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CYTOTOXIC CONSTITUENTS FROM *Wikstroemia indica*

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Wikstroemia indica (L.) C. A. Mey, a shrub of the Thymelaeaceae family, has been used in Chinese folk medicine for the treatment of syphilis, arthritis, whooping cough, and cancer [1]. It was reported that the methanol extract of *W. indica* has significant inhibitory activity *in vivo* against Ehrlich ascites carcinoma growth in mice (97% inhibition) as well as P-388 lymphocytic leukemia growth in mice (T/C = 180%) at 50 $mg \cdot kg^{-1} \cdot day^{-1}$ after intraperitoneal injection [2]. The coumarins and lignans from *W. indica* were proven to have antitumor activity. Matairesinol and arctigenin, which are lignans, have inhibitory activity against human HL-60 leukemia [3], and daphnoretin, a coumarin compound, inhibits Ehrlich ascites carcinoma growth by inhibiting the synthesis of nucleic acid and protein [4]. Activity-guided fractionation of the ethanol extract of stems and roots of *Wikstroemia indica* led to the isolation of 13 compounds. The structures of the isolated compounds were elucidated on the basis of their spectral data and chemical evidence [5–13]. Compounds **1**, **6**, **7**, and **9** were isolated for the first time from this plant, and **8** was a new compound identified in a previous study. Its structure was reported in [14], but its antitumor activity has not been estimated. Moreover, the bioactivity components responsible for the anticancer activity of *Wikstroemia indica* are not well understood.

According to previous literature, compound **2** exhibited efficient antitumor activity against U937, 95D, AGS, and LA795 cell lines [15, 16]. Literature [17] on the cytotoxicity of compound **4** against CNE and HeLa showed that it is concentration dependent. It has also inhibitory activity against A549 and HEp-2 cells. Compound **5** showed activity against E–J and B16 cell lines [18]. Compound **6** has anti-tumor activity towards many cell lines, such as SK-BR-3, MCF-10A, MCF-7, Huh7, HeLa, and HepG2 cell lines [19–22]. At the same time, it also exhibited activity against A2780, BxPC-3, MiaPaca-2v, and AsPC-1 cell lines [23, 24]. Compound **7** revealed significant inhibitory effects against H460, A2780/CP70, A2780/wt, OVCAR-3, HL-60, MDA-MB-231, BT-549, MIA PaCa-2, Panc-1, PC-3, and MCF-7 cell lines [25–31]. Compound **10** has been reported to show inhibitory activity against MCF-7, K562, A431, and PC-3 cell lines [32]. Compound **11** exhibited efficient activity against HL-60, K562, A431, and PC-3 cells [33]. Compound **12** exhibited anticancer activity against HL-60, K562, AGC, and Hepa 1c1c7 cell lines [34, 35]. Compound **13** revealed inhibitory effects against AGC and Hepa 1c1c7 cells [35].

The 95% EtOH extract of *W. indica* exhibited moderate cytotoxic activity against HeLa and SGC-7901 cell lines and no activity against Bel-7402 cell lines (Table 1). The petroleum ether fraction and chloroform fraction had antitumor activity against three tumor cell lines, the ethyl acetate fraction showed antitumor activity against HeLa and SGC-7901 cell lines in a concentration-dependent manner, and the *n*-butanol fraction had no cytotoxic activity against three tumor cell lines. As for LY.I–LY.XVI of the EtOAc fraction, fractions LY.II–LY.X exhibited significant dose-dependent cytotoxic activity for HeLa and SGC-7901 cells, whereas LY.I, LY.XI–LY.XVI showed no cytotoxic activity. As for LC.I–LC.XI of the chloroform fraction, fractions LC.II–LC.XI showed significant dose-dependent cytotoxic activity for HeLa and SGC-7901 cells, whereas LC.I showed no cytotoxic activity.

