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Constituents of *Peucedanum zenkeri* seeds and their antimicrobial effects

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The methanol extract of *Peucedanum zenkeri* L. seeds showed antimicrobial activity which is concentrated in the *n*-hexane fraction. Bioactivity-guided chromatographic fractionation of the seeds of *P. zenkeri* led to the isolation and characterization of five major coumarins, umbelliprenin, imperatorin, bergapten, isopimpinellin and byakangelicin, as well as two minor coumarins, 7-methoxy coumarin and 5-hydroxy-8-methoxy psoralen. Amongst the isolated compounds only imperatorin, bergapten and isopimpinellin were found to possess anti-microbial activity.

1. Introduction

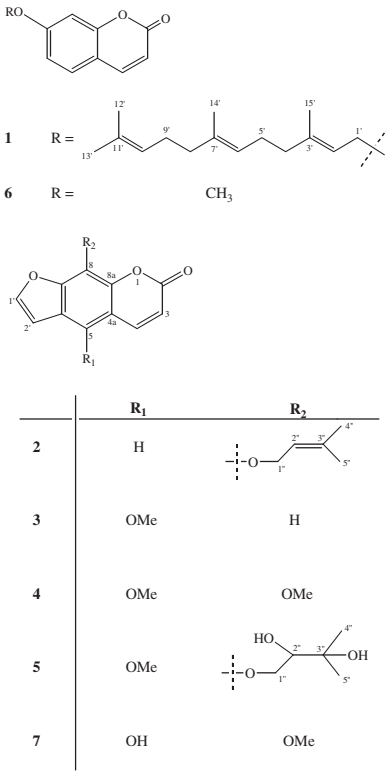
Peucedanum zenkeri is an herb found in the West-Central African equatorial forest. The roots of *Peucedanum* species, known as Qian-Hu, are widely used in Chinese folk medicine as antitussive, expectorant, antipyretic and stomachic [1]. Earlier investigations performed on *Peucedanum* species resulted in the isolation of coumarins [1–4], volatile constituents [5], chromones [6], and butenolides [7]. No phytochemical or pharmacological investigation has yet been carried out on *Peucedanum zenkeri*. As part of our ongoing biological evaluation of West African medicinal plants, we undertook a bioactivity-guided phytochemical investigation of the seeds of *P. zenkeri* that resulted in the isolation of seven coumarins; umbelliprenin, imperatorin, bergapten, isopimpinellin, byakangelicin, 7-methoxy coumarin and 5-hydroxy-8-methoxy psoralen [8–13].

2. Investigations, results and discussion

During our preliminary biological screening studies of West African medicinal plants, we observed antimicrobial activity for the methanol extract of *P. zenkeri* concentrated in the *n*-hexane fraction (Table 1). Therefore, we focused our attention on the *n*-hexane extract. Bioactivity-guided fractionation of aforementioned extract resulted in the isolation of four coumarins (**1**–**4**). Further chromatographic studies performed on the less active chloroform extract gave compounds **5**–**7**. The structure of the compounds were identified by comparison of their spectral data (see experimental) with those reported for umbelliprenin (**1**)

Table 1: Antimicrobial activities of the extracts of *P. zenkeri* seeds

Extract	% Growth Inhibition				
	C. albicans	*C. neoformans*	*S. aureus*	MRS	*M. intracellulare*
MeOH	100	57	100	100	100
H_2O	8	0	0	0	0
$CHCl_3$	75	74	63	57	84
n-Hexane	100	100	100	100	90



	R_1	R_2
2	H	
3	OMe	H
4	OMe	OMe
5	OMe	
7	OH	OMe

Table 2: Antimicrobial activities of the *n*-hexane extract fractions

Fr. code	IC_{50} (μg/mL)				
	C. albicans	*C. neoformans*	*S. aureus*	MRS	*M. intracellulare*
A	90	NA	NA	NA	100
D	NA	NA	90	NA	NA
E	NA	NA	100	NA	NA
J	NA	NA	80	150	NA
K	90	NA	95	90	15
L	150	70	65	60	NA
M	200	150	45	20	NA
N		150	75	80	NA

