuranosyl (1 → 6)-*β*-D-glucopyranoside (Figure 1).

Compound **2** was obtained as white amorphous powder. The positive-mode HR-ESI-MS data showed a quasi-molecular ion peak $[M + H]^+$ at *m/z* 475.1865, indicating a molecular mass of 474.1768 and the molecular formula of **2** as $C_{21}H_{30}O_{12}$. In the ^{1}H NMR spectrum (Table S2 and Figure S7), it showed that AA′BB′-type aromatic proton signals at δ_H 7.98 (2H, d, J = 8.9 Hz) and 7.19 (2H, d, J = 8.9 Hz) implying the presence of *p*-substituted phenyl. The ^{1}H NMR data of **2** appeared one terminal methyl protons at δ_H 1.17 (3H, t, J = 7.2 Hz) and one connected methylene protons at δ_H 3.02 (2H, q, J = 7.2 Hz). The ^{1}H NMR data also showed two anomeric proton signals [δ_H 5.02 (d, J = 7.6 Hz, H-1′), δ_H 4.37 (d, J = 7.7 Hz, H-1″)] suggesting the presence of two *β*-glucopyranose units. The ^{13}C NMR data (Table S2 and Figure S8) and DEPT (Table S2 and Figure S9) of **2** showed 21 different carbons signals, comprised 1 carbonyl carbon, 6 aromatic carbons, 3 methylene aliphatic carbons, 10 methane aliphatic carbons and 1 methyl carbon. The above data also revealed that **2** contained two typical glucopyranose units [δ_C 101.5, 75.2, 78.0, 71.4, 77.9, 69.8] and [δ_C 104.7, 74.8, 78.0, 71.6, 77.8, 62.7], which were confirmed by acid hydrolysis and HPLC as the same as **1**. HMBC data(Table S2 and Figure S11) of **2**, revealed the presence of the propionyl group in the aglycone as correlations between the signals: (i) δ_H 3.02 (2H, q, J = 7.2 Hz) to the carbonyl carbon at δ_C 202.2 (C-7) and one methyl group at δ_C 8.8 (C-9); (ii) the methyl proton at δ_H 1.17 (H-9) to δ_C 202.2 (C-7) and 32.4 (C-8). HMBC correlations also showed the sugar unit connectivities as correlations between the signals: the anomeric proton δ_H 5.02 (1H, d, J = 7.6 Hz) of Glc I to δ_C 162.8 (C-4) of the aglycone indicating Glc I H-1 to be linked to C-4 of the aglycone; δ_H 4.37 (1H, d, J = 7.7 Hz, Glc II H-1) to δ_C 69.8 (Glc I C-6) showing Glc II H-1 to be connected to Glc I C-6. The sugar moieties of **2** linked at 4-*O*- were assigned as 4-*O*-*β*-D-glucopyranosyl (1→6)-*β*-D-glucopyranoside. Thus, the structure of **2** was identified as 4-hydroxypropiophenone-4-*O*-*β*-D-glucopyranosyl (1→6)-*β*-D-glucopyranoside (Figure 1).

The identity of the known naphthalenyl glucosides **3**–**8** (Figure 2) was established by comparison with previously reported spectroscopic data as follows: 1, 4, 8-trihydroxy-

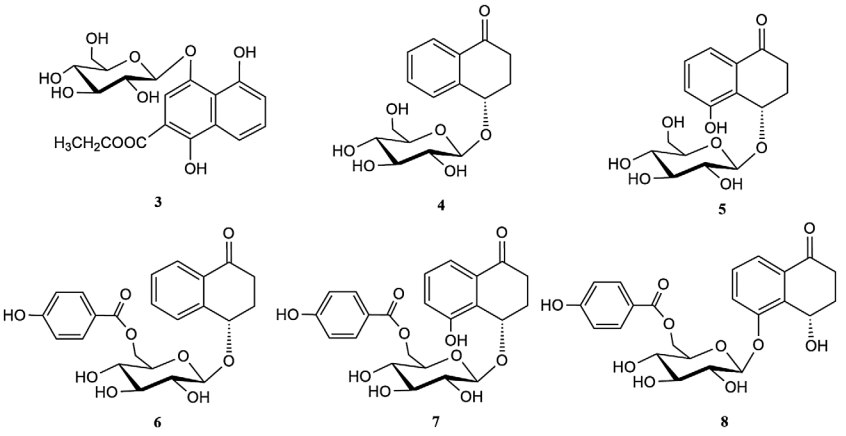

Figure 2. Structures of compounds **3**–**8**.

