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Antimicrobial Effects of Ocotillone Isolated from Stem Bark of *Ailanthus altisshima*

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Abstract Bioassay-directed chromatographic fractionation of a methylene chloride extract of *Ailanthus altisshima* indicated the presence of 20(*S*), 24(*R*), epoxy-25-hydroxydammarane-3-one (compound **1**, ocotillone) which was isolated from this plant, for the first time. Antimicrobial activity of compound **1** was measured by inhibition of bacterial and fungal cells growth and by a hemolytic assay with human erythrocytes, respectively. The results revealed that compound **1** had potent antibacterial activity against Gram-negative bacteria, *P. aeruginosa* and *S. typhimurium*, that were without hemolytic activity, whereas it had weak antimicrobial activity against Gram-positive bacteria and fungi. These results demonstrated that the compound **1** has more antibacterial activity against Gram-negative bacteria, which have no hemolytic activity, than Gram-positive bacteria and fungi. This is the first report on the biological activities of the compound **1**.

Key words: Antibiotic activity, *Ailanthus altisshima*, ocotillone

Antibiotics have become indispensable in the modern health care system, assisting and complementing the natural immune system. However, with the appearance of resistant strains, a continuous search for more potent and efficient antibiotic agents is necessary. By taking advantage of a long experience in the use of herbal medicines, the development of new drugs derived from herbal medicines may avoid side effects or toxicities that synthetic drugs might have. In a search for antibacterial compounds from natural products, an antibacterial assay was carried out on about 83 species of plants [1, 9]. The results showed that crude methylene chloride extracts of the stem bark of *A. altisshima* had antibacterial activity.

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The stem bark of *Ailanthus altisshima* Swingle (Simaroubaceae) was purchased from Kyoung-Dong Herb Market in Seoul, Korea in 2000, and authenticated by our Pharmacognosy Department. Voucher specimens were deposited in the Herbarium of the College of Pharmacy, Chosun University (773-15). The methanol extract of the stem bark of *A. altisshima* was successively extracted with CH_2Cl_2, EtOAc, and *n*-BuOH. The concentrated CH_2Cl_2-soluble phase showed antibacterial activity against Gram-negative bacteria. The CH_2Cl_2 extract was purified by repeated column chromatography over Silica gel, Sephadex LH 20, and LiChrosorb RP18 to give a compound designated **1**.

The HR-FABMS and NMR spectra were measured using a JMS 700 (JEOL) and Varian Unity Inova 500 (500 MHz) spectrophotometer, respectively. Compound **1** was obtained as an amorphous powder. The HR-FABMS of **1** showed a molecular ion peak at *m/z* 481.3660 $(M+Na)^+$, which provided the elemental formula $C_{30}H_{50}O_3$ (calcd. 481.3658). The IR spectrum showed absorption bands at 3,400 (OH) and 1,710 cm^{-1} (C=O). The NMR spectra, including ^1H NMR, ^{13}C NMR; Distortionless Enhancement of Polarization Transfer (DEPT) [6], Heteronuclear Multiple Quantum Coherence (HMQC) [2], and Heteronuclear Multiple Bond Correlation (HMBC) [3] were all measured in a $CDCl_3$ solvent system. The ^1H NMR spectrum showed that **1** had a 3-oxo dammarane type triterpene skeleton: eight angular methyl peaks (δ 0.88, δ 0.94, δ 0.99, δ 1.04, δ 1.12, δ 1.14, δ 1.21), the methylene peak neighboring a C-3 carbonyl group (δ 2.45), and there was an oxymethine peak (δ 3.73, *t*, *J*=7.5 Hz). The ^{13}C NMR and DEPT spectrum of **1** revealed thirty carbon signals: one carbonyl (δ 218.11), ten methylenes, five methines [one of them bearing an oxygen atom (δ 83.81)], seven quaternary carbons [two of them bearing an oxygen atom (δ 86.36, δ 71.43)], and eight methyl carbons. In the HMQC spectrum, eight angular methyl carbons at δ_C 15.12, 16.04,

Table 1. Antibacterial activity of compound **1**.

