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α-Tetralone glycosides from the green walnut husks of *Juglans mandshurica* Maxim. and their cytotoxic activities

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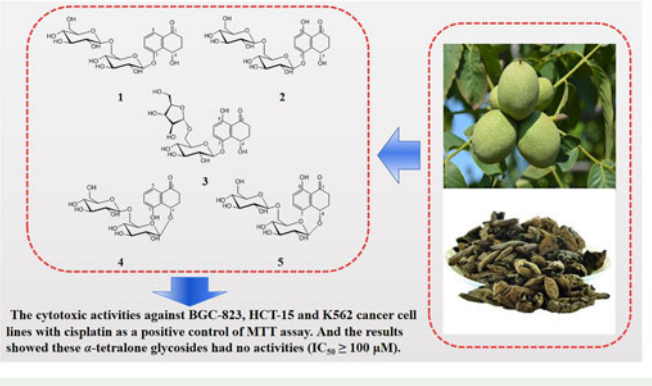
ABSTRACT

Five new α-tetralone glycosides, juglanbiosides A-E (**1–5**), together with an α-tetralone derivative (**15**) and nine known 1,4-naphthoquinones (**6–14**) were isolated from the 95% EtOH extract of green walnut husks of *Juglans mandshurica* Maxim. Their structures were elucidated by comprehensive spectroscopic methods (^{1}H, ^{13}C NMR, DEPT, HSQC, HMBC, CD, HR-ESI-MS). In *vitro* cytotoxicities of all the isolated compounds were evaluated against BGC-823, HCT-15 and K562 cancer cell lines.



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1. Introduction

Juglans mandshurica Maxim is a deciduous tree, which belongs to the Juglandaceae family, and widely distributed in the Korea, Far East Russia and northeast of China (Zhao et al. 2018). The kernels of the nuts are edible. The green walnut husks of *J. mandshurica*, as a kind of Chinese folk medicines, has been used to treat dermatosis, uterine prolapse and leukopenia. In addition, modern researches have shown that the

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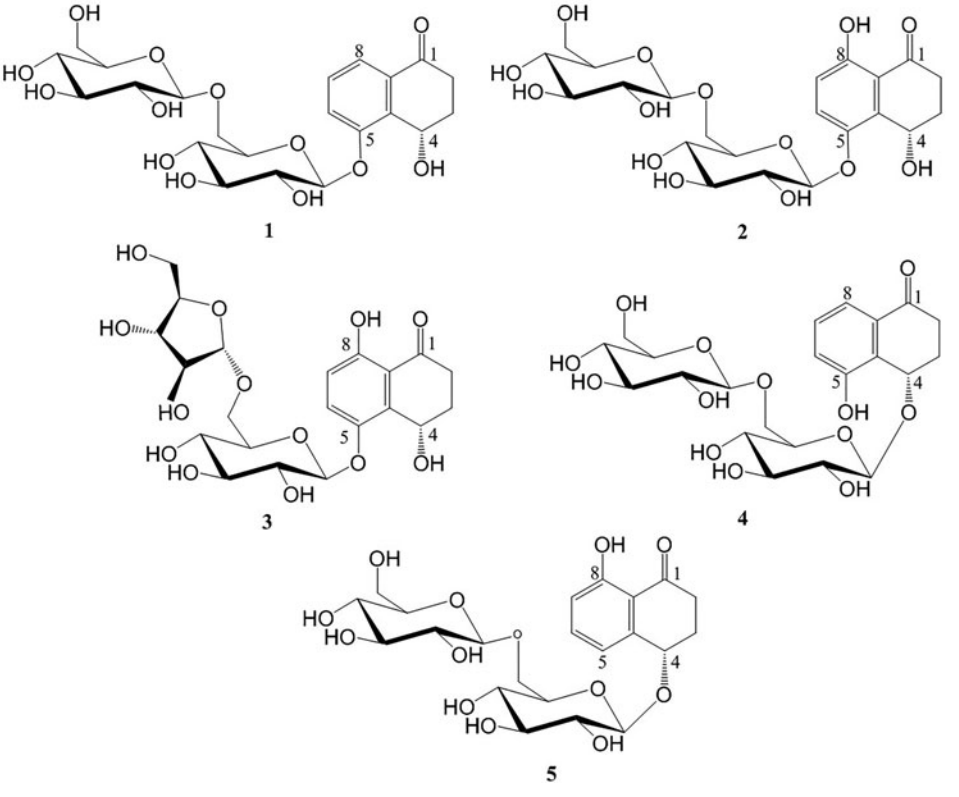