3-naphthalene-carboxylic acid-1-*O*-*β*-D-glucopyranoside ethyl ester (**3**) (Zhou et al. 2015a), (4*S*)-4-hydroxy-*α*-tetralone-4-*O*-*β*-D-glucopyranoside (**4**) (Liu et al. 2004), (4*S*)-45-dihydroxy-*α*-tetralone-4-*O*-*β*-D-glucopyranoside (**5**) (Liu et al. 2004) (4*S*)-4-hydroxy-*α*-tetralone-4-*O*-*β*-D-(6′-*O*-4″-hydroxylbenzoyl)glucopyrano-side (**6**) (Zhou et al. 2015a), (4*S*)-45-dihydroxy-*α*-tetralone-4-*O*-*β*-D-(6′-*O*-4″-hydro-xylbenzoyl) glucopyranoside (**7**) (Zhou et al. 2015a), (4*S*)-458-thihydroxy-*α*-tetralone-5-*O*-*β*-D-(6′-*O*-4″-hydroxylbenzoyl) glucopyranoside (**8**) (Zhou et al. 2015a). Among them, **3** and **6**–**7** were first discovered by our research group.

The isolated compounds (**1**–**8**) were assessed for cytotoxic activity against three human tumour cell lines, BGC-823 (gastric carcinoma), HepG-2 (liver carcinoma), MCF-7 (breast carcinoma) and cisplatin as positive control using the MTT method. The *in vitro* cytotoxic activity of these compounds and cisplatin was evaluated at different concentrations against above-mentioned tumour cells, and the IC_{50} values are shown (Table S3). Among all the compounds tested, only the new compounds **1** and **2** showed superior cytotoxic activity against tumour cell, but the remaining compounds of naphthalenyl glucosides **3**–**8** had less or no activity. These results were in accordance with previous reports (Liu et al. 2010).

3. Experimental

3.1. Plant material

The green husks of *J. mandshurica* were collected in Changbai Mountains (Jilin, China), in late July 2014 and identified by one of us (Z.Y.Y.). The dried samples were grounded into fine powder (60 mesh), dried thoroughly in an oven at 40 °C for 3 days. A voucher specimen (QLY 20140112) has been stored at the College of Pharmacy, Heilongjiang University of Chinese Medicine.

3.2. Chemicals and general experiments procedures

High-resolution electrospray ionisation (HR-ESI) mass spectra were obtained on a micromass LCT spectrometer (Waters, Milford, USA). The IR spectra were obtained on a Shimadzu

FTIR-8400S spectrometer (Shimadzu, Tokyo, Japan). Melting points are uncorrected and were obtained on Hoover capillary melting point (Thomas-Hoover, New Jersey, USA). ^{1}H, ^{13}C NMR, DEPT, HSQC, HMBC were obtained on Bruker DPX 400 NMR instrument (Bruker, Rheinstetten, Germany). Chemical shifts (δ) are expressed in parts per million (ppm) using TMS as an internal standard. HPLC chromatograms were obtained with an Agilent Technologies 1260 infinity HPLC system (Agilent Technologies, Germany) and semi-preparative HPLC (Waters, 515–2414, USA) was performed using a Hypersil-ODS II column (300 × 20 mm i.d., 10 µm, Ylite, Dalian, China). Deionised water was prepared a Milli-Q system (Milford, MA, USA). An automatic polarimeter (WZZ-2B, Chengdu, China) was used for the determination of specific rotation. A multiscan microplate reader (Thermo Labsystems, Helsinki, Finland) was used for the MTT assays. BGC-823, HepG-2 and MCF-7 cell lines obtained from Institute of biochemistry and cell biology (Shanghai, China) were grown in Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) from Sigma (St. Louis, MO, USA). Column chromatography (CC) was performed using Si gel (80–300 mesh) from HaiYang Co. Ltd (Qingdao, China) and ODS from DAISOGEL (Beijing, China). All solvents used were of analytical grade.

