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A pair of new tirucallane triterpenoid epimers from the stems of *Picrasma quassioides*

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[ABSTRACT] A pair of new tirucallane triterpenoid epimers, picraquassins M and N (**1** and **2**), were isolated from the stems of *Picrasma quassioides* (D. Don) Benn. Their structures were determined based on comprehensive spectroscopic and X-ray crystallographic analyses. In addition, their AChE inhibitory activity, cytotoxicity against five human tumour cell lines (SW480, MCF-7, HepG2, Hela, and PANC-1), and antimicrobial activity against two bacteria (*Staphylococcus. aureus* 209P and *Escherichia coli* ATCC0111) and two fungi (*Candida albicans* FIM709 and *Aspergillus niger* R330) were evaluated.

[KEY WORDS] Tirucallane triterpenoid; Epimers; *Picrasma quassioides*; AChE inhibitory activity; Cytotoxicity; Antimicrobial activity

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Introduction

The plants of *Picrasma quassioides* (D. Don) Benn, known as "Ku Mu" in China, are an important member of family Simaroubaceae. Their stems, branches, or leaves are traditionally used as folk medicines for the treatment of anemopyretic cold, sore throat, dysentery, and so on [1]. The chemical constituents of *P. quassioides* were reported with diverse bioactivities, such as inhibiting cAMP phosphodiesterase [2], inhibiting acetylcholinesterase (AChE) [3-5], anti-inflammatory [6], antihypertensive [7], antibacterial [8], and anti-angiogenic activities [9]. Previous investigation showed that alkaloids and quassinoids were the major components of *P. quassioides* [10]. In recent years, dozens of tirucallane triterpenoids were also obtained from *P. quassioides* [11-12], which play an important role in plant chemotaxonomy of *P. quassioides*. In the present study, a pair of new tirucallane triterpenoid epimers [picraquassins M (**1**) and N (**2**)] were isolated from the stems of *P. quassioides*. In addition, their AChE inhibitory activity, cytotoxicity, and antimicrobial activity were evaluated. Herein, details of the isolation, structural elucidation, and biological activities of **1** and **2** are reported.

Results and Discussion

Compound **1** was obtained as a colorless block-shaped crystal. Its molecular formula was established as $C_{32}H_{50}O_3$ (eight degrees of unsaturation) from its HRESI-MS data (*m/z* 505.3646 $[M + Na]^+$, Calcd. for $C_{32}H_{50}O_3Na$ 505.3658). In the ^1H NMR spectrum of **1**, the characteristic signals of two olefinic protons [δ_H 5.25 (1H, dd, J = 8.1, 3.0 Hz) and δ_H 5.10

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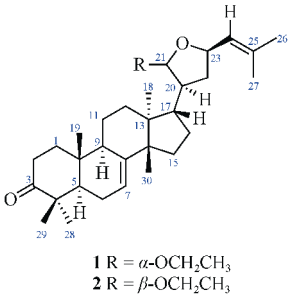
These authors have no conflict of interest to declare.
Dedicated to Professor SUN Han-Dong on the Occasion of His 80th Birthday

(1H, br d, J = 8.2 Hz)], two *O*-methine protons [δ_H 4.82 (1H, d, J = 3.0 Hz) and δ_H 4.60 (1H, ddd, J = 9.0, 8.2, 5.2 Hz)], two *O*-methylene protons [δ_H 3.72 (1H, dq, J = 14.1, 7.0 Hz), and 3.36 (1H, dq, J = 14.1, 7.0 Hz)], and eight methyl group protons [δ_H 1.67 (3H, br s), 1.64 (3H, br s), 1.16 (3H, t, J = 7.0 Hz), 1.05 (3H, s), 0.98 (3H, s), 0.96 (6H, s), and 0.82 (3H, s)] were observed. Combined with the DEPT-135 spectrum, 32 signals were observed in the ^{13}C NMR spectrum, which can be assigned to five sp^2 carbons [including a ketone carbonyl carbon (δ_C 216.4)], six sp^3 methine carbons [including two oxygenated carbons (δ_C 107.0 and 73.5)], nine sp^3 methylene carbons [including an oxygenated carbon (δ_C 63.3)], four sp^3 quaternary carbons, and eight sp^3 methyl carbons. The proton signals were associated with the directly attached carbon atoms in the HSQC experiment. An analysis of the ^{1}H-^{1}H COSY spectrum revealed the presence of the subunits C-1–C-2, C-5–C-6–C-7, C-9–C-11–C-12, C-15–C-16–C-17–C-20(C-21)–C-22–C-23–C-24, C-1′–C-2′ (Fig. 2). Combined with the ^{1}H-^{1}H COSY analyses, the HMBC correlations as shown in Fig. 2 revealed the planar structure of **1**. The assignments of all proton and carbon resonances are provided in Tables 1 and 2.