	MIC : μM			
	Gram positive		Gram negative	
	B. subtilis	*S. epidermidis*	*P. aeruginosa*	*S. typhimurium*
Compound **1**	25	50	6.25-12.5	6.25-12.5
Melittin	0.39	1.56	1.56	<0.097

16.35, 20.99, 23.57, 24.27, 26.71, 27.47 gave correlations with eight methyl proton signals at δ_H 1.08 (*s*, H-19), 0.94 (*s* , H-18), 0.99 (*s*, H-30), 1.04 (*s*, H-29), 1.12 (*s*, H-21), 1.14 (*s*, H-26), 1.21 (*s*, H-27), and 0.88 (*s*, H-28), respectively, and δ_C 83.31 gave a correlation with δ_H 3.73 (*t*, *J*=7.5 Hz, H-24). In the HMBC spectrum, the carbon signal (δ 218.11) showed ^{1}H-^{13}C long-range correlations with H-1, H-2, and H-29. Comparing these ^{1}H-NMR and ^{13}C-NMR data with those reported in the literature, compound **1** was identified as 20(*S*), 24(*R*), epoxy-25-hydroxydammarane-3-one (ocotillone), which had been previously isolated from *Panax pseudo-ginseng* subsp. *himalaicus* by Tanaka *et al.* [7-8, 13-16, 20, 21].

The antibacterial activity of compound **1** was measured. *Pseudomonas aeruginosa* (KCTC 1637) and *Salmonella typhimurium* (KCTC 1926) were supplied from the Korea Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience & Biotechnology (KRIBB) in Taejon, Korea. Melittin, a honeybee venom toxin, was used as a positive control, since it has been reported to possess a potent antimicrobial activity with a broad spectrum [10-11, 18]. *B. subtilis*, *S. epidermidis*, *P. aeruginosa*, and *S. typhimurium* were grown to the mid-phase in a medium (g/l) [10 bactotryptone/5 yeast extract/10 NaCl (pH 7.0)]. Compound **1** was filtrated through a 0.22 μm filter and stepwise-diluted in a medium of 1% bactopeptone. The tested organism [final bacterial suspension: 2×10^6 colony forming units (CFU)/ml], suspended in growth medium (100 μl) was mixed with 100 μl of a two-fold diluted serial compound **1** solution in a microtiter-well plate with three replicates. The serially diluted sample solution (100.0, 50.0, 25.0, 12.50, 6.25, 3.125, 1.56, 0.78 μM) of compound **1** or melittin were added to each well, and the cell suspension was incubated for 18 h at 37°C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of sample which gave no visible growth on the plate [19]. In order to measure the antifungal activity, the fungal strains, *Saccharomyces cerevisiae* (KCTC 7296), *Trichosporon beigelii* (KCTC 7707), and *Candida albicans* (TIMM 1768), were separately seeded on 96-well plates (NUNC, U.S.A.) at a density of 2×10^4 cells per well in a volume of 100 μl of YPD media. *S. cerevisiae* and *T. beigelii* were obtained from the KCTC of the KRIBB. *C. albicans* was obtained from the Center for Academic Society in Osaka, Japan. To these fungal cells were added 100 μl each of serially diluted compound **1** or melittin, and the cell suspension was incubated for 24 h at 30°C. After incubation, 5 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (5 mg/ml MTT in PBS, pH 7.4) was added to each well, and the plates were incubated at 37°C for another 4 h. The optical density of each well was measured at 580 nm by a microtiter ELISA reader (Molecular Devices Emax, U.S.A.) [12].

Table 2. Antifungal activity of compound **1**.