NA: No activity

Table 3: Antimicrobial activities of the compounds isolated from *P. zenkeri*

Compound	IC_{50} (μg/mL)	
	C. neoformans	*M. intracellulare*
Imperatorin (**2**)	20	50
Bergapten (**3**)	50	35
Isopimpinellin (**4**)	40	NA

[8], imperatorin (**2**) [9], bergapten (**3**) [10], isopimpinellin (**4**) [11], byakangelicin (**5**) [12], 7-methoxy coumarin (**6**) [13] and 5-hydroxy-8-methoxy psoralen (**7**).

All the compounds tested (**1**–**6**) were inactive against *Candida albicans*, *Staphylococcus aureus*, *MRS*, *Aspergillus fumigatus*, and *Pseudomonas aeruginosa*. Although coumarins tested were from the same chemical class, only compounds **2**–**4** exhibited activity against *Cryptococcus neoformans* and *Mycobacterium intracellulare* shown in Table 3. These results indicate that there are critical structural features responsible for antimicrobial activity. While the compounds possessing basic coumarin skeleton (**1** and **6**) were inactive, three of four furano-coumarins (**2**–**4**) were reasonably active. In contrast, compound **5**, which is also a furano-coumarin, unexpectedly lacked activity. The chemical difference between **4** and **5** is the presence of a prenyl side chain at C-5 for **5**. Compound **7** was not tested because of the paucity of the sample.

Based on the results, the furano-coumarin framework seems very important for antimicrobial effects of these compounds. Further studies are required to confirm the above assumptions regarding structure-activity relationship of coumarins.

3. Experimental

3.1. Plant material

Peucedanum zenkeri was collected in November 2001 from the cultivation farm of Dr. Wirmum Claire, Director of Medicinal Foods and Plants in Bamenda and a Botanist with the ICBG Project.

3.2. Extraction and isolation

P. zenkeri (600 g) seeds were extracted twice with 6.0 L MeOH at 40 °C for 5 h. The filtrate was concentrated under reduced pressure to obtain 66.4 g of oily greenish concentrate. The bulk extract was suspended in 200 mL of water and washed five times with 300 mL portions of *n*-hexane. The organic portion yielded 15.0 g of oily greenish extract upon concentration. The aqueous portion was further washed four times with 300 mL $CHCl_3$ to give 19.8 g extract. The hexane fraction (15.0 g) was mounted on a column (80 × 4 cm) packed with 230 g of SiO_2 and eluted with increasing polarities of *n*-hexane-EtOAc-MeOH mixtures. Fractions were pooled into 18 major fractions (Frs. A-R) on the basis of their TLC profiles. Fraction H precipitated overnight into white crystals that were washed with hexane to obtain umbelliprenin (**1**, 400 mg). Imperatorin (**2**, 1.9 g) also precipitated from fraction J during the concentration process. The pale yellow crystals were also washed with hexane. A mixture of crystals containing three substances (350 mg) precipitated from fraction K. These were purified on chromatotron (using *n*-hexane-EtOAc solvent systems), followed with gel filtration column (Sephadex LH-20, using MeOH) and finally with preparative TLC (using *n*-hexane-EtOAc; 6 : 4) to give bergapten (**3**, 11.6 mg), isopimpinellin (**4**, 15.8 mg), and more of imperatorin (**2**, 25 mg).