1 R = α-OCH_2CH_3
2 R = β-OCH_2CH_3

Fig. 1 Chemical structures of 1 and 2

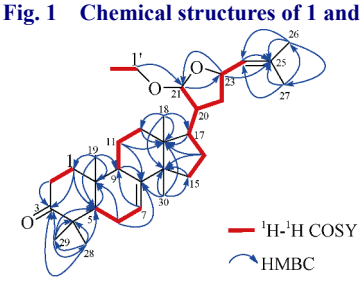


Fig. 2 The key ^{1}H-^{1}H COSY and HMBC correlations for 1

The relative configuration of **1** was determined based on the NOESY experiment (Figs. 3A and 3B). In the NOESY experiment, the observed correlations between H_3-19 and Ha-2/H_3-29/Hb-6, and between H-5 and H-9/Hb-1 indicated the β-orientation of CH_3-19 and CH_3-29, and the α-orientation of H-5 and H-9. Similarly, the α-orientation of CH_3-18 and the β-orientation of H-17 and CH_3-30 were deduced from the NOESY correlations between H_3-18 and H-9/Hb-16, and between H_3-30 and Ha-16/H-17. H-20 was established as α-orientation by the conformation analysis based on the NOESY correlations between H-21 and H-17, between H-17 and Hb-22, between Hb-22 and Ha-16, and between Hb-16 and H-20 (Fig. 3B). The NOESY correlations between H-20 and H-23, between H-21 and Hb-22/H-24, and between H-24 and H-22b suggested that H-23 was α-orientation, and H-21 was β-orientation. The X-ray crystallographic analysis of **1** (Fig. 4) confirmed the above deduction, and the values of the Flack parameter [0.1 (2)] and Hooft parameter [0.09 (7)] allowed the absolute configuration of **1** to be assigned as 5*R*, 9*R*, 10*R*, 13*S*, 14*S*, 17*S*, 20*S*, 21*R*, and 23*R*. Thus, **1** was structurally established and named as picraquassin M.

Table 1 ^{1}H NMR data (δ in ppm, J in Hz) for 1 and 2 in $CDCl_3$ at 600 MHz

Position	1*	2*
1	1.93, ddd (13.2, 5.3, 3.0), Ha	1.96, ddd (15.0, 5.3, 3.1), Ha
	1.42, ddd (13.2, 10.5, 4.4), Hb	1.43, ddd (15.0, 9.1, 4.0), Hb
2	2.70, ddd (14.5, 10.5, 5.3), Ha	2.73, ddd (14.5, 9.1, 5.3), Ha
	2.18, ddd (14.5, 4.4, 3.0), Hb	2.22, ddd (14.5, 4.0, 3.1), Hb
5	1.68	1.71
6	2.04, Ha	2.07, Ha
	2.05, Hb	2.05, Hb
7	5.25, dd (8.1, 3.0)	5.28, dd (8.3, 3.2)
9	2.28	2.29
11	1.55, Ha	1.55, Ha
	1.53, Hb	1.53, Hb
12	1.73, Ha	1.93, Ha
	1.51, Hb	1.35, Hb
15	1.50, Ha	1.53, Ha
	1.43, Hb	1.48, Hb
16	1.82, Ha	1.84, Ha
	1.23, Hb	1.29, Hb
17	1.73	2.05
18	0.82, s	0.81, s
19	0.96, s	0.99, s
20	2.16	1.96
21	4.82, d (3.0)	4.77, d (3.7)
22	2.01, Ha	1.95, Ha
	1.16, Hb	1.51, Hb
23	4.60, ddd (9.0, 8.2, 5.2)	4.68, ddd (9.3, 9.0, 6.3)
24	5.10, br d (8.2)	5.14, br d (9.0)
26	1.67, br s	1.69, br s
27	1.64, br s	1.64, br s
28	0.98, s	1.02, s
29	1.05, s	1.09, s
30	0.96, s	1.00, s
1′	3.72, dq (14.1, 7.0), Ha	3.71, dq (14.2, 7.2), Ha
	3.36, dq (14.1, 7.0), Hb	3.32, dq (14.2, 7.2), Hb
2′	1.16, t (7.0)	1.16, t (7.2)

