Acridone alkaloids with cytotoxic and antimalarial activities from *Zanthoxylum simullans* Hance

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ABSTRACT

Background: *Zanthoxylum simullans* Hance is a popular natural spice belonging to the Rutaceae family and it is one of the common prescribed herbs in traditional Chinese medicine. **Materials and Methods:** The chemical constituents were mainly isolated and purified by silica gel column chromatography and semi-preparative High Performance Liquid Chromatography. Their structures were identified by comparing the spectral data with those reported in the literature. Cytotoxic activities for the isolated acridone alkaloids were evaluated against two prostate cancer cell lines PC-3M and Lymph Node Carcinoma of Prostate (LNCaP), and their antimalarial activities were tested against two different strains of the parasite *Plasmodium falciparum* 3D7, and Dd2. **Results:** The root bark MeOH extract of *Z. simullans* Hance afforded β-sitosterol, 4-methoxy benzoic acid, daucosterol, and five acridone alkaloids, normelicopidine, normelicopine, melicopine, melicopidine, and melicopicine. All five acridone alkaloids were isolated from this plant for the first time and exhibited certain cytotoxic and antimalarial activities *in vitro*. **Conclusion:** Normelicopidine was the most active against PC-3M, LNCaP and Dd2 with IC_{50} values of 12.5, 21.1, and 18.9 ug/mL respectively.

Key words: Acridone alkaloids, antimalarial, cytotoxity, *Zanthoxylum simullans* Hance

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INTRODUCTION

The genus *Zanthoxylum*, family Rutaceae comprises about 250 species of deciduous, evergreen trees and shrubs distributed world-wide around the tropical and subtropical areas. It is economically important because of their alimentary, industrial, and medicinal applications.[1] *Zanthoxylum simullans* Hance, a popular natural spice, has long been prescribed in traditional Chinese medicine for the treatment of stomach ache, toothache, intestinal worms, eczema, and pruritus.[2] Extensive phytochemical investigations have been carried out on this plant, along with the study of its biological activities, especially, antiplatelet aggregation.[3-7] Most alkaloids isolated from this plant exhibited strong antiplatelet aggregation activities, except for zanthosimuline, a new acridone alkaloid found in the root bark. It has demonstrated a general cytotoxic response when evaluated against a variety of human cancel cell lines and P388 cells.[4] In this paper, we conducted a search for more bioactive constituents from *Z. simullans* Hance.

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MATERIALS AND METHODS

Plant material

The root barks of *Z. simullans* Hance were collected in September 2009 at Jingxi county, Guangxi province, China and identified by Dr. Jingquan Yuan, Associate Professor of Guangxi Medicinal Herb Garden. A voucher specimen (No. 20090018Z) has been deposited in the Herbarium of South-Central University for Nationalities, Wuhan, P. R. China.

General

Melting points were determined on a Kofler melting point apparatus and are uncorrected. Utlraviolet (UV) spectra were determined on a Shimadzu UV-250 spectrometer. ^{1}H and ^{13}C NMR (Carbon-13 Nuclear Magnetic Resonance) spectra were recorded on a Bruker DRX-400 spectrometer with Tetramethylsilane (TMS) as an internal standard. The chemical shifts (δ) were reported in ppm, and coupling constants (J) were given in Hz. The ESI-MS and EI-MS (Electrospray Ionization Mass Spectrometry and Electron Impact Mass Spectrometry) data were recorded on the Quadrupole Time-of-flight (Q-TOF) Mass Spectrometry Micro Liquid Chromatography-Tandem Mass Spectrometry (LC-MS-MS) and Finnigan MAT-95

mass spectrometers. Semi-preparative HPLC system: Two PrepStar SD-1 solvent delivery modules, a ProStar UV-Vis 320 detector and a ProStar 701 Fraction Collector (Varian, Walnut Creek, CA, USA); a Sunfire RP-18 column (5 μm; 250 mm × 20 mm, waters) for semi-preparative isolation. The following types of silica gel were used: 300-400 mesh for column chromatography and GF_{254} for Thin Layer Chromatography (TLC) (Qing Dao Hai Yang Chemical Group Co.). Sephadex LH 20 (GE Healthcare) was also used for column chromatography. Cell lines PC-3M, LNCaP and HEK293 were purchased from the American Type Culture Collection. Parasite strains Dd2 and 3D7 were from the Queensland Institute of Medical Research.