Figure 1. Structures of compounds 1–5.

herb possesses extensive pharmacological activities, such as anti-tumor (Lou et al. 2018; Lou et al. 2017), antioxidant (Meshkini and Tahmasbi 2017), anti-inflammatory (Dong and Yuan 2018) and antimicrobal effects (Wianowska et al. 2016). Previous phytochemical studies on the green walnut husks of *J. mandshurica* led to the isolation of chemical constituents including naphthoquinones (Jiang et al. 2018), diarylheptanoids (Jin et al. 2015), triterpenoids (Zhou et al. 2015b) and phenolic acids (Huo et al. 2017), etc. It is well to be mentioned that the derivates of 1,4-naphthoquinone and α-tetralone possess the capacity of killing cancer cells (Chen et al. 2015; Guo et al. 2015). To discover hit or lead compounds possessing anticancer activity from traditional Chinese medicine herbs, we carried out the investigation concerning 1,4-naphthoquinones and α-tetralones of the green walnut husks of *J. mandshurica*, which resulted in the isolation of 15 compounds, including 5 new α-tetralone glycosides (Figure 1). In addition, the antitumour activity of compounds **1–15** in *vitro* was evaluated using MTT assay on the BGC-823, HCT-15, K562 human cancer cells.

2. Results and discussion

Compound **1** was obtained as a yellow amorphous powder. Its molecular formula, $C_{22}H_{30}O_{13}$, was determined by analyzing the peak at *m/z* 525.2070 $[M+Na]^+$ in the HR-ESI-MS, which indicating 8 degrees of unsaturation. ^{1}H NMR spectrum of **1** showed

two methylene protons in the high field at δ_H 2.26 (2H, m, H-3), 3.00 (1H, ddd, $J = 17.0$, 12.4, 5.6 Hz, H-2a) and 2.51 (1H, brd, $J = 17.0$ Hz, H-2b), and an oxymethine at δ_H 5.33 (1H, brs, H-4). Besides, ^{1}H NMR also displayed three protons of an ABC spin system at δ_H 7.58 (1H, brd, $J = 8.0$ Hz, H-6), 7.45 (1H, t, $J = 8.0$ Hz, H-7) and 7.66 (1H, brd, $J = 8.0$ Hz, H-8), indicating the presence of a tri-substituted benzene ring. The ^{13}C NMR spectrum, in combination with HMBC and HSQC spectrum (Figures S7 and S8) showed signals for a carbonyl carbon at δ_C 200.0 (C-1), two methylene carbons at δ_C 30.7 (C-3), 33.7 (C-2) and a tertiary carbon with hydroxyl group at δ_C 61.4 (C-4). All the analysis mentioned above indicated that compound **1** was a derivative of α-tetralone (Wu et al. 2010). The substituent position of the hydroxyl group was deduced to be C-5 through the correlations between δ_H 5.33 (H-4) and δ_C 156.9 (C-5) in HMBC spectra. In sugar region of ^{1}H NMR spectrum, two doublets at δ_H 4.98 (1H, d, $J = 7.5$ Hz, H-1′) and 4.37 (1H, d, $J = 7.6$ Hz, H-1′′) belonging to the anomeric glucose protons were appeared and pointed out a β-configuration of glycosyl linkages. The former signal gave cross-peak in the HMBC experiment (Figure S2) with C-5 (δ_C 156.9) which was an evidence confirming that the sugar moiety was attached at position 5 of aglycone. The cross-peaks between δ_H 4.18 (1H, brd, $J = 11.6$ Hz, H-6′a) and δ_C 104.8 (C-1′′), δ_H 4.37 (H-1′′) and δ_C 69.9 (C-6′) in the HMBC spectrum indicated that the connection type of glucopyranose was 1→6. The absolute configurations of two glucoses were both determined as D-form by acid hydrolysis, derivatization and GC analysis. Furthermore, the CD spectrum of its aglycone was measured to determine the absolute configuration of the chiral center at C-4. It was identified to be *S* configuration by comparing its CD spectrum data with those of the references (Machida et al. 2005), where a positive Cotton effect at 227 nm was observed (Figure S3). Accordingly, the structure of **1** was determined to be 4(*S*)-4,5-dihydroxy-α-tetralone 5-O-β-D-glucopyranosyl(I′′→6′)-β-D-glucopyranoside, named as juglanbioside A.