*Indiscernible signals owing to overlapping or having complex multiplicity are reported without designating multiplicity

Table 2 ^{13}C NMR data (δ in ppm) for 1 and 2 in $CDCl_3$ at 150 MHz

Position	1	2
1	38.3, CH_2	38.4, CH_2
2	34.7, CH_2	34.8, CH_2
3	216.4, C	216.7, C
4	47.7, C	47.8, C
5	52.2, CH	52.3, CH
6	24.4, CH_2	24.3, CH_2
7	117.9, CH	117.8, CH
8	145.5, C	145.8, C
9	48.2, CH	48.3, CH
10	35.0, C	35.0, C
11	17.6, CH_2	17.7, CH_2
12	31.5, CH_2	31.0, CH_2
13	43.6, C	43.5, C
14	50.8, C	50.7, C
15	33.7, CH_2	34.2, CH_2
16	27.2, CH_2	27.3, CH_2
17	50.6, CH	44.9, CH
18	22.5, CH_3	23.1, CH_3
19	12.6, CH_3	12.7, CH_3
20	49.0, CH	47.4, CH
21	107.0, CH	102.4, CH
22	39.3, CH_2	36.0, CH_2
23	73.5, CH	75.2, CH
24	124.8, CH	128.3, CH
25	136.7, C	136.6, C
26	25.7, CH_3	25.8, CH_3
27	18.2, CH_3	17.8, CH_3
28	24.2, CH_3	24.4, CH_3
29	21.4, CH_3	21.5, CH_3
30	27.3, CH_3	27.4, CH_3
1′	63.3, CH_2	61.9, CH_2
2′	15.3, CH_3	15.2, CH_3

Compound **2** was obtained as a colorless needle-like crystal. Its molecular formula was established as $C_{32}H_{50}O_3$ on the basis of the positive HRESI-MS ion at *m/z* 505.3650 $[M + Na]^+$ ($C_{32}H_{50}O_3Na$, Calcd. for 505.3658), which was the same as that of **1**. IR spectra indicated these two compounds had the same functional groups. The ^{13}C NMR data of **2** (Tables 1 and 2) highly resembled those of **1** with the main differences at C-21 (δ_C 102.4 for **2** and 107.0 for **1**) and C-17 (δ_C 44.9 for **2** and 50.6 for **1**). The chemical shifts of C-21 and C-17 were both upfield-shifted, which maybe for the presence of the *γ*-gauche effect from 21-OCH_2CH_3. The above observation implied that compound **2** was probably the epimer of **1** at C-21. The NOESY correlations between H-20 and H-23, and H-24 and Ha-1' confirmed the above deduction. The absolute configurations of **2** were determined by X-ray crystallographic analysis (Fig. 4) with the value of the Flack parameter 0.0 (4). Thus, **2** was structurally established and named as picraquassin N.

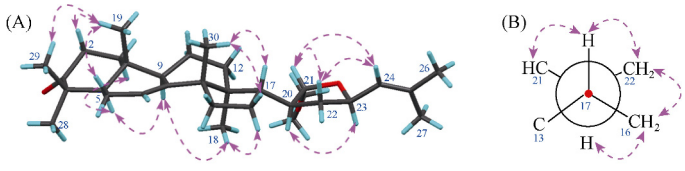
Compounds **1** and **2** were evaluated for the AChE inhibitory activity, cytotoxicity against five human tumour cell lines (SW480, MCF-7, HepG2, Hela, and PANC-1), and antimicrobial activity against two bacteria (*Staphylococcus. aureus* 209P and *Escherichia coli* ATCC0111) and two fungi (*Candida albicans* FIM709 and *Aspergillus niger* R330). None of them possessed potent activities.

Experimental

General experimental procedures

Methanol (MeOH) was purchased from Yuwang Industrial Co., Ltd. (Yucheng, China). Ethanol (EtOH) was purchased from Dongju Experimental Apparatus Co., Ltd. (Guangzhou, China). Tetrahydrofuran (THF) was got from Fuchen Chemical Reagents Factory (Tianjin, China). Cyclohexane, ethyl acetate (EtOAc) and chloroform ($CHCl_3$) were purchased from Fine Chemical Co., Ltd. (Tianjin, China).