Extraction

Air-dried root barks of *Z. simullans* Hance (200 g) were ground and then extracted by maceration at room temperature with MeOH in a 1 L conical flask mounted on a shaking table (3 × 0.6 L, 4 h each). The solvents were evaporated at reduced pressure to yield 18.6 g MeOH extract.

Isolation

The MeOH extract was subjected to CC over silica gel (500 g) eluted gradiently with CH_2Cl_2-MeOH (25:1-1:2) to afford fractions 1-7. Fraction 2 (2.1 g) was rechromatographed on silica gel (120 g) with CH_2Cl_2-MeOH (20:1-10:1) to give compounds 1 (135 mg), 2 (27 mg) and 5 (87 mg). The crude alkaloids (0.9 g) derived from fraction 3 (1.6 g) after filtration over Sephadex LH 20 with MeOH were further submitted to semi-preparative HPLC eluted with a linear gradient of 10-70% aq. MeOH in 0.5% TFA at 9.0 mL/min for 120 min to yield successively compounds 4 (12 mg, t_R 32.5 min), 5 (39 mg, t_R 36.1 min), 6 (5 mg, t_R 47.7 min), 7 (16 mg, t_R 64.6 min), 8 (78 mg, t_R 68.9 min).

Compound 1, colorless crystal (MeOH), mp, 140-142°C; Electron Impact Mass Spectrometry (EIMS) *m/z* 414 $[M]^+$, 396, 381, 354, 329, 303, 273, 255; ^{1}H-NMR ($CDCl_3$): δ 5.33 (1H, d, J = 5.0 Hz, H-6), 3.50 (1H, m, H-3), 0.99 (3H, s, H-19), 0.92 (3H, d, J = 8.1 Hz, H-21), 0.84 (3H, t, J = 7.7 Hz, H-29), 0.82 (3H, d, J = 8.7 Hz, H-27), 0.80 (3H, d, J = 8.7 Hz, H-26), 0.66 (3H, s, 18-Me).

Compound 2, colorless crystal (EtOH), mp, 183-185°C; EIMS *m/z* 152 $[M]^+$, 135, 122, 109, 92, 84, 56; ^{1}H-NMR ($CDCl_3$): δ 8.42 (2H, dd, J = 8.6, 2.0 Hz, H-2, H-6), 7.06 (2H, dd, J = 8.6, 2.0 Hz, H-3, H-5), 3.68 (3H, s, OMe); ^{13}C-NMR ($CDCl_3$): 125.1 (C-1), 132.3 (C-2, C-6), 114.1 (C-3, C-5), 163.6 (C-4), 168.8 (C-7), 55.4 (OMe).

Compound 3, white powder; ESIMS *m/z* 599 $[M + Na]^+$, 576 $[M]^+$, 413 [M-glu-1]$^+$; EIMS *m/z* 414 $[M]^+$, 396, 381, 354, 329, 303, 273, 255, 231, 213; ^{1}H-NMR (DMSO-d_6): δ 5.34 (1H, m, H-6), 4.92 (1H, d, J = 8.0 Hz, Glu, H-1'), 3.50 (1H, m, H-3), 0.99 (3H, s, H-19), 0.92 (3H, d, J = 8.1 Hz, H-21), 0.84 (3H, t, J = 7.7 Hz, H-29), 0.82 (3H, d, J = 8.7 Hz, H-27), 0.80 (3H, d, J = 8.7 Hz, H-26), 0.66 (3H, s, 18-Me); ^{13}C-NMR (DMSO-d_6): 36.7 (C-1), 29.3 (C-2), 76.8 (C-3), 38.2 (C-4), 140.3 (C-5), 120.1 (C-6), 31.3 (C-7, C-8), 50.5 (C-9), 36.1 (C-10), 22.5 (C-11), 41.7 (C-12), 45.0 (C-13), 56.1 (C-14), 24.8 (C-15), 27.7 (C-16), 55.3 (C-17), 11.6 (C-18), 19.0 (C-19), 35.4 (C-20), 18.8 (C-21), 33.0 (C-22), 25.3 (C-23), 49.5 (C-24), 28.5 (C-25), 19.6 (C-26), 12.9 (C-27), 23.8 (C-28), 12.0 (C-29), 100.6 (Glu, C-1'), 73.3 (C-2'), 76.8 (C-3'), 69.9 (C-4'), 76.6 (C-5'), 60.9 (C-6').