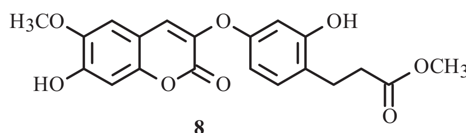

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TABLE 1. The Cytotoxic Activity of Extract against Tumor Cell Lines, IC_{50} ($\mu g \cdot mL^{-1}$)

Extract	HeLa	SGC-7901	Extract	HeLa	SGC-7901
95% Ethanol	169.45 ± 2.81	369.89 ± 5.46	LY.X	44.68 ± 2.24	209.2 ± 3.11
P.E. Fr.*	93.86 ± 5.42	72.57 ± 0.21	LY.XI–LY.XVI	> 300	> 300
$CHCl_3$ Fr.**	112.06 ± 3.20	72.93 ± 6.29	LC.I	> 300	> 300
EtOAc Fr.	101.21 ± 4.68	63.66 ± 1.52	LC.II	94.63 ± 2.17	109.1 ± 4.41
BuOH Fr.	–	–	LC.III	59.66 ± 3.04	101.2 ± 3.01
LY.I	–	–	LC.IV	85.28 ± 3.24	86.12 ± 3.19
LY.II	56.49 ± 1.28	86.52 ± 3.66	LC.V	46.78 ± 2.52	86.12 ± 3.09
LY.III	79.17 ± 4.92	94.01 ± 4.57	LC.VI	92.28 ± 4.26	102.6 ± 4.05
LY.IV	56.53 ± 4.68	69.07 ± 0.56	LC.VII	74.98 ± 3.84	108.2 ± 5.11
LY.V	56.57 ± 8.53	47.53 ± 8.68	LC.VIII	39.58 ± 1.25	79.32 ± 1.91
LY.VI	59.36 ± 4.46	49.26 ± 3.54	LC.IX	94.63 ± 3.08	112.2 ± 3.89
LY.VII	88.43 ± 3.32	62.78 ± 9.85	LC.X	79.65 ± 4.16	99.12 ± 5.02
LY.VIII	41.86 ± 4.30	107.41 ± 0.92	LC.XI	194.5 ± 4.06	212.2 ± 5.23
LY.IX	49.86 ± 8.49	88.35 ± 9.14	5-Fluorouracil	1.72 ± 0.33	4.38 ± 2.02

IC_{50} is defined as the concentration resulting in a 50% decrease in cell number; –: means not detected.
Bel-7402: IC_{50} *278.68 ± 1.56; **116.44 ± 5.85.

TABLE 2. Inhibitory Effect of Compounds from *Wikstroemia indica* against Several Tumor Cell Lines, IC_{50} (μM)

Compound	HeLa	SGC-7901	Bel-7402	HepG2	MCF-7
Genkwanin (**3**)	–	162.1 ± 21.1	–	–	–
Chamaejasim A (**9**)	29.0 ± 0.4	54.0 ± 7.6	43.7 ± 1.6	29.2 ± 3.2	46.1 ± 1.5
5-Fluorouracil	13.2 ± 2.5	33.7 ± 15.5	19.3 ± 5.2	21.2 ± 0.6	7.2 ± 2.6

IC_{50} is defined as the concentration resulting in a 50% decrease in cell number; –: means not detected.

By activity-guided fractionation using cytotoxicity assay against HeLa and SGC-7901 cell lines, 13 compounds were isolated from *W. indica*. The 50% growth inhibition (IC_{50}) values are listed in Table 2. Compound **3** showed weak cytotoxic activity against SGC-7901 cell line but no cytotoxic activity against HeLa, MCF-7, Bel-7402, and HepG2 cell lines. The cytotoxic activity of compound **9** against HeLa and HepG2 cell lines was strong and concentration dependent. Compound **9** showed moderate cytotoxic activity against MCF-7, SGC-7901, and Bel-7402 cell lines. The cytotoxicities of compounds **8** and **4** against the five cell lines above were studied, but they showed no activity. To the best of our knowledge, this is the first report for the cytotoxic activity of compounds **3** and **9**.