The melting points were determined on an X-5 micro-melting point apparatus (Beijing TECH instrument Co., Ltd., Beijing, China) without corrected. Optical rotations were recorded on a JASCO P1020 digital polarimeter (Jasco International Co., Ltd., Tokyo, Japan). UV data were measured using a JASCO V-550 UV/Vis spectrometer (Jasco International Co., Ltd., Tokyo, Japan). IR data were tested on a JASCO FT/IR-480 plus spectrometer (Jasco International Co., Ltd., Tokyo, Japan). The HRESI-MS spectra were obtained on a Waters Synapt G2 TOF mass spectrometer (Waters Corporation, Milford, USA). 1D and 2D NMR spectra were acquired with Bruker AV 600 spectrometers (Bruker BioSpin Group, Faellanden, Switzerland), and $CDCl_3$ (δ_H 7.26/δ_C 77.0) was used as solvent. Semi-preparative HPLC was carried out on a Shimadzu LC-6AD liquid chromatography (Shimadzu Inc., Kyoto, Japan) with an SPD-20A detector with a Phenomenex Gemini C_{18} column (10.0 mm × 250 mm, 5 μm;



1

Fig. 3 (A) Key NOESY correlations for 1; (B) Conformation analysis for the C-17 and C-20 segment in 1

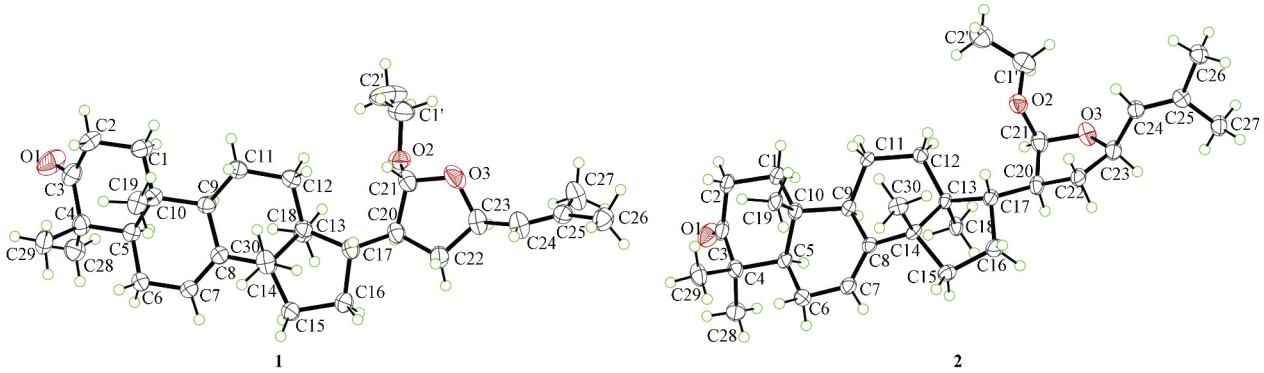


Fig. 4 X-ray structures of 1 and 2

Phenomenex Inc., Los Angeles, USA). Medium pressure liquid chromatography (MPLC) was equipped with a dual pump gradient system, an UV preparative detector, and a Dr Flash II fraction collector system (Shanghai Lisui E-Tech Co., Ltd., Shanghai, China). Column chromatography (CC) was carried out on silica gel (200−300 mesh, Qingdao Haiyang Chemical Group Corporation, Qingdao, China), ODS (50 μm, YMC Co., Ltd., Kyoto, Japan) and Sephadex LH-20 (Amersham Pharmacia Biotech Co., Ltd., Atlanta, USA).

Plant materials

The dried stems of *P. quassioides* (D. Don) Benn. were collected from Hubei Province, China, in December 2014 and identified by Professor ZHOU Guang-Xiong, College of Pharmacy, Jinan University, Guangzhou, China. A voucher specimen was deposited in the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou, China.