3.3. Extraction and isolation

The air-dried parts of materials (10.0 kg) were powdered and soaked in 80 L of EtOH for 7 days. The extraction was repeated three times and then concentrated under reduced pressure to afford the EtOH extract (637 g). The extracts were concentrated and then suspended in H_2O followed by successive partitioning with n-hexane, CH_2Cl_2, EtOAc and n-BuOH, respectively. The BuOH extract (122 g) was subjected to silica gel (200–300 mesh) column chromatography (CC), eluted with CH_2Cl_2–MeOH (30 : 1 → 1 : 1, *v*/*v*), to afford six fractions (Fr1-Fr6). Fraction 3 (5.15 g) was fractionated and purified by ODS CC with MeOH/H_2O (2 : 8 → 1 : 0) to yield **1** (10.8 mg) and **3** (5.5 mg). Fraction 4 (4.88 g) was separated twice by ODS CC with MeOH/H_2O (2 : 8 → 1 : 0) to afford a number of subfractions 4a, 4b, 4c and 4d. Subfraction 4b (0.56 g) was purified by semi-preparative HPLC chromatography (MeOH/H_2O 45 : 55, *v*/*v*, flow rate 3 mL/min) to yield **2** (6.4 mg), **6** (10.2 mg), **7** (8.1 mg) and **8** (12.4 mg). Subfraction 4c (0.56 g) was purified by semi-preparative HPLC chromatography (MeOH/H_2O 55 : 45, *v*/*v*, flow rate 3 mL/min) to yield **4** (4.9 mg), **5** (9.3 mg).

3.3.1. *Hexyl-1-O-α-L-arabinofuranosyl (1 → 6)-β-D-glucopyranoside* (1)

White amorphous powder, $[\alpha]_D^{25}$ − 22.4 (*c* = 0.50, MeOH); IRν_{max} (film) cm^{-1}: 3395, 2961, 2928, 2855, 1453, 1078, 1004. ^{1}H NMR (MeOH, 400 MHz) δ: 4.95 (1H, d, *J* = 1.1 Hz, H-1″), 4.24 (1H, d, *J* = 7.8 Hz, H-1′), 4.04 (1H, dd, *J* = 11.0, 2.3 Hz, H-6′a), 3.98 (1H, m, H-4″), 3.96 (1H, m, H-2″), 3.86(1H, dt, *J* = 9.6, 6.8 Hz, H-1a), 3.81 (1H, dd, *J* = 6.2, 3.6 Hz, H-3″), 3.73 (1H, dd, *J* = 11.6, 5.3 Hz, H-5″a), 3.63 (1H, dd, *J* = 11.6, 3.4 Hz, H-5″b), 3.53 (1H, dt, *J* = 9.6, 6.8 Hz, H-1b), 3.50 (1H, dd, *J* = 11.0, 5.0 Hz, H-6′b), 3.42 (1H, ddd, *J* = 9.0, 5.9, 2.3 Hz, H-5′), 3.34 (1H, t, *J* = 8.8 Hz, H-3′), 3.25 (1H, t, *J* = 9.0 Hz, H-4′), 3.15 (1H, dd, *J* = 8.8, 7.8 Hz, H-2′), 1.61 (2H, dt, *J* = 14.9, 6.8 Hz, H-2), 1.28–1.35 (6H, m, H-3, 4, 5), 0.90 (3H, t, *J* = 6.8 Hz, H-6). ^{13}C NMR (125 MHz, MeOH): 110.0 (C-1″), 104.4 (C-1′), 86.0 (C-2″), 83.3 (C-4″), 78.1 (C-3′), 76.7 (C-5′), 75.1 (C-2′), 72.0 (C-4′), 71.1 (C-1), 68.1 (C-6′), 63.1 (C-5″), 32.9 (C-4), 30.8 (C-2), 26.8 (C-3), 23.7 (C-5), 14.4 (C-6). HR-ESI-MS (positive): *m*/*z* 419.4137 [M + Na]$^+$ (Calcd for $C_{17}H_{32}O_{10}$, 396.2011).

