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Antibacterial Activity of Continentalic Acid from *Aralia continentalis*

Ja-Young Kim · Seo-Jin Oh · Seung-Il Jeong
Dept. of Herbology, College of Oriental Medicine, Woosuk University

獨活(*Aralia continentalis*)추출물 Continentalic Acid의 항균활성 연구

김자영 · 오서진 · 정승일

독활(Aralia continentalis)로부터 항균활성 물질을 찾아내기 위한 일환으로 항균실험과 분리실험을 병행하여 실시하였다. 항균활성물질 분리는 독활을 클로로포름으로 추출하여 실리카겔(SiO_2)과 분취용액체크로마토그래피(prep-HPLC)법으로 2종의 화합물을 분리하여 핵자기공명(NMR) 등 분광학적인 기법으로 이용하여 구조동정을 하였다. 이때 2종의 화합물은 (-)-pimara-8(1),15-diene-19-oic acid와 (24E)-stagmasta-5,22-dien-3β-ol임이 확인되었다. 2종의 화합물에 대한 메티실린 내성 황색포도상구균(MRSA) 및 메티실린 감응 황색포도상구균(MSSA)의 표준균주와 임상분리균주(MRSA)에서의 최소엑제농도(MIC)가 8-16μg/mL로 나타났다. 그러므로 본 연구의 결과로부터 화합물 (-)-pimara-8(1),15-diene-19-oic acid은 항생제 내성균에 대한 치료제로서 개발 가능성을 확인할 수 있었다.

Key words : Aralia continentalis, Antibacterial Activity, Continentalic Acid

Ⅰ. Introduction

Staphylococcus aureus (S. aureus) is one of the most important human pathogen, causing suppuration, pneumonia, septicemia, endocarditis and osteomyelitis (Pan et al., 2002). S. aureus was associated with a high mortality rate in the preantibiotic era, and then was proved to be susceptible to the earliest antimicrobial substance (Romero Vivas et al., 1995; You et al., 1999). Staphylococcal resistance to wide spectrum β lactam antibiotics, such as methicillin and oxacillin, emerged soon after the introduction of the first drug in this class and there has been a steady rise in the incidence of methicillin resistant S. aureus (MRSA) clinical isolates. Particularly worrying is the increasing in incidences of multidrug resistant organisms

교신저자 : 김자영, 전북 완주군 삼례읍 후정리 490 우석대학교 대학원 한의학과 본초학교실
(Tel : 063-290-1561, E-mail : jayashin@hanmail.net)
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such as MRSA. Therefore, new agents are needed to treat the MRSA associated infection. To meet this need, several natural products are candidates to be new antibiotic substances (Hur et al., 2004; Kim et al., 2004; 2005).

On the other hand, Aralia continentalis Kitagawa (Araliaceae), known as 'Dokwhal' in Korea, has been widely used in traditional Korean medicine to treat analgesia, antirheumatic, neuralgia, and as a cure for arthralgia, rheumatism, lumbago and lameness (Kim et al., 1998). In addition, there are several reports, suggesting that A. continentalis have pharmacological properties to analgesic and anti inflammation (Han et al., 1983; Okuyama et al., 1991; Park et al., 2005). However, little is known about the antibacterial effects of A. continentalis on MRSA. In the course of our ongoing project on the detection of bioactive compounds from medicinal plants, the $CHCl_3$ soluble extract of roots of A. continentalis was found to exhibit distinctive antimicrobial activity at the 16 μg/mg level. Bioassay directed further purification of the extract by various chromatographic methods afforded the active compound (continentalic acid, Fig. 1). Details of the isolation and biological activities of this compound will be discussed.

II. Materials and Methods

General. Optical rotation was measured on a Bellingham and Stanley ADP 2000 polarimeter. IR spectra was recorded on an FT IR spectrophotometer. NMR spectrum was recorded in $CDCl_3$ on a Bruker AVANCE 300 and 500 MHz spectrometers. Chemical shifts values (δ) are reported in part per million (ppm) relative to NMR solvent $CDCl_3$ (δ H=7.20, δC=77.0). Coupling constants (J values) are given the Hertz. 1H 1H COSY, HMBC and HMQC experiments were recorded with gradient enhancements using sine shaped gradient pulses. ESI mass spectra was obtained on a Macro Mass Quatro LC with electro spray ionization method. TLC was performed using Kieselgel 60 F254 (Merck) pre coated plates and spots were visualized by spraying with vanillin sulfuric acid spray and followed by heating.

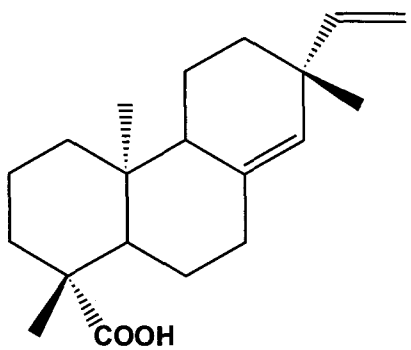
Plant materials. The plant materials, roots of A. continentalis were collected in Imsil, Korea, in November 2004. A voucher specimen (No. JSI 53) has been deposited in Dept. of Herbology, College of Oriental Medicine, Woosuk University.

