Antifungal and Antibacterial Activities of *Pentanema divaricatum* and Its Active Constituent

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The antimicrobial activity of ethanol and chloroform extracts of *Pentanema divaricatum* Cass. was studied using the conventional disk diffusion method. The extracts' highest antimicrobial activity was observed against *Aspergillus niger*. Bioassay-guided fractionation of the crude extract by preparative thin layer chromatography (PTLC) showed one antimicrobial fraction which was especially effective against *Aspergillus niger*. By conventional spectroscopy the active fraction was identified as 4α,5α-epoxy-10α,14*H*-1-epi-inuviscolide. This compound represented the most potent antimicrobial candidate, with MIC values of < 25 μg/disk against *A. niger* strains and 200 μg/disk against *Bacillus cereus* and *Staphylococcus aureus*.

Key words: Antimicrobial, Inuviscolide Derivative, *Pentanema divaricatum*

Introduction

Life-threatening fungal and bacterial infections have increased dramatically in immunocompromised patients during the recent decades (Ghannoum, 1997; Trevejo *et al.,* 2005). Antimicrobial agents currently available are limited either by their low effectiveness or by their toxicity due to the prolonged treatments often required, which can become expensive (Freixa *et al*., 1998). Therefore, there is a constant need for more effective antimicrobial agents. In our program of searching for potent antifungal agents we have selected an extract prepared from *Pentanema divaricatum* Cass. (Compositae). There are few reports on biological and phytochemical properties of this species in the literature (Muhammad *et al*., 2003; Mossa *et al*., 1997). This paper reports the bioactivity-guided fractionation of the total extract of *P. divaricatum*, leading to the isolation of one potent anti-*Aspergillus niger* compound. The antifungal and antibacterial effects of ethanol and chloroform extracts of *P. divaricatum*, and of the isolated active compound, were also determined against eight species of microorganisms: *Bacillus cereus* (ATCC 1274), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 29737), *Pseudomonas aeruginosa* (ATCC 9027), *Aspergillus niger* (PIM), *Aspergillus niger* (ATCC 1624), *Aspergillus fumigatus* (ATCC 204305) and *Candida albicans* (ATCC 10231).

Materials and Methods

General experimental procedures

IR spectra were recorded as KBr pellets or films on a Bomen MB-154 Fourier Transform spectrometer (Bomen, Quebec, Canada). ^{1}H, ^{13}C, HMQC, HMBC and ^{1}H-^{1}H-COSY spectra of compound **1** were recorded on a Bruker FT 500 instrument (500 MHz for ^{1}H and 125 MHz for ^{13}C). Electron-impact mass spectra were taken on an Agilent Technologies model 5973 spectrometer (Agilent Technologies, Palo Alto, CA, USA). Column chromatography was conducted using silica gel 70–200 mesh (Merck, Darmstadt, Germany) as the ab-

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sorbing phase. Preparative TLC was performed on silica gel 60 GF_{254} plates (Merck); the plates were observed under a UV CAMAG cabinet (254 nm; Desaga, Wiesloch, Germany).

Plant collection

Pentanema divaricatum (Compositae) was collected at Kamaroj, Fars Provice, Iran at an altitude of 1700 m in May 2003, and was identified by Dr. F. Attar. A voucher specimen of the plant (No. 37301-TEH) was deposited in the Central Herbarium of Tehran University, Tehran, Iran.

Extraction, chromatography, and spectroscopy

The whole plant (100 g) was air-dried at room temperature and pulverized. The ethanol and chloroform extracts were prepared separately by macerating the powder for 72 h at room temperature. Bioassay-guided fractionation was carried out by preparative thin layer chromatography (PTLC) on silica gel 60 F_{254} (Merck) using hexane/ethyl acetate (60:40 v/v) as the solvent system (Momen-Roknabadi, 2007). The fractions were visualized under UV light at 254 nm and eluted using ethanol. Furthermore, the active constituent was abundantly purified using the following method. The chloroform extract was subjected to column chromatography on silica gel (1× 70 cm) with hexane/ethyl acetate (60:40 v/v), yielding five fractions (A–E). Fraction A (1.39 g) was submitted to silica gel column chromatography eluted with hexane/ethyl acetate (60:40) to obtain seven fractions (A1–A7). Fraction A3 (30 mg) was purified again with the same solvent to obtain compound **1** (8 mg, R_f = 0.47). The pure compound was identified as 4α,5α-epoxy-10α,14*H*-1-epi-inuviscolide using spectroscopic methods such as ^{1}H NMR, ^{13}C NMR, HMQC, HMBC, ^{1}H-^{1}H-COSY and EI-mass spectroscopy.