	C. albicans	*T. beigelii*	*S. cerevisiae*
Compound **1**	N.D.	N.D.	N.D.
Melittin	3.125	3.125-6.25	3.125

The hemolytic activity of compound **1** was evaluated by determining the released hemoglobin from 8% suspensions of fresh human erythrocytes at 414 nm [4]. Human red blood cells were centrifuged and washed three times with PBS (PBS: 35 mM phosphate buffer/0.15 M NaCl, pH 7.0). One hundred μl of human red blood cells suspended in 8% (v/v) PBS were plated into 96-well plates, and then 100 μl of compound **1** or melittin were added to each well. The plates were incubated for 1 h at 37°C and centrifuged at 150 ×*g* for 10 min. One hundred μl aliquots of the supernatant were transferred to the 96-well plates. Hemolysis was measured by absorbance at 414 nm with an ELISA plate reader (Molecular Devices Emax, Sunnyvale, CA, U.S.A.). Zero percent and 100% hemolysis were determined

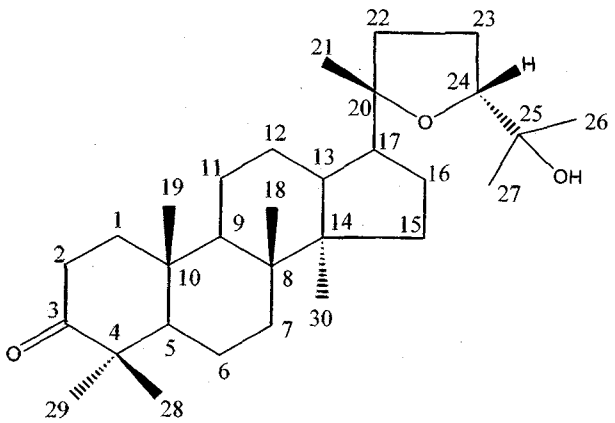

Fig. 1. Chemical structure of compound **1** isolated from *Ailanthus altisshima*.

Table 3. Hemolytic activity of compound **1**.

	% Hemolysis (μM)							
	100	50	25	12.5	6.25	3.125	1.56	0.78
Compound **1**	0	0	0	0	0	0	0	0
Melittin	100	100	100	100	100	93	63	28

in PBS and 0.1% Triton X-100, respectively. The hemolysis percentage was calculated using the following equation:

% hemolysis
=[($Abs_{414\,nm}$ in the peptide solution−$Abs_{414\,nm}$ in PBS)
/(Abs_{414nm} in 0.1% Triton-X 100-$Abs_{414\,nm}$ in PBS)]×100.

The antimicrobial activity of compound **1** (ocotillone) is summarized in Tables 1 and 2. Although compound **1** showed negligible activity against Gram-positive bacteria and fungi, it showed potent antibacterial activity against Gram-negative bacteria. The MIC value of compound **1** for *P. aeruginosa* and *S. typhimurium* were 6.25−12.5 μM, whereas it was 25−50 μM for *B. subtilis* and *S. epidermidis*. These results indicate that compound **1** is about 4-fold more potent in antibacterial activity against Gram-negative bacteria than against Gram-positive bacteria, but less potent than melittin which was used as a positive control. It is almost equivalent in activity to cefotaxime, which showed a broad-spectrum of antibiotic activity [5]. The hemolysis at various concentrations of compound **1** was measured against human erythrocyte cells, however, compound **1** showed no hemolytic activity, while melittin exhibited strong hemolytic activity (Table 3). These results demonstrate that compound **1** has a remarkable antibacterial activity against Gram-negative bacteria with no hemolytic activity.

Most of the bacterial infections caused by Gram-negative bacteria were generally regarded as more troublesome than those caused by Gram-positive bacteria. In Gram-positive bacteria, there is a single membrane system, which encloses the cytoplasm, but the membrane is double in most Gram-negative bacteria [17]. The inner and outer membrane of Gram-negative bacteria prevents the influx of drug into the bacteria. Compound **1** (ocotillone), which showed considerable selectivity against Gram-negative bacteria, with no hemolytic activity, might possibly be developed as a leading antimicrobial compound.

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