Compound 4, orange needles ($CHCl_3$), mp, 208-210°C; UV (EtOH) λ_{max} (nm): 219, 250, 272, 304, 322, 393; ESI-MS: *m/z* 621 $[2M + Na]^+$, 599 $[2M + H]^+$, 300 $[M + H]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz): 14.7 (1H, s, OH), 8.32 (1H, d, J = 8.9 Hz, H-8), 7.79 (1H, dd, J = 8.7, 7.6 Hz, H-6), 7.76 (1H, d, J = 8.7 Hz, H-5), 7.64 (1H, d, J = 8.9, 7.6 Hz, H-7), 6.05 (2H, s, O-CH_2-O), 4.03 (3H, s, OCH_3), 3.86 (3H, s, N-CH_3); ^{13}C-NMR (DMSO-d_6, 100 MHz): 175.9 (C-9), 145.1 (C-3), 144.4 (C-5a), 137.3 (C-4a), 136.6 (C-1), 135.4 (C-2), 133.5 (C-6), 130.1 (C-4), 126.3 (C-8), 124.1 (C-8a), 122.1 (C-7), 117.1 (C-5), 113.5 (C-1a), 102.7 (O-CH_2-O), 61.2 (4-OMe), 42.0 (N-Me).

Compound 5, red needles ($CHCl_3$), mp, 234-236°C; UV (EtOH) λ_{max} (nm): 222, 250, 277, 313, 423; ESI-MS: *m/z* 322 $[M + Na]^+$, 300 $[M + H]^+$; EIMS *m/z*: 299 $[M]^+$ (83), 285 (8), 284 (100), 254 (31), 170 (16), 158 (31), 115 (17); ^{1}H-NMR (DMSO-d_6, 400 MHz): 14.9 (1H, s, OH), 8.37 (1H, d, J = 7.9 Hz, H-8), 7.82 (1H, dd, J = 8.4, 7.7 Hz, H-6), 7.78 (1H, d, J = 7.7 Hz, H-5), 7.67 (1H, d, J = 8.4, 7.9 Hz, H-7), 5.99 (2H, s, O-CH_2-O), 4.01 (3H, s, OCH_3), 3.97 (3H, s, N-CH_3); ^{13}C-NMR (DMSO-d_6, 100 MHz): 176.2 (C-9), 147.9 (C-1), 143.5 (C-3), 144.1 (C-5a), 133.9 (C-6), 133.1 (C-2), 132.1 (C-4), 127.5 (C-4a), 127.3 (C-8), 122.5 (C-8a), 121.8 (C-7), 116.2 (C-5), 115.1 (C-1a), 102.4 (O-CH_2-O), 61.1 (2-OMe), 40.6 (N-Me).

Compound 6, light yellow needles ($CHCl_3$), mp, 176-178°C; UV (EtOH) λ_{max} (nm): 215, 251, 271, 302, 404; EIMS *m/z*: 313 $[M]^+$ (75), 299 (28), 298 (100), 284 (21), 280 (10), 270 (50), 268 (13), 255 (10), 254 (8), 169 (8); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_H 8.53 (1H, dd, J = 9.0 Hz, H-8), 7.90 (1H, dd, J = 8.7, 7.7 Hz, H-6), 7.75 (1H, d, J = 8.7 Hz, H-5), 7.58 (1H, d, J = 9.0, 7.7 Hz, H-7), 6.08 (2H, s, O-CH_2-O), 4.01, 3.98 (2 CH.H, s, 2OCH_3), 3.97 (3H, s, N-CH_3); ^{13}C-NMR (DMSO-d_6, 100 MHz): 176.1 (C-9), 149.8 (C-1), 143.8 (C-3), 143.1 (C-5a), 134.1 (C-6), 133.6 (C-2), 131.9 (C-4), 127.1 (C-4a), 127.0 (C-8), 122.9 (C-8a), 121.6 (C-7), 115.5 (C-5), 114.3 (C-1a), 102.3 (O-CH_2-O), 62.4 (1-OMe), 61.4 (2-OMe), 39.2 (N-Me).