Compound **2** was obtained as a yellow amorphous powder, which assigned the molecular formula to be $C_{22}H_{30}O_{14}$ according to HR-ESI-MS data at *m/z* 541.1979 $[M + Na]^+$ (calcd for 541.1954), and the unsaturation was same as for **1**. A comparison of its NMR data with those of **1** indicated that an aromatic proton at C-8 in **1** was substituted by a hydroxyl group at the same position in **2**. Thus, the AB-type aromatic proton signals at δ_H 7.58 (1H, d, $J = 9.2$ Hz, H-6) and 6.91 (1H, d, $J = 9.2$ Hz, H-7) were observed in place of ABC-type in the aryl ring. The remaining substructure of **2** was elucidated to be identical to that of **1** by detailed analysis of HMQC and HMBC NMR spectrum of **2**. Accordingly, the structure of **2** was elucidated to be 4(*S*)-4,5,8-trihydroxy-α-tetralone 5-O-β-D-glucopyranosyl(I′′→6′)-β-D-glucopyranoside, and named as juglanbioside B.

Juglanbioside C (**3**), yellow amorphous powder, possessed a molecular formula of $C_{21}H_{28}O_{13}$ determined by positive HR-ESI-MS at *m/z* 511.1886 ($[M + Na]^+$, calcd for 511.1894), indicating 8 degrees of unsaturation. By comparison of its NMR data with that of **2** revealed that their structures were the same, except for one of the glucopyranosyl unit replaced by arabinofuranosyl unit in sugar moiety, and the absolute configuration of arabinofuranosyl unit and glucopyranosyl unit in **3** were determined D-form and L-form respectively by acid hydrolysis, derivatization and GC analysis. The ^{1}H NMR spectrum showed β-anomeric configuration for glucopyranosyl unit due

to the large terminal constant values of the proton at δ_H 4.78 (1H, d, $J = 7.6$ Hz, H-1′), and the α orientation of the anomeric configuration for arabinofuranosyl unit was deduced from the small coupling constant of the anomeric proton at δ_H 4.93 (1H, d, $J = 1.2$ Hz, H-1″). Accordingly, the structure of **3** was assigned to be 4(*S*)-4,5,8-trihydroxy-α-tetralone 5-O-α-L-arabinopyranosyl(l″→6′)-β-D-glucopyranoside.

Compound **4** was obtained as a yellow amorphous powder and determined to have the molecular formula $C_{22}H_{30}O_{13}$ based on its HR-ESI-MS data at *m/z* 525.2047 $[M + Na]^+$ (calcd for 525.2064), corresponding to 8 degrees of unsaturation. A detailed comparison of NMR data revealed that **4** is differed from **1** mainly at C-4 and C-5. The chemical shift of H-6 (δ_H 7.10, 1H, dd, $J = 7.9$, 1.1 Hz) of **4** was shifted upfield by −0.48 ppm, and C-4 at δ_C 69.5 was shifted down field by 8.1 ppm than that of **1**, respectively. It was also clearly determined by the long range correlation between δ_H 4.57 (1H, d, $J = 7.9$ Hz, H-1′) and δ_C 69.5 (C-4), δ_H 5.43 (1H, t, $J = 3.0$ Hz, H-4) and δ_C 103.7 (C-1′) in the HMBC spectra (Figure S2), together with the NMR data as described above, suggesting the position of glycosylation at C-4 (δ_C 69.5) in **4** and the hydroxyl located at C-5 (δ_C 156.9). Accordingly, the structure of **4** was established to be 4(*S*)-4,5-dihydroxy-α-tetralone 4-O-β-D-glucopyranosyl(l″→6′)-β-D-glucopyranoside, and named as juglanbioside D.