Extraction and isolation

The dried stems of *P. quassioides* (100.2 kg) were reflux extracted two times with 95% EtOH to afford a crude extract (2.0 kg). Then the extract was suspended in water and extracted with $CHCl_3$ three times to yield $CHCl_3$-soluble fraction (749.1 g). The $CHCl_3$-soluble fraction was subjected to a silica gel column (11.0 cm × 70.5 cm), which was successively eluted with cyclohexane/EtOAc (99 : 1, 95 : 5, 90 : 10; 80 : 20, 70 : 30, 60 : 40, 50 : 50, and 0 : 100, *V/V*) to afford eleven fractions (F1−F11). F3 (5.5 g) was subjected to MPLC on ODS CC using a successive elution of MeOH/H_2O (65 : 35, 70 : 30, 80 : 20, 100 : 0, *V/V*) to yield fractions 3.1−3.6. F3.3 (2.0 g) was subjected to Sephadex LH-20 column eluted with CH_3OH to afford five fractions (F3.3.1−F3.3.5). F3.3.2 (481.7 mg) was isolated using semi-preparative HPLC (MeOH/H_2O, 88 : 12, *V/V*) at a flow rate of 3 mL·min^{-1} to yield **1** (t_R 28.0 min, 110.2 mg) and **2** (t_R 33.0 min, 50.2 mg).

Picraquassin M (1)

Colorless block-shaped crystals (MeOH/THF); mp 136.2−145.8 °C; $[\alpha]_D^{27}$ −62.3 (*c* 1.0, CH_3OH); UV (MeOH) λ_{max} (log ε) 205 (4.10) nm; IR (KBr) ν_{max} 2966, 2943, 2873, 2856, 1706, 1450, 1370, 1360, 1101, 1014, 980, 871 cm^{-1}. ESI-MS (positive) *m/z* 483 $[M + H]^+$, *m/z* 505 $[M + Na]^+$. HRESI-MS (positive) *m/z* 505.3646 $[M + Na]^+$ (Calcd. for $C_{32}H_{50}O_3Na$, 505.3658); ^1H and ^{13}C NMR see Tables 1 and 2.

Picraquassin N (2)

Colorless needle-like crystals (MeOH/THF); mp 155.0−156.6 °C; $[\alpha]_D^{27}$ +1.7 (*c* 1.0, CH_3OH); UV (MeOH) λ_{max} (log ε) 205 (4.16) nm; IR (KBr) ν_{max} 2972, 2952, 2890, 2856, 1708, 1448, 1387, 1370, 1118, 1006, 988, 863 cm^{-1}. ESI-MS (positive) *m/z* 483 $[M + H]^+$, *m/z* 505 $[M + Na]^+$. HRESI-MS (positive) *m/z* 505.3650 $[M + Na]^+$ (Calcd. for $C_{32}H_{50}O_3Na$, 505.3658); ^1H and ^{13}C NMR see Tables 1 and 2.

X-ray crystallographic analysis of 1

Upon crystallization from MeOH/THF using the vapor diffusion method, colorless block-shaped crystals of **1** were obtained. Data were collected using a Sapphire CCD with graphite monochromated Cu Kα radiation, λ = 1.54184 Å at 173.00 (10) K. Crystal data: $C_{32}H_{50}O_3$, M = 482.72, space group $P2_12_12_1$; unit cell dimensions were determined to be *a* = 10.1541 (3) Å, *b* = 11.9206 (3) Å, *c* = 23.7635 (7) Å, α = 90.00°, β = 90.00°, γ = 90.00°, *V* = 2876.41 (14) Å^3, *Z* = 4, *Dx* = 1.115 mg·m^3, *F* (000) = 1064.0, μ(Cu Kα) = 0.531 mm^{-1}. 13207 reflections were collected (7.44° ≤ θ ≤ 125.5°), in which independent unique 4597 reflections (R_{int} = 0.0186, R_{sigma} = 0.0200) were used in all calculations. Using Olex2 [13], the structure was solved by direct methods using the SHELXS program, and refined by the SHELXL program. In the structure refinements, hydrogen atoms were fixed geometrically at the calculated distances and allowed to ride on their parent atoms. The final refinement gave R_1 = 0.0323 (I > 2σ(I)), $_wR_2$ = 0.0805 (all data), *S* = 1.070, Flack = 0.1 (2), and Hooft = 0.09 (7). Crystallographic data for picraquassin M (**1**) been deposited in the Cambridge Crystallographic Data Center as supplementary publication No. CCDC 1957403. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44-(0)1223-336033, or E-mail: deposit@ccdc.cam. ac.uk).