Compound 7, light yellow prismatic crystal (EtOEt), mp, 121-122°C; UV (EtOH) λ_{max} (nm): 221, 252, 276, 302, 325, 399; ESI-MS: *m/z* 649 $[2M + Na]^+$, 336 $[M + Na]^+$, 314 $[M + H]^+$; MS *m/z*: 313 $[M]^+$ (100), 298 (59), 285 (25), 280 (28), 271 (13), 270 (74), 269 (9), 268 (25), 255 (16), 253 (10), 252 (50), 240 (9), 224 (14), 212 (7); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_H 8.26 (1H, d, *J* = 8.7 Hz, H-8), 7.89 (1H, dd, *J* = 8.5, 7.4 Hz, H-6), 7.81 (1H, d, *J* = 8.5 Hz, H-5), 7.63 (1H, d, *J* = 8.7, 7.4 Hz, H-7), 6.02 (2H, s, O-CH_2-O), 4.05, 3.84 (2 CH.H, s, 2OCH_3), 3.82 (3H, s, N-CH_3); ^{13}C-NMR (DMSO-d_6, 100 MHz): 176.3 (C-9), 145.6 (C-3), 144.7 (C-5a), 137.6 (C-1), 137.2 (C-4a), 135.7 (C-2), 133.3 (C-6), 129.4 (C-4), 126.1 (C-8), 123.8 (C-8a), 121.7 (C-7), 116.9 (C-5), 113.9 (C-1a), 103.1 (O-CH_2-O), 61.4 (1-OMe), 61.1 (4-OMe), 42.2 (N-Me).

Compound 8, yellow prismatic crystal (Acetone), mp 133-134°C; UV λ_{max} (nm): 203 (sh), 218, 268, 310 (sh), 397; EIMS *m/z*: 329 $[M]^+$, 314 (100), 270, 256, 228, 157, 149, 143, 77; ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_H 8.41 (1H, d, *J* = 8.9 Hz, H-8), 7.85 (1H, dd, *J* = 8.4, 7.7 Hz, H-6), 7.73 (1H, d, *J* = 8.4 Hz, H-5), 7.59 (1H, d, *J* = 8.9, 7.7 Hz, H-7), 4.11, 4.02, 3.97, 3.80 (4 80.H, s, 4OCH_3), 3.91 (3H, s, NCH3); ^{13}C-NMR (DMSO-d_6, 100 MHz): 176.4 (C-9), 152.6 (C-1), 149.7 (C-3), 144.9 (C-5a), 139.4 (C-4), 138.5 (C-4a), 136.7 (C-2), 134.1 (C-6), 126.7 (C-8), 123.5 (C-8a), 121.9 (C-7), 117.2 (C-5), 114.2 (C-1a), 62.2 (1-OMe), 61.9 (2-OMe), 61.9 (3-OMe), 62.1 (4-OMe), 42.1 (N-Me).

Identification of the isolated compounds [Figure 1] was based on comparison of UV, MS, ^{1}H-NMR and ^{13}C-NMR data to previously reported ones as β-sitosterol 1,[8] 4-methoxy benzoic acid 2,[8] daucosterol 3[9] and five acridone alkaloids, normelicopidine 4,[10] normelicopine 5,[11] melicopine 6,[11] melicopidine 7[11] and melicopicine 8.[12] It is noteworthy to mention that all these acridone alkaloids were isolated from this plant for the first time.

Cytotoxity assay

Compounds 4-8 were added to Falcon 384 well black/clear tissue treated assay plates containing 3000 adherent cells/well (HEK293) in an assay volume of 45 uL. The plates were incubated for 72 h at 37°C and 5% CO_2. After incubation the supernatant was aspirated from the wells and 40 uL of 10% Alamar Blue added per well. Plates were incubated for a further 5-6 h and measured for fluorescence at 535 nm excitation and 590 nm emission using a VICTOR II (PerkinElmer, Waltham, MA). The % inhibition of cell proliferation was calculated using Dimethylsulfoxide (DMSO) and 10 uM 5-Fluorouracil control data. IC_{50} values were obtained by plotting % inhibition against log dose using the Prizm4 graphing package and nonlinear regression with variable slope plot.