Juglanbioside E (**5**), a yellow amorphous powder, was assigned the same molecular formula, $C_{22}H_{30}O_{13}$, as **1** and **4** by HR-ESI-MS at *m/z* 525.1993 $[M + Na]^+$ (calcd for 525.2026). Comparison of the NMR and HR-ESI-MS data of **5** with those of **4** revealed the only difference was that the substitution site of a hydroxyl group at C-5 in **4** was replaced by C-8 in **5**. A group of obvious ABC-type aromatic proton signals at δ_H 7.18 (1H, dd, $J = 8.0$, 0.8 Hz, H-5), 7.50 (1H, t, $J = 8.0$ Hz, H-6) and 6.89 (1H, dd, $J = 8.0$, 0.8 Hz, H-7) were comfirmed by ^{1}HNMR of compound **5**. Meanwhile the aromatic carbon at C-4 (δ_C 74.7) had HMBC correlation signals with H-5 (δ_H 7.18), and it clearly indicated that the C-5 has not been substituted. Thus, it indicated the substituted position of the hydroxyl group was at C-8 (δ_C 163.9) on the aromatic ring. Accordingly, the structure of **5** was established to be 4(*S*)-4,8-dihydroxy-α-tetralone 4-O-β-D-glucopyranosyl(l″→6′)-β-D-glucopyranoside, and named as juglanbioside E.

The five new α-tetralone glycosides were named as juglanbiosides A-E (**1-5**). The known compounds were identified as juglone (**6**) (Yamashita et al. 2009), 5-methoxy-1,4-naphthoquinone (**7**) (Dong et al. 2011), 2-hydroxy-1,4-naphthoquinone (**8**) (Zhou et al. 2015a), 3-methoxy juglone (**9**) (Lee et al. 2016), 2-ethoxy juglone (**10**) (Li et al. 2006), 3-ethoxy juglone (**11**) (Li et al. 2006), 5,8-dihydroxy-1,4-naphthoquinone (**12**) (Dong et al. 2011), 2,5-dihydroxy-1,4-naphthoquinone (**13**) (Zhou et al. 2015a), 3,5-dihydroxy-1,4-naphthoquinone (**14**) (Dong et al. 2011) and (*S*)-regiolone (**15**) (Lee et al. 2016) (Figure S1).

Several 1,4-Naphthoquinone and α-tetralone derivatives from *J. mandshurica* have been reported to have cytotoxic properties in various cancer cell lines (Yao et al. 2014). Therefore we evaluated for the cytotoxic activities of compounds **1-15** against three human cancer cell lines (BGC-823, HCT-15 and K562) (Table S1) with cisplatin as a positive control of MTT assay. The IC_{50} values were expressed as the means ± SD based on three independent experiments. The aglycones isolated from the CH_2Cl_2 extract layer showed varying degrees of cytotoxicity, of which compound **6** showed

extremely strong activities with IC_{50} values of 9.6 ± 2.35, 27.8 ± 2.66 and 35.5 ± 5.11 μM. And we found that any changes in the structure resulted in significantly reduced cytotoxicity. Moreover, these α-tetralone glycosides isolated from the *n*-butanol layer had no activities ($IC_{50} \geq 100$ μM). It is reported that the antineoplastic activity of α-tetralone homologues appeared to be much less efficacy than 1,4-naphthoquinones (Yang et al. 2018). And this conclution is basically consistent with our experimental findings.

3. Experimental section

3.1. General

Optical rotations were determined with a JASCO P2000 digital polarimeter (Jasco, Tokyo, Japan). Circular dichroism (CD) spectra were performed on an Applied Photophysics Spectropolarimeter (Agilent, USA). HR-ESI-MS spectra were acquired on a micromass LCT spectrometer (Waters, Milford, USA). The NMR spectra were measured with a Bruker DPX 400 spectrometer (Bruker, Rheinstetten, Germany), using TMS as an internal standard. Silica gel H (200-300 mesh) (Qingdao Haiyang Chemical Co. Ltd., Qingdao, China), C18 reversed-phase (RP-18) silica gel (50 μm, YMC Ltd., Kyoto, Japan) and sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) were used for open column chromatography. Thin-layer chromatography (TLC) was performed on precoated silica gel GF_{254} (Qingdao Haiyang Chemical Co. Ltd., Qingdao, China). HPLC chromatograms were obtained with an Agilent Technologies 1260 infinity HPLC system (Agilent Technologies, Germany) and SunFire C18 column (Waters, 150 × 4.6 mm, 5 μm, USA). Semi-preparative HPLC (Waters, 515-2414, Milford, MA, USA) was carried out with a Hypersil-ODS II column (300 × 20 mm i.d., 10 μm, Ylite, Dalian, China). All solvents were used of analytical grade (Tianjin Chemical Reagents Company Ltd., Tianjin, China).

3.2. Plant material

The green walnut husks of JM were collected from Changbai Mountain, Jilin Province, China in 2016, and identified by Professor Bingyou Yang of College of Pharmacy of the Heilongjiang University of Chinese Medicine. The voucher specimen (No. 20160902) was preserved in the Herbarium Laboratory of Heilongjiang University of Chinese Medicine.