The NMR spectra were recorded with a Bruker-ARX 300 or 600 spectrometer operating at 300 or 600 MHz for ^1H and 75 or 150 MHz for ^{13}C NMR, respectively. Chemical shifts were reported in parts per million on the δ scale with TMS as the internal standard. ESI-MS were recorded on a Finnigan LCQ LC-MS analyzer. HR-ESI-MS/MS were recorded on a Bruker ESI-Q-TOF-MS/MS analyzer. The melting points were obtained by a thermal values analysis with a microscope (Beijing Taike Chemical Apparatus Company, Beijing, China). Column chromatography was performed with silica gel (Qingdao Haiyang Chemical Group Co. Ltd., Qingdao China), TLC was performed on precoated silica gel G plates, and spots were detected by spraying with 10% ethanolic H_2SO_4 reagent.

Plant Material. The stems and roots of *W. indica* were collected from Guangdong Province and authenticated by Prof. Qi-Shi Sun at Shenyang Pharmaceutical University, where a voucher specimen (No. 071001) was deposited.

Extraction and Isolation. The dried and crushed stems and roots of *W. indica* (13 kg) were extracted with 95% EtOH three times under refluxing. The crude extract (870 g) obtained after vacuum concentration was suspended in H_2O and then partitioned successively with petroleum ether, chloroform, ethyl acetate, and *n*-butanol to give the petroleum ether fraction (30 g), the chloroform fraction (124 g), the ethyl acetate fraction (145 g), and the *n*-butanol fraction (314 g). The extract of 95% EtOH and its extracted fractions were evaluated for their cytotoxic activity against three tumor cell lines (Table 1). Then, the ethyl acetate fraction (140 g) was subjected to silica gel column eluted with chloroform–methanol (100:0–0:100) to afford 16 fractions (LY.I–LY.XVI). All fractions were evaluated for their cytotoxic activity against HeLa and SGC-7901 cell lines

(Table 1). Fraction LY.II was applied to a silica gel column using petroleum ether–acetone (100:0–0:100) as eluent to obtain compounds **1** (12 mg), **2** (10 mg), **3** (20 mg), **4** (2 g), and **5** (15 mg). Fraction LY.V was applied on a silica gel column eluted with chloroform–methanol (100:0–0:100) and dichloromethane–ethyl acetate–glacial acetic acid (80:1:1–0:100:0) to give compounds **6** (9 mg) and **7** (5 mg). Fraction LY.VI was chromatographed over a silica gel column with petroleum ether–acetone (100:0–0:100) and methylene chloride–absolute ethanol (80:1–20:1) as eluent, followed by recrystallization from chloroform, to give compounds **8** (15 mg) and **9** (20 mg). The chloroform fraction (120 g) was applied to a silica gel column eluted with petroleum ether–acetone (100:0–0:100) as the eluting solvent to give 11 fractions (LC.I–LC.XI). All the fractions were evaluated for their cytotoxic activity against HeLa and SGC-7901 cell lines (Table 1). Fraction LC.V was applied to a silica gel column using chloroform–methanol (80:1–0:100) as eluent to afford 13 fractions (LC.V-I–LC.V-XIII). Isolation of fraction LC.V-II on a silica gel column using methylene chloride–ethyl acetate (30:1–0:1) and TLC (petroleum ether–ethyl acetate) afforded compounds **10** (12 mg) and **11** (10 mg). Fraction LC.VIII was applied on a silica gel column with hexamethylene–acetone (80:1–0:1) to give four fractions (LC.VIII-I–LC.VIII-IV). Fraction LC.VIII-IV was chromatographed over RP-HPLC (ODS, 60% MeOH) to give compounds **12** (14 mg) and **13** (25 mg).

Eicosanoic Acid (1). White amorphous powder, mp 59–60°C. ESI-MS *m/z* 311.1 $[M – H]^–$ (calcd for $C_{20}H_{40}O_2$, 312.1.) ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 179.3 (C-1), 29.7, 29.4, 29.2 and 29.1 (a set of methyl and methylene group signals).