X-ray crystallographic analysis of 2

Upon crystallization from MeOH/THF using the vapor diffusion method, colorless needle-like crystals of **2** were obtained. Data were collected using a Sapphire CCD with graphite monochromated Cu Kα radiation, λ = 1.54184 Å at

100.0 (10) K. Crystal data: $C_{32}H_{50}O_3$, M = 482.72, space group $P2_12_12_1$; unit cell dimensions were determined to be a = 7.04268 (9) Å, b = 12.92163 (12) Å, c = 30.5927 (3) Å, α = 90.00°, β = 90.00°, γ = 90.00°, V = 2784.03 (5) Å^3, Z = 4, Dx = 1.152 mg·m^3, F (000) = 1064.0, μ(Cu Kα) = 0.549 mm^{-1}. 47757 reflections were collected (7.42° ≤ θ ≤ 147.32°), in which independent unique 5571 reflections (R_{int} = 0.0509, R_{sigma} = 0.0224) were used in all calculations. Using Olex2 [13], the structure was solved by direct methods using the SHELXS program, and refined by the SHELXL program. In the structure refinements, hydrogen atoms were fixed geometrically at the calculated distances and allowed to ride on their parent atoms. The final refinement gave R_1 = 0.0693 (I > 2σ(I)), $_wR_2$ = 0.1900 (all data), S = 1.155, Flack = 0.0 (4), Hooft = 0.03 (7). Crystallographic data for picraquassin N (**2**) have been deposited in the Cambridge Crystallographic Data Center as supplementary publication No. CCDC 1957404. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44-(0)1223-336033, or E-mail: deposit@ccdc.cam.ac.uk).

Acetylcholinesterase (AChE) inhibitory assay

The AChE inhibitory activity of compounds **1** and **2** were evaluated according to Ellman's method [14-15] with slight modifications. Firstly, 20 μL of 0.2 U·mL^{-1} AChE, 3 mmol·L^{-1} acetylthiocholine iodide (ATCh) (20 μL), and 40 μL of 0.2 mmol·L^{-1} samples were put into 96-well plate. The mixture was incubated at 37 °C for 30 min. Then 10 μL of 1 mol·L^{-1} HCl and 120 μL of 0.7 mmol·L^{-1} 5, 5'-dithio-bis-(2-nitrobenzoic) acid (DTNB) was added into the mixture to stop the reaction. Huperzine A (Hup A) was used as a positive control. The mixture was monitored at 405 nm using the Synergy HT microplate reader to get the absorbance values. All of the samples were tested in triplicate. The relative inhibitory activity (I%) = [(E − S)/E] × 100 (S and E represented the absorbance of the incubated AChE solution with and without the tested sample, respectively). The 50% inhibitory concentration (IC_{50}) value of Hup A was calculated in GraphPad Prism V5.01 (GraphPad Software Inc., San Diego, CA, USA) using a weighted regression of the plot.

Cytotoxicity assay

The cytotoxicity assay was performed using the CCK-8 assay according to the manufacturer's protocol [16]. Five human cancer cell lines (human colon cancer SW480, human breast cancer MCF-7, human liver cancer HepG2, human cervical cancer Hela, and human pancreatic cancer PANC-1) were used in the cytotoxicity assays. All the cells were cultured in DMEM medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. Briefly, 1 × 10^4 cells were seeded into each well of the 96 well plate and treated with 40 μmol·L^{-1} of the compounds with cisplatin as the positive control for 48 h. Then, CCK-8 solution (10 μL) was added to each well and incubated for 2 h at 37°C. The absorbance (A) of the samples at 450 nm was measured using a microtiter plate reader. The cell viability of each compound was calculated by the following formula: [(A_{sample} − A_{blank})/(A_{DMSO} − A_{blank})] × 100%.

Antimicrobial assay

The antimicrobial activities against two bacteria (*Staphylococcus. aureus* 209P and *Escherichia coli* ATCC0111) and two fungi (*Candida albicans* FIM709 and *Aspergillus niger* R330) were measured in sterile 96-well plates using the broth microdilution method [17-18]. Beef extract agar medium for bacteria and sabouraud's dextrose agar medium for fungi were used, respectively. These two media were sterilized by high temperature. Final sample concentrations were prepared from serial dilutions and ranged from 128 to 0.07 μg·mL^{-1} (128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.13, 0.07 μg·mL^{-1}) in the growth medium, and the continuous 2-fold dilution method was used to evaluate the minimal inhibitory concentrations (MICs). The MICs were defined as the lowest concentration at which no microbial growth could be observed. Tobramycin and itraconazole were used as the positive controls for the antibacterial and antifungal assays, respectively.

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