The $CHCl_3$ extract (19.8 g) was chromatographed on a column (55 × 7.5 cm) packed with 700 g of SiO_2 and eluted with increasing polarities of *n*-hexane-EtOAc-MeOH mixtures. Fractions pooled into eight major fractions (Frs. A′–H′) following their TLC profiles. Fraction F′ precipitated on standing overnight to yield cream white crystals of byakangelicin (**5**, 94.0 mg). Gel filtration chromatography (Sephadex LH-20) of fraction C′ (1.4 g) using MeOH yielded white crystals of 7-methoxy coumarin (**6**, 3.5 mg). Fraction B′ (1.7 g) on a column packed with 150 g of SiO_2 eluted with *n*-hexane-EtOAc mixtures to yield pale yellow crystals of 5-hydroxy-8-methoxy psoralen (**7**, 3.0 mg) including more of imperatorin (97.5 mg), bergapten (10 mg) and isopimpinellin (99.8 mg).

3.2.1. Umbelliprenin (1)

White powder; ^{1}H NMR data (300 MHz, $CDCl_3$): δ 6.22 (1 H, d, J = 9.4 Hz, H-3), 7.61 (1 H, d, J = 9.5 Hz, H-4), 7.33 (1 H, d, J = 8.4 Hz, H-5), 6.83 (1 H, s, H-6), 6.80 (1 H, s, H-8), 4.57 (2 H, d, J = 6.5 Hz, H-1′), 5.45 (1 H, t, J = 6.1 Hz, H-2′), 1.74 (3 H, s, Me-15′), 1.95 (2 H, m, H-4′), 2.10 (2 H, m, H-5′), 5.06 (1 H, dd, J = 6.9, 13.2 Hz, H-6′), 1.65 (3 H, s, Me-14′), 2.01 (2 H, m, H-8′), 2.11 (2 H, m, H-9′), 5.06 (1 H, dd, J = 6.9, 13.2 Hz, H-10′), 1.57 (6 H, s, Me-12′ and Me-13′); ^{13}C NMR data (75 MHz, $CDCl_3$): δ 162.5 (C-2), 113.3 (C-3), 143.2 (C-4), 112.8 (C-4a), 129.1 (C-5), 113.6 (C-6), 161.6 (C-7), 102.0 (C-8), 156.2 (C-8a), 65.9 (C-1′), 118.9 (C-2′), 142.7 (C-3′), 26.1 (C-15′), 40.1 (C-4′), 39.9 (C-5′), 124.7 (C-6′), 135.9 (C-7′), 18.1 (C-14′), 27.1 (C-8′), 26.5 (C-9′), 123.9 (C-10′), 131.7 (C-11′), 16.4 (C-12′), 17.2 (C-13′); HRESIFTMS: *m/z* 367.2206 $[M + H]^+$.

3.2.2. Imperatorin (2)

Pale yellow powder; ^{1}H NMR data (300 MHz, $CDCl_3$): δ 6.33 (1 H, d, J = 9.6 Hz, H-3), 7.74 (1 H, d, J = 9.6 Hz, H-4), 7.34 (1 H, s, H-5), 6.80 (1 H, d, J = 2.1 Hz, H-2′), 7.66 (1 H, d, J = 2.1 Hz, H-1′), 4.97 (2 H, d, J = 7.2 Hz, H-1″), 5.66 (1 H, t, J = 7.1 Hz, H-2″), 1.71 (6 H, s, Me-4″ and Me-5″); ^{13}C NMR data (75 MHz, $CDCl_3$): δ 160.9 (C-2), 115.9 (C-3), 144.0 (C-4), 116.9 (C-4a), 113.6 (C-5), 126.3 (C-6), 149.0 (C-7), 132.0 (C-8), 147.0 (C-1′), 107.1 (C-2′), 70.5 (C-C-1″), 120.2 (C-2″), 140.1 (C-3″), 18.5 (C-4″), 26.2 (C-5″); HRESIFTMS: *m/z* 271.0922 $[M + H]^+$.