Disk diffusion susceptibility test

In order to identify the most active antifungal fractions, approx. 4 mg of each TLC-fractioned compound was re-dissolved in ethanol (Merck). Antifungal activity was determined using disk diffusion bioassays with *A. niger* ATCC 1624 and 400 μg of compound per disk. Disks containing ethanol were used as negative controls in all experiments.

In order to investigate the MIC values of the ethanol and chloroform extracts and compound **1**, a collection of test fungi and bacteria, shown in Table I, was used. All test strains were obtained from the Laboratory of General Microbiology, Department of Food and Drugs, Faculty of Pharmacy, Medical Sciences/University of Tehran, Tehran, Iran. The organisms were tested on Sabouraud dextrose agar (SDA) for fungi and on Müller-Hinton agar (MHA) for bacteria. Excess moisture was absorbed for 10 min prior to application of 6 mm paper disks containing different amounts of total extracts (500, 1000, 2000, 4000, 8000 μg) and the active fraction (25, 50, 100, 200, 400 μg). The MIC was defined as the lowest drug concentration resulting in a clear zone of growth inhibition around the disk after a conventional incubation period (Pettit *et al.*, 2002). Paper disks containing different concentrations of fluconazole (Pfizer AG, Zurich, Switzerland) and gentamycin (Sigma, St. Louis, MO, USA) were applied on the test plates as comparative positive controls.

Results and Discussion

Table I shows the antimicrobial effects of *P. divaricatum* extracts against the test organisms. The

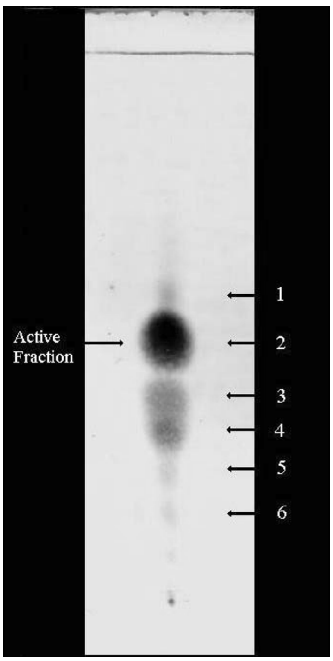

Fig. 1. The thin layer chromatogram of the chloroform extract of *Pentanema divaricatum* shows at least six distinct compounds after visualization by vanillin-sulfuric acid at 110 °C. Line number 2 shows the active compound (R_f = 0.47).

Table I. Minimum inhibitory concentrations (MICs) of crude extracts of *Pentanema divaricatum* and compound **1** against different test strains.

Species	MIC [μg/disk]				
	Chloroform extract	Ethanol extract	**1**	Gentamycin	Fluconazole
Candida albicans ATCC 10231	> 8000	> 8000	400	ND	10
Aspergillus niger PIM	< 1000	< 1000	< 25	ND	10
Aspergillus niger ATCC 1624	< 1000	< 1000	< 25	ND	> 40
Aspergillus fumigatus ATCC 204305	4000	2000	> 400	ND	40
Bacillus cereus ATCC 1274	1000	< 1000	200	16	ND
Echerichia coli ATCC 8739	> 8000	8000	> 400	8	ND
Staphylococcus aureus ATCC 29737	1000	1000	200	16	ND
Pseudomonas aeruginosa ATCC 9027	> 8000	> 8000	> 400	32	ND

ND, not determined.

Table II. Antifungal activity of different TLC fractions of the *Pentanema divaricatum* chloroform extract against *Aspergillus niger* (ATCC 1624).

Sample	Diameter of growth inhibition zone[a]
TLC fraction No. 2 (400 μg)	33 ± 1
Other TLC fractions (400 μg)	–
Fluconazole (10 μg)	9 ± 0.5

[a] Mean value in mm, $n = 3$.
– Negative.