***β*-Sitosterine (2)**. White amorphous powder, mp 142°C.

Genkwanin (3). Yellow powder, mp 293°C, ESI-MS *m/z* 283.3 $[M – H]^–$.

Daphnoretin (4). Light yellow needles, mp 239–240°C. ESI-MS *m/z* 351.6 $[M – H]^–$. ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 3.80 (3H, s, OC$\underline{H}_3$), 6.36 (1H, d, J = 9.6, H-3′), 6.85 (1H, s, H-8), 7.10 (1H, dd, J = 9.1, 2.1, H-6′), 7.17 (1H, s, H-8′), 7.20 (1H, s, H-5), 7.69 (1H, d, J = 9.6, H-5′), 7.86 (1H, s, H-4), 8.03 (1H, d, J = 9.6, H-4′), 10.29 (1H, s, OH). ^{13}C NMR (75 MHz, DMSO-d_6, δ): 157.0 (C-2), 135.8 (C-3), 130.9 (C-4), 110.3 (C-4a), 109.5 (C-5), 145.8 (C-6), 159.8 (C-7, 7′), 102.9 (C-8), 147.5 (C-8a), 160.1 (C-2′), 114.0 (C-3′), 144.1 (C-4′), 114.5 (C-4a′), 130.0 (C-5′), 113.5 (C-6′), 104.1 (C-8′), 56.1 (O$\underline{C}H_3$).

Umbelliferone (5). Colorless needles, mp 210–211°C.

Apigenin (6). Yellow powder, mp 348–350°C. ESI-MS *m/z* 269.2 $[M – H]^–$.

Kaempferol (7). Yellow needles in $CHCl_3$.

Wikstrocoumarin (8). Colorless needles in $CHCl_3$, mp 180–182°C. ^{1}H NMR (300 MHz, acetone-d_6, δ, ppm, J/Hz): 2.60 (2H, t, J = 7.5, H-2), 2.87 (2H, t, J = 7.5, H-3), 6.58 (1H, d, J = 2.5, H-3′), 6.50 (1H, dd, J = 8.4, 2.5, H-5′), 7.10 (1H, d, J = 8.4, H-6′), 7.38 (1H, s, H-4″), 7.16 (1H, s, H-5″), 6.84 (1H, s, H-8″), 3.88 (3H, s, 6″-OCH_3), 3.63 (3H, s, 1-OCH_3), 8.63 (1H, s, 7″-OH), 8.63 (1H, s, 2′-OH). ^{13}C NMR (75 MHz, acetone-d_6, δ): 173.1 (C-1), 33.7 (C-2), 25.2 (C-3), 122.7 (C-1′), 156.0 (C-2′), 104.9 (C-3′), 156.2 (C-4′), 108.6 (C-5′), 130.9 (C-6′), 157.0 (C-2″), 139.0 (C-3″), 126.2 (C-4″), 111.0 (C-4a″), 108.6 (C-5″), 145.5 (C-6″), 149.5 (C-7″), 102.7 (C-8″), 147.1 (C-8a″), 55.9 (6″-OCH_3), 50.8 (1-OCH_3).

Chamaejasmenin A (9). Yellow powder, mp 193–195°C. ESI-MS *m/z* 569.8 $[M – H]^–$. ^{1}H NMR (300 MHz, acetone-d_6, δ, ppm, J/Hz): 3.38 (1H, d, J = 12.3, H-3, 3″), 3.83 (6H, s, 4′, 4‴-OC$\underline{H}_3$), 5.17 (1H, s, H-8, 8″), 5.75 (1H, s, H-6, 6″), 5.87 (2H, d, J = 12.3, H-2, 2″), 6.85 (4H, d, J = 8.6, H-3′, 3‴), 7.15 (4H, d, J = 8.6, H-2′, 2‴). ^{13}C NMR (75 MHz, DMSO-d_6, δ): 80.6 (C-2, 2″), 47.5 (C-3, 3″), 194.9 (C-4, 4′), 163.6 (C-5, 5′), 96.4 (C-6, 6″), 166.8 (C-7, 7′), 95.2 (C-8, 8″), 162.4 (C-9, 9″), 101.2 (C-10, 10″), 126.7 (C-1′, 1‴), 129.6 (C-2′, 2‴), 114.3 (C-3′, 3‴), 158.5 (C-4′, 4‴), 113.9 (C-5′, 5‴), 127.2 (C-6′, 6‴), 55.4 (4′, 4‴-O$\underline{C}H_3$).