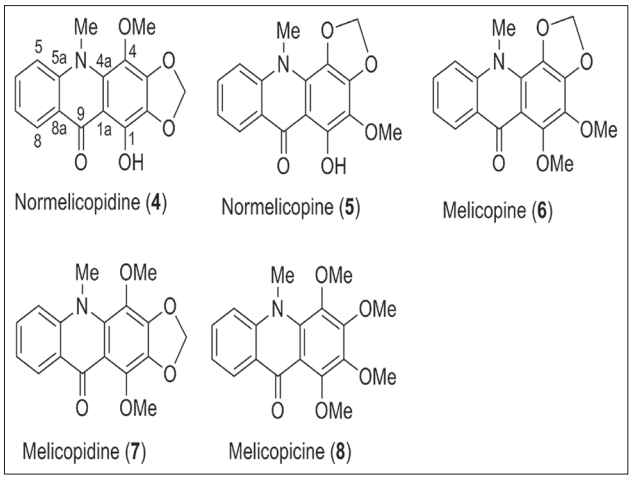

Figure 1: Structures for compounds 4-8

Antimalarial assay

Compounds 4-8 were incubated in the presence of 2 or 3% parasitemia (3D7 or Dd2) and 0.3% hematocrit in a total assay volume of 50 uL, for 72 h at 37°C and 5% CO_2, in poly-Dlysine-coated Cell Carrier imaging plates. After incubation plates were stained with DAPI in the presence of saponin and Triton X-100 and incubated for a further 5 h at RT in the dark before imaging on the Evotec OPERA High Throughput Screening confocal imaging system (PerkinElmer, Waltham, MA). The digital images obtained were analyzed using the Perkin-Elmer Acapella spot detection software, where fluorescent spots that fulfilled the criteria established for a stained parasite were counted. The % inhibition of parasite replication was calculated using DMSO and 2 uM artemisinin control data.

RESULTS AND DISCUSSION

Cytotoxity [Table 1] of five acridone alkaloids isolated from this herb was evaluated against two prostate cancer cell lines PC-3M and LNCaP. Preliminary toxicity toward human cells was investigated using a human embryonic kidney cell line (HEK293). The results revealed that all five compounds exhibited potential cytotoxity against PC-3M and LNCaP with IC_{50} value range between 12 ug/mL and 65 ug/mL, and showed no cytotoxity against HEK293 up to 100 ug/mL. Compound 4 was the most active against PC-3M and LNCaP with IC_{50} values of 12.5 and 21.1 ug/mL respectively.

Antimalarial activity [Table 2] for the acridone alkaloids has also been tested against a chloroquine-sensitive (3D7) and a chloroquine-resistant (Dd2) *Plasmodium falciparum* strain. All Compounds displayed IC_{50} values in a range of 18-42 ug/mL

Table 1: Cytotoxic activities of compounds 4-8 (IC_{50}, ug/mL; mean±SD, *n*=3)

Compound	Cell lines		
	PC-3M	LNCaP	HEK293
4	12.5±1.9	21.1±2.6	>100
5	36.3±2.4	44.6±2.7	>100
6	47.9±3.6	37.8±3.4	>100
7	36.1±2.2	28.9±3.1	>100
8	51.4±4.3	64.2±4.7	ND
5-FUa	7.5±1.8	9.3±1.5	14.7±0.4

5-FU: a5-fluorouracil was used as positive control; ND: Not determined; SD: Standard deviation . PC-3M and LNCaP: two prostate cancer cell lines; IC50: 50% inhibitory concentration

Table 2: Antimalarial and cytotoxic activity for compounds 4-8 (IC_{50}, ug/mL; mean±SD, *n*=3)

Compound	IC_{50} (ug/mL)			Selectivity index
	Dd2	3D7	HEK293	
4	18.9±2.2	NT	>100	>5.0 (Dd2)
5	25.9±2.6	31.2±3.3	>100	>3.8 (Dd2), >3.2 (3d7)
6	33.7±1.9	29.7±2.8	>100	>2.9 (Dd2), >3.3 (3d7)
7	21.6±2.5	25.5±1.9	>100	>4.6 (Dd2), >3.9 (3d7)
8	41.2±2.4	37.7±3.1	>100	>2.4 (Dd2), >2.6 (3d7)
Artemisinin	0.0056	0.0056	0% @ 0.56	>100 (Dd2, 3D7)
Chloroquine	0.067	0.013	0% @ 1.03	>16 (Dd2), >80 (3D7)

NT: Not tested; SD: Standard deviation

against 3D7 and Dd2. Compound 4 showed the strongest activity against Dd2 with IC_{50} value of 18.9 ug/mL.

CONCLUSION

Acridone alkaloids are abundant in the Rutaceae family, especially occurring in citrus plants, and many of them showed interesting pharmaceutical properties such as anticancer, antitumor, antiviral, antimalarial, and antiallergic activities. Our investigation on the root bark of *Z. simullans* Hance has led to the isolation of five acridone alkaloids, normelicopidine, normelicopine, melicopine, melicopidine, and melicopicine from this plant for the first time. All tested compounds exhibited certain cytotoxic and antimalarial activities *in vitro*, and compound 4 was the most active against PC-3M, LNCaP and Dd2 with IC_{50} values of 12.5, 21.1 and 18.9 ug/mL respectively.

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