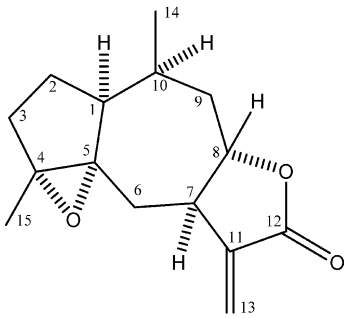

Fig. 2. Chemical structure of 4α,5α-epoxy-10α,14*H*-1-epi-inuviscolide.

strongest antimicrobial activity was observed for both total extracts against *A. niger*. TLC analysis of the chloroform extract showed at least six distinct fractions, which were visualized by vanillin/sulfuric acid at 110 °C (Fig. 1). The antifungal activities of each of the fractions were determined against *A. niger* (ATCC 1624) by a disk diffusion method (Table II). Bioactivity-guided fractionation of this extract led to the isolation of one compound (see Fig. 2). The active component of the

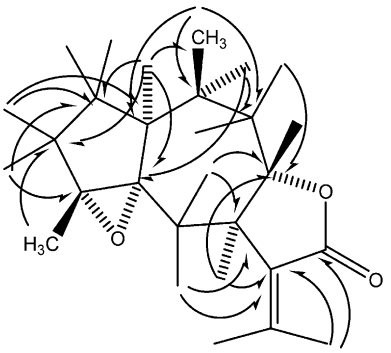

Fig. 3. Important HMBC NMR correlations observed for compound **1.**

Table III. NMR data of compound **1**.

No.	^{1}H	^{13}C
1	2.63 (*d*, 1H, *J* = 8.9)	48.1
2	1.30 (*m*, 2H)	30.1
3	1.74 (*m*, 1H)	33.1
	1.93 (*m*, 1H)	
4	–	70.4
5	–	70.2
6	1.93 (*m*, 1H)	31.0
	2.13 (*d*, 1H, *J* = 15.6)	
7	3.06 (*m*, 1H)	44.8
8	4.10 (*m*, 1H)	83.0
9	2.34 (*m*, 1H)	40.8
	1.85 (*m*, 1H)	
10	2.13 (*d*, 1H, *J* = 15.6)	35.0
11	–	139.5
12	–	170.4
13	6.27 (*d*, 1H, *J* = 3.3)	120.2
	5.54 (*d*, 1H, *J* = 3.0)	
14	0.99 (*d*, 3H, *J* = 7.4)	15.0
15	1.42 (*s*, 3H)	15.9

total extract involved in the antifungal activity had an R_f value of 0.47. The compound's structure was confirmed by ^{1}H NMR, ^{13}C NMR and 2D NMR spectra (Figs. 2 and 3). The spectral data (^{1}H NMR, ^{13}C NMR) of this antifungal compound revealed (Table III) that it was 4α,5α-epoxy-10α,14*H*-1-epi-inuviscolide (**1**): Gum; EI-MS: *m/z* (%) = 248 [M+](100), 233(11), 207(56), 203(82), 189(51), 133(54), 109(52), 95(53), 81(45), 55(46); IR (KBr): ν_{max} = 3434, 3017, 2923, 1737, 1455, 1360, 1241, 1147, 1038, 750 cm^{-1}. The identification was carried out by comparing the spectroscopic data to those reported in the literature (Mossa *et al*., 1997).

The inhibitory effect of **1** against numerous test organisms is also shown in Table I. As shown in the table, **1** represented the most potent antimicrobial candidate, with MIC values of $< 25\,\mu$g/disk against *A. niger* strains and 200 μg/disk against *B. cereus and S. aureus*. However, this sesquiterpene lactone from *P. divaricatum* showed no activity at a concentration of 400 μg/disk against the strains *A. fumigatus, E. coli* or *P. aeruginosa* (Table I). The chloroform and ethanol extracts of *F. persica* exhibited weaker activity against all test strains and, in particular, against *C. albicans* and *P. aeruginosa* even at a higher tested concentration (8000 μg/disk). Most of these crude extracts' antimicrobial activity was observed against filamentous fungi and Gram-positive bacterial test strains.

Acknowledgements

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