3.3. Extraction and isolation

The air-dried powdered green walnut husks of *Juglans mandshurica* (10.0 kg) were extracted with 95% EtOH at room temperature three times, seven days each time. The solvent was evaporated to dryness, and then yielded a crude extract (684.3 g). The extract was suspended in H_2O and partitioned successively with CH_2Cl_2 (3 × 1.5 L), EtOAc (3 × 1.5 L) and *n*-butanol (3 × 1.5 L), respectively. The CH_2Cl_2 part (97.0 g) was fractionated by silica gel column chromatography (CC) with a gradient of pet. ether-EtOAc (1:0→0:1, v/v) to obtain eleven fractions (Fr.A-Fr.K). Fr.C (5.6g) was further separated by silica gel CC with pet. ether-EtOAc (50:1→1:1, v/v) to yield six subfractions

(Fr.C_1-C_6). The compound **6** (80.5 mg) was crystallized from Fr.C_1 (201.6 mg). Fr.C_2 (215.6 mg) was further purified by silica gel CC eluted with pet. ether-EtOAc (30:1, v/v) to yield **7** (12.7 mg), **8** (8.4 mg), **9** (7.5 mg), **10** (7.2 mg) and **11** (5.6 mg). Fr.C_3(262.9 mg) was purified by preparative TLC developed with pet. ether-EtOAc (25:1, v/v) to yield **15** (6.6 mg). Fr.E (3.2 g) was purified by the silica gel CC eluting with CH_2Cl_2-MeOH (35:1→0:1, v/v) to afford five subfractions (Fr.E_1-E_5). Fr.E_2 (118.0 mg) was purified by preparative TLC eluting with CH_2Cl_2-MeOH (25:1, v/v) to give **12** (6.3 mg), **13** (5.4 mg) and **14** (7.1 mg). The *n*-butanol part (148.0 g) was subjected by silica gel CC with a gradient of CH_2Cl_2-MeOH (1:0→0:1, v/v) to afford five fractions (Fr.L-Fr.P). Fr.N (8.6 g) was fractionated by ODS CC eluting with MeOH-H_2O (2:8→10:0, v/v) to offer nine subfractions (Fr.N_1-Fr.N_9). Fr.N_6 (766.3 mg) was purifed by Sephadex LH-20 CC eluting with MeOH-H_2O (40:60, v/v) to yield six subfracions (Fr.$N_{6\text{-}1}$-Fr.$N_{6\text{-}6}$). Fr.$N_{6\text{-}2}$ (99.6 mg) was finally purifed by semi-preparative HPLC eluting with MeOH-H_2O (30:70, v/v) to give **4** (4.7 mg, t_R = 22 min), **1** (6.9 mg, t_R = 25 min). Fr.$N_{6\text{-}3}$ (152.0 mg) was separated by semi-preparative HPLC eluting with MeOH-H_2O (25:75, v/v) to offer **3** (6.0 mg, t_R = 26 min) and **5** (3.4 mg, t_R = 30 min). Compound **2** (7.9 mg, t_R = 33 min) was then obtained from Fr.$N_{6\text{-}4}$ (212.0 mg) by semi-preparative HPLC eluted with MeOH-H_2O (20:80, v/v).

3.3.1. Juglanbioside A (1)

Yellow amorphous powder; $[\alpha]_D^{25}$-30.2 (*c* 0.10, MeOH); CD (*c* 1.15×10^{-5}, MeOH) $\triangle\varepsilon$ (nm): +9.60 (227), -1.12 (287); HR-ESI-MS *m/z* 525.2070 $[M+Na]^+$ (calcd for $C_{22}H_{30}O_{13}Na$, 525.2096). ^1H NMR and ^{13}C NMR data see the supplementary material.

3.3.2. Juglanbioside B (2)

Yellow amorphous powder; $[\alpha]_D^{25}$-28.6 (*c* 0.10, MeOH); CD (*c* 1.02×10^{-5}, MeOH) $\triangle\varepsilon$ (nm): +6.65 (212), -2.45 (340); HR-ESI-MS *m/z* 541.1979 $[M+Na]^+$ (calcd for $C_{22}H_{30}O_{14}Na$, 541.1954). ^1H NMR and ^{13}C NMR data see the supplementary material.