Physcion (10). Orange needles in $CHCl_3$.

Chrysophanol (11).

Arctigenin (12). ^{1}H NMR (300 Hz, $CDC1_3$, δ, ppm, J/Hz): 2.50–2.66 (4H, m, H-2, 3, 7″), 2.95 (2H, m, H-7′), 3.82 (6H, s, OCH_3), 3.86–3.92 (4H, m, OCH_3, H-4a), 4.17 (1H, dd, J = 9.2, 7.2, H-4b), 6.47 (1H, d, J = 1.8, H-2″), 6.57 (1H, dd, J = 8.1, 2.1, H-6″), 6.63 (1H, dd, J = 7.8, 1.8, H-6′), 6.65 (1H, d, J = 1.8, H-2′), 6.77 (1H, d, J = 8.1, H-5″), 6.85 (1H, d, J = 8.1, H-5′). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 178.7 (C-1), 46.6 (C-2), 40.9 (C-3), 71.3 (C-4), 129.5 (C-1′), 111.5 (C-2′), 146.7 (C-3′), 144.5 (C-4′), 114.1 (C-5′), 122.1 (C-6′), 34.5 (C-7′), 130.5 (C-1″), 111.7 (C-2″), 149.0 (C-3″), 147.8 (C-4″), 111.2 (C-5″), 120.6 (C-6″), 38.2 (C-7″), 55.8 ($OCH_3 \times 3$).

Matairesinol (13). ^{1}H NMR (300 MHz, $CDC1_3$, δ, ppm, J/Hz): 6.83 (1H, d, J = 8.4, H-5″), 6.80 (1H, d, J = 8.0, H-5′), 6.61 (2H, m, H-2″), 6.59 (1H, d, H-6″), 6.49 (1H, dd, J = 16.5, 7.8, H-6′), 6.40 (1H, d, J = 1.5, H-2′), 4.15 (1H, dd, J = 9.0, 7.0, H-4b), 3.89 (1H, dd, J = 9.0, 7.0, H-4′), 3.81 (6H, s, OCH_3), 2.83–2.97 (2H, m, H-7′b), 2.45–2.62 (4H, m, H-2, H-3, H-7″a, H-7″b). ^{13}C NMR (75 MHz, $CDC1_3$, δ): 178.8 (C-1), 46.5 (C-2), 40.9 (C-3), 71.2 (C-4), 129.5 (C-1′), 111.4 (C-2′), 146.7 (C-3′),

144.5 (C-4′), 114.0 (C-5′), 122.0 (C-6′), 34.5 (C-7′), 129.7 (C-1″), 110.9 (C-2″), 146.6 (C-3″), 144.3 (C-4″), 114.4 (C-5″), 121.3 (C-6″), 38.2 (C-7″), 55.7 (OCH_3), 55.8 (OCH_3).

Cell Culture. HeLa (human uterine carcinoma), HepG2 (hepatoma carcinoma cell), MCF-7 (human breast cancer), SGC-7901 (human gastric cancer), and Bel-7402 (human hepatocellular carcinoma) cell lines were cultured in RPMI-1640 medium including 10% fetal bovine serum. All cells were maintained at 37°C and 5% CO_2 in a humidified-atmosphere incubator. The confluent cells were used for bioactivity assay. All cell lines were purchased from China Medical University (Shenyang, China).

Cytotoxic Assay. Inhibition of cellular growth was estimated using 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) assay as described by Mosmann [6].

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