3.3.2. 4-hydroxypropiophenone-4-O-β-D-glucopyranosyl (1 → 6)-β-D-glucopyranoside (2)

White amorphous powder, $[\alpha]_D^{25}$ − 13.8 (*c* = 0.75, MeOH); IRν_{max} (film) cm^{-1}: 3287, 2923, 2916, 2915, 1725, 1688, 1095. ^{1}H NMR (MeOH, 400 MHz) *δ*: 7.98 (2H, d, *J* = 8.9 Hz, H-2, 6), 7.19 (2H, d, *J* = 8.9 Hz, H-3, 5), 5.02 (1H, d, *J* = 7.6 Hz, H-1′), 4.37 (1H, d, *J* = 7.7 Hz, H-1′′), 4.17 (1H, dd, *J* = 11.6, 1.5 Hz, H-6′a), 3.81(1H, dd, *J* = 11.6, 5.8 Hz, H-6′b), 3.74 (1H, dd, *J* = 11.1, 5.3 Hz, H-6′′a), 3.64 (1H, m, H-6′′b), 3.49 (2H, m, H-2′′, 5′′), 3.39 (1H, m, H-4′), 3.29 (3H, m, H-3′, 3′′, 4′′), 3.22 (1H, m, H-2′), 3.17 (1H, m, H-5′), 3.02 (2H, q, *J* = 7.2 Hz, H-8), 1.17 (3H, t, *J* = 7.2 Hz, H-9). ^{13}C NMR (125 MHz, MeOH) *δ*: 202.2 (C-7), 162.8 (C-4),132.4 (C-1), 131.4 (C-2, 6), 117.4 (C-3, 5), 104.7 (C-1′′), 101.4 (C-1′), 78.0 (C-3′, 3′′), 77.9 (C-5′), 77.8 (C-5′′), 75.2 (C-2′), 74.8 (C-2′′), 71.6 (C-4′′), 71.4 (C-4′), 69.8 (C-6′), 62.7 (C-6′′), 32.4 (C-8), 8.8 (C-9). HR-ESI-MS (positive): *m/z* 475.1865 $[M + H]^+$ (Calcd for $C_{21}H_{30}O_{12}$, 474.1768).

3.4. Acid hydrolysis

Compounds **1** and **2** (each about 1.5 mg) were refluxed with 1.0 mol/L HCl (5 mL, dioxane-H_2O, v/v) for 7 h under 80 °C water bath. After cooling down to room temperature, the reaction mixture was extracted three times with CH_2Cl_2:H_2O (each 1 : 1, v/v, 1 mL). The aqueous layer was neutralised with 5% NaOH and desalted with Sephadex LH-20 to obtain the sugar residue (0.8 mg). The monosaccharides in **1** were first determined as L-arabinose and D-glucose by co-TLC with authentic sugar, eluting with chloroform/n-BuOH/MeOH/acetic acid/water 17 : 10 : 6 : 2 : 3 [$Rf_{(L\text{-}Ara)}$ = 0.41, $Rf_{(D\text{-}Glc)}$ = 0.38). In the same way, the monosaccharide in **2** was identified as D-glucose. Then, the PMP derivative of the monosaccharide mixture was prepared as the past report (Honda et al. 1989). The resulted PMP-monosaccharide was dissolved in EtOH/n-hexane solution (1 : 4, v/v) and analysed by HPLC using CHRIALPAK AD-H column, with an elution solvent system of n-hexane/2-propanol (87 : 13), at 25 °C, and detected by DAD detector. The standard sugars were operated with the same methods. The monosaccharides in **1** were determined to be L-arabinose (t_R = 11.3 min) and D-glucose (t_R = 9.6 min). The monosaccharide in **2** was determined to be D-glucose (t_R = 9.6 min).

3.5. Cell cultures

HepG-2, BGC-823 and MCF-7 cell lines were maintained in DMEM supplemented with 10% foetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco-BRL). The cells were incubated in 5% CO_2 humidified at 37 °C for growth.

3.6. Cytotoxicity assay

Tumour cells in logarithmic growth phase were seeded in a 96-well microtiter plates and kept overnight for attachment. Eight compounds and positive control (cisplatin), dissolved in dimethyl sulfoxide and PB (phosphate buffer), were added at various concentrations from 200 to 0.5 µM for 24 h. The optical density was measured at 570 nm using a multiscan microplate reader. All experiments were performed in triplicate. Data were expressed as the concentration required for inhibiting growth of tumour cells by 50% (IC_{50}).

Supplementary material

Supplementary material relating to this paper is available online, alongside Table S1–S3 and Figure S1–S11.

Disclosure statement

No potential conflict of interest was reported by the authors.

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