3.2.3. Bergapten (3)

Pale yellow powder; ^{1}H NMR data (300 MHz, DMSO-d_6): δ 6.34 (1 H, d, J = 9.8 Hz, H-3), 8.17 (1 H, d, J = 9.8 Hz, H-4), 4.24 (3 H, s, 5-OMe), 8.02 (1 H, d, J = 2.3 Hz, H-1′), 7.38 (1 H, d, J = 1.4 Hz, H-2′), 7.32 (1 H, s, H-8); ^{13}C NMR data (75 MHz, DMSO-d_6): δ 160.3 (C-2), 112.8 (C-3), 139.4 (C-4), 106.7 (C-4a), 149.6 (C-5), 61.1 (5-OMe), 113.0 (C-6), 158.2 (C-7), 94.0 (C-8), 152.7 (C-8a), 145.0 (C-1′), 105.3 (C-2′); HRESIFTMS: *m/z* 239.0028 $[M + Na]^+$.

3.2.4. Isopimpinellin (4)

White powder; ^{1}H NMR data (300 MHz, DMSO-d_6): δ 6.36 (1 H, d, J = 9.8 Hz, H-3), 8.15 (1 H, d, J = 9.8 Hz, H-4), 4.01 (3 H, s, 5-OMe), 4.15 (3 H, s, 8-OMe), 8.07 (1 H, d, J = 2.3 Hz, H-1′), 7.37 (1 H, d, J = 2.2 Hz, H-2′); DEPT data (75 MHz, DMSO-d_6) δ 113.4 (C-3), 140.6 (C-4), 61.6 (5-OMe), 62.1 (8-OMe), 147.2 (C-1′), 106.5 (C-2′); HRESIFTMS: *m/z* 247.0312 $[M + H]^+$.

3.2.5. Byakangelicin (5)

Pale yellow powder; ^{1}H NMR data (500 MHz, DMSO-d_6): δ 6.33 (1 H, d, J = 9.7 Hz, H-3), 8.18 (1 H, d, J = 9.8 Hz, H-4), 8.09 (1 H, d, J = 2.0 Hz, H-1′), 7.37 (1 H, d, J = 2.1 Hz, H-2′), 4.18 (3 H, s, 8-OMe), 4.46 (1 H, dd, J = 1.7, 9.9 Hz, H_a-1″), 4.19 (1 H, dd, J = 1.7, 9.9 Hz, H_b-1″), 3.65 (1 H, m, H-2″), 4.95 (1 H, d, J = 5.6 Hz, 2″-OH), 4.35 (1 H, s, 3″-OH), 1.06 (3 H, s, Me-4″), 1.15 (3 H, s, Me-5″); ^{13}C NMR data (125 MHz, DMSO-d_6): δ 160.5 (C-2), 113.4 (C-3), 140.6 (C-4), 107.8 (C-4a), 144.0 (C-5), 115.3 (C-6), 150.4 (C-7), 127.7 (C-8), 144.9 (C-8a), 61.7 (8-OMe), 147.1 (C-1′), 106.4 (C-2′), 76.7 (C-1″), 77.5 (C-2″), 71.6 (C-3″), 25.3 (C-4″), 28.1 (C-5″); HRESIFTMS: *m/z* 357.1018 $[M + Na]^+$.

3.2.6. 7-Methoxy coumarin (6)

Light brown powder; ^{1}H NMR data (500 MHz, DMSO-d_6): δ 6.18 (1 H, d, J = 9.5 Hz, H-3), 7.94 (1 H, d, J = 9.5 Hz, H-4), 7.54 (1 H, d, J = 8.5 Hz,

H-5), 6.77 (1 H, dd, J = 1.2, 7.9 Hz, H-6), 4.11 (3 H, s, 7-OMe), 6.71 (1 H, s, H-8); ^{13}C NMR data (125 MHz, DMSO-d_6): δ 162.2 (C-2), 130.5 (C-3), 145.3 (C-4), 112.1 (C-4a), 103.0 (C-5), 112.2 (C-6), 161.3 (C-7), 114.0 (C-8), 156.4 (C-8a), 61.9 (OMe).