3.3.3. Juglanbioside C (3)

Yellow amorphous powder; $[\alpha]_D^{25}$-12.2 (*c* 0.10, MeOH); CD (*c* 1.37×10^{-5}, MeOH) $\triangle\varepsilon$ (nm): +7.05 (215), -1.85 (341); HR-ESI-MS *m/z* 511.1886 $[M+Na]^+$ (calcd for $C_{21}H_{28}O_{13}Na$, 511.1894). ^1H NMR and ^{13}C NMR data see the supplementary material.

3.3.4. Juglanbioside D (4)

Yellow amorphous powder; $[\alpha]_D^{25}$-22.9 (*c* 0.10, MeOH); CD (*c* 1.28×10^{-5}, MeOH) $\triangle\varepsilon$ (nm): +8.94 (224), -2.18 (284); HR-ESI-MS *m/z* 525.2047 $[M+Na]^+$ (calcd for $C_{22}H_{30}O_{13}Na$, 525.2064). ^1H NMR and ^{13}C NMR data see the supplementary material.

3.3.5. Juglanbioside E (5)

Yellow amorphous powder; $[\alpha]_D^{25}$-18.7 (*c* 0.10, MeOH); CD (*c* 1.85×10^{-5}, MeOH) $\triangle\varepsilon$ (nm): +12.17 (211), -3.30 (265); HR-ESI-MS *m/z* 525.1993 $[M+Na]^+$ (calcd for $C_{22}H_{30}O_{13}Na$, 525.2026). ^1H NMR and ^{13}C NMR data see the supplementary material.

3.4. Acid hydrolysis

Compounds **1-5** (each about 1.5 mg) were treated with 4.0 N TFA (trifluoroacetic acid, aqueous solution, 3 mL) at 95 °C for 6 h. After cooling to room temperature, the reaction mixture was extracted with CH_2Cl_2 (5 mL × 3). The aqueous phases were evaporated to dryness. The residue was analyzed by TLC, with silica gel G as adsorbents, 10% H_2SO_4 in 95% EtOH as detection reagent for spraying, followed by heating the plate to develop the colors, and with CH_2Cl_2-MeOH-HOAc-H_2O (25:10:2:2) as solvent systems for the development of sugars. The R_f values of D-glucose and L-arabinose were determined, by interactive comparison with the authentic D-glucose and L-arabinose, in the order of 0.24 and 0.53, respectively. Subsequently, the residues were dissolved in pyridine. And then 0.5 mL hexamethyldisilane and 0.3 mL trimethylchlorosilane were added, followed by heating at 60 °C for 2 h. Finally, the thiazolidine derivative was analysed by GC (Lin et al. 2018), and the retention times of D-glucose and L-arabinose were 14.525, 10.687 min, respectively.

3.5. In vitro antitumor activities assay

The human gastric cancer (BGC-823), colorectal cancer (HCT-15) and erythroleukemic (K562) cell lines were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (Gibco, New York, USA) and 1% antibiotic mixture comprising penicillin-streptomycin (Sigma, Missouri, USA). Three cell lines were seeded at 4×10^3 cells per well in 96-well culture plates and allowed to incubate for 24 h in a humidified environment containing 5% CO_2 at 37 °C. The cells were then treated with various concentrations of compounds (10 μL) for 48 h. Cisplatin was used as a positive control. The concentrations of cisplatin and the test compounds were 0.04, 0.2, 1.0, 5, 25 and 125 μM in medium, respectively. At the end of the incubation, MTT (10 μL, 5 mg/mL) solution was added into each well. Plates were further incubated another 4 h under the above conditions. After decanting the MTT solution, the formazan crystals were dissolved by 100 μL DMSO with gently shaking of 5 min at room temperature. The optical density (OD) was measured at 490 nm using a multiscan microplate reader (Thermo Labsystems, Helsinki, Finland). All the experiments were performed independently in triplicate. The antitumor activities were expressed as the 50% inhibition concentration (IC_{50} value).

4. Conclusion

In this study, we mainly reported the isolation, structure elucidation and cytotoxicities of five new compounds, named juglanbiosides A-E (**1–5**), together with ten known compounds **6–15**. The cytotoxic activities of the isolated compounds were evaluated against three human cancer cell lines. In the meantime, we preliminarily summarized the structure-activity relationship of them. The information rendered by structure-activity relationship may lead to a better understanding of structural requirements of antitumor activity and maybe also can help in the design of novel potent antitumor activity.

Disclosure statement

No potential conflict of interest was reported by the authors.

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