3.2.7. 5-Hydroxy-8-methoxypsoralen (7)

Cream white powder; ^{1}H NMR data (500 MHz, DMSO-d_6): δ 6.32 (1 H, d, J = 9.8 Hz, H-3), 8.18 (1 H, d, J = 9.8 Hz, H-4), 10.11 (1 H, s, 5-OH), 4.10 (3 H, s, 8-OMe), 8.04 (1 H, d, J = 2.1 Hz, H-1′), 7.29 (1 H, d, J = 2.3 Hz, H-2″); ^{13}C NMR data (125 MHz, DMSO-d_6): δ 160.8 (C-2), 113.3 (C-3), 140.7 (C-4), 108.0 (C-4a), 126.3 (C-5), 115.7 (C-6), 148.0 (C-7), 140.5 (C-8), 142.1 (C-8a), 62.0 (8-OMe); HRESIFTMS: *m/z* 233.0436 $[M + H]^+$.

3.3. Antimicrobial bioassay

Fungal organisms employed in the OIGM assay include: *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906. Bacteria include *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 43300 (MRS), *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. All organisms are stored on agar slants at 4 °C until needed (*C. albicans* and *C. neoformans* on Sabouraud Dextrose agar (Difco, Detroit), *S. aureus*, MRS and *P. aeruginosa* on Eugon agar (Difco, Detroit), *M. intracellulare* on Lowenstein-Jensen agar (BBL, Maryland), and *A. fumigatus* on YM agar). Susceptibility testing is performed using a modified version of the NCCLS methods. Excluding *A. fumigatus* (which is prepared on the day of the assay), all microorganisms are subcultured prior to the assay by suspending cells from the slant in the appropriate broth and incubating at varying temperatures and times: *C. albicans* in Sabouraud dextrose broth (Difco, Detroit) for 24 h at 37 °C, *C. neoformans* in Sabouraud dextrose broth for 72 h at 30 °C, *S. aureus* and MRS in Eugon broth (BBL, Maryland) for 24 h at 37 °C, *M. intracelluare* in Middlebrook broth with OADC enrichment (BBL, Maryland) for 72 h at 37 °C, *P. aeruginosa* in Eugon broth for 6 h at 37 °C. For the assay, the microbial inocula, excluding *A. fumigatus*, are prepared by diluting the subcultured organism in its incubation broth: *S. aureus* and MRS = 1 : 50 dilution, *P. aeruginosa* = 1 : 1000 dilution, *C. albicans* = 1×10^4 cells/ml determined by a hemacytometer count of the saline-washed overnight culture, *C. neoformans* = 2×10^5 cells/ml determined turbidimetrically, *M. intracellulare* = 1 : 30 dilution. The *A. fumigatus* inoculum is prepared by gently removing the growth from a slant and transferring to 50 ml YPD broth. Prepared test compounds/extracts are dissolved in DMSO, serially-diluted using normal saline, and transferred in duplicate to 96 well microtiter plates (flat bottom plates for *C. albicans*, *C. neoformans*, *S. aureus*, MRS and *P. aeruginosa* and round bottom plates for *M. intracelluare* and *A. fumigatus*). The microbial inoculum is added to achieve a final volume of 200 µl and final concentrations starting with 500 µg/ml for crude extracts and 50 µg/ml for pure compounds. Drug (tetracycline (Sigma, St. Louis) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi as well as growth and blank (media only) controls are added to each test plate. Except for *M. intracellulare* and *A. fumigatus*, which are inspected visually, all other organisms are read turbidimetrically at 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) prior to and after incubation: *C. albicans*, *S. aureus*, MRS and *P. aeruginosa* at 37 °C for 24 h, *C. neoformans* and *A. fumigatus* at 30 °C for 72 h, and *M. intracellulare* at 37 °C for 72 h). For turbidimetrically-read organisms, percent growth is calculated and plotted versus concentration to afford the IC_{50}/MIC. Minimum fungicidal or bactericidal concentrations (MFC/MBC) are determined by removing 5 µl of each duplicate, transferring to agar and incubating at previously-mentioned times and temperatures. The MFC/MBC is defined as the lowest concentration of sample to allow no growth [14, 15].

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