Extraction and isolation. The air dried roots (3.6 kg) of A. continentalis were crushed and extracted three times with MeOH under reflux. The MeOH extract was concentrated, suspended in H_2O and sequentially partitioned with $CHCl_3$, EtOAc and n BuOH. The bioactive $CHCl_3$ soluble fraction (60 g) was subjected to silica gel (Merck Kieselgel 60; 0.063 0.2 mm particle size; 3×20 cm) column chromatography. The column was eluted with a 10:1 (1 L), 5:1 (0.5 L), 2:1 (0.5 L) and 1:1 (0.5 L) mixture of n hexane EtOAc (84

fraction of 30 mL), followed by EtOAc (10 fraction of 250 mL). Fractions of similar composition as determined by TLC analysis were pooled. Further purification of the fraction numbers 14 21 (elution volume: 240 mL) and 46 49 (elution volume: 120mL) using recycling preparative HPLC [1H column, $CHCl_3$, 3 mL/min, detection at 254 nm] yielded compound 1(328 mg, tr = 58.2 min) and . The structures of ( ) pimara 8(14),15 diene 19 oic acid (continentalic acid) {[α]D25: 120.1° (c 0.8, $CHCl_3$)} and 24E stigmasta 5,22 dien 3β ol (stigmasterol) {[α]D25: 48.3° (c 0.28, $CHCl_3$)} were identified by the comparison of its spectral data (MS, 1D NMR, and 2D NMR) with those in the literature (Han et al., 1983). Copies of the original spectra for compound are obtainable from the author of correspondence.

Preparation of bacterial cells. Staphylococcal strains listed in Table 1 were 12 clinical isolates (MRSA) from Wonkwang University Hospital and the standard strain of methicillin resistant Staphylococcus aureus (MRSA) ATCC 33591 and Staphylococcus aureus ATCC 25923, which is methicillin susceptible Staphylococcus aureus (MSSA). The MRSA strains were defined on the basis of the occurrence of the mecA gene Standards (NCCLS, 1997). After culturing all strains on Mueller Hinton agar (Difco, Detroit, MI), the cells were resuspended in Mueller Hinton broth (Difco) to give 108 colony forming units/mL; the resuspended cells were then incubated.

Detection of *mecA* gene. Detection of the mecA gene in strains of MRSA and MSSA was performed by PCR amplification. Total genomic DNA was obtained from Staphylococcus aureus by the phenol chloroform extraction method as described earlier in previous report (Tsen and Chen, 1992). Bacteria collected from 5 mL of the 18 h culture in Mueller Hinton broth were used for DNA extraction after treatment with lysostaphin and RNase (Sigma Chemical Co., St. Louis, MO, USA). The PCR assay was performed in a DNA thermal cycler, GeneAmp



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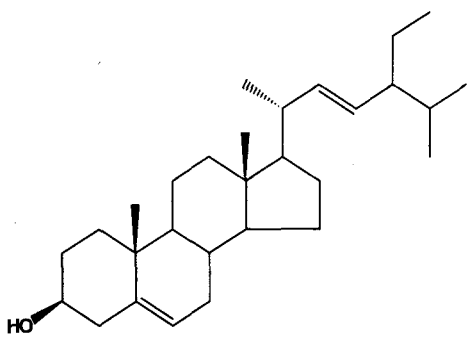

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Figure 1. The structure of compound 1 and 2 isolated from roots of A. continentalis.

PCR system 9700 (PE Applied Biosystems, Mississauga, Ont., Canada), by using a Gene Taq Amplifying Kit (Wako Pure Chemicals Industries Ltd., Japan), according to the manufacturer' s recommendations. Synthetic oligonucleotides used as primers were 5′ ATGAGATT AGGCATGTCGTTCC 3′ and 5′ TGGATGACGTACCTGAGCC 3′ (Ryffel et al., 1990).

Determination of anti MRSA activity. All strains were grown in tryptic soy broth for 24 h at 37℃. After incubation, they were diluted with the same medium to give a concentration of approximately 108 colony forming units (CFU/mL). Isolated compound was dissolved in dimethyl sulfoxide (DMSO) and 2 fold serial dilutions were made, and added to tryptic soy agar plates. Bacterial cell suspensions were inoculated onto the plates using a bacteria planter (5 μL). The final inoculum of CFU inoculated onto the agar plates was 5×105 for all strains. Inoculated plates were incubated at 37℃ for 24 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which no colonies were observed after incubation. The agar plate containing only DMSO served as a control.

Ⅲ. Results and Discussion

Infection caused by methicillin resistant Staphylococcus aureus (MRSA) in compromised hosts poses a serious problem all over the world, because MRSA strains are resistant to many antibiotics in the hospital environment. The emergence of multi drug resistance in pathogenic bacteria has created an urgent need for new antibiotics and new approaches to the treatment of bacterial infections. Natural products from plants and microorganisms traditionally have provided many useful compounds leading to new drugs and medicine (Sato et al., 2000; Gibbons et al., 2003). Hence, a research program was embarked on to search for new biologically active compounds against antibiotic resistant bacteria. The 80% MeOH extract of dried roots of A. continentalis was fractionated into hexane, chloroform, ethyl acetate and n butanol soluble fraction. Among them, only the chloroform extract showed notable antibacterial activity against the MRSA strains.

Therefore, aiming to identify the active substances, the chloroform fraction was submitted to column chromatography in silica gel using eluent mixtures of hexane and ethyl acetate of increasing polarity. A needle white compound was purified by repeated recycling prep HPLC as the active constituent against the bacteria. The assignments of the 1H and 13C NMR chemical shift values of compound were based on the HMQC and HMBC correlations.

On the basis of the foregoing findings (Han et al., 1983; Kang et al., 1996), compound 1 was determined to be a (-) pimara 8(14),15 diene 19 oic acid, named continentalic acid. Also, compound 2 was determined to be a 24E stigmasta 5,22 dien 3β ol, named stigmasterol.

Table 1. Correlation of carriage of the mecA[a] gene and MICs of the ampicillin and methicillin against 12 S. aureus strains, standard MSSA and MRSA strains

Strains	mecA[a]	MIC (μg/mL)	
		Ampicillin	Methicillin
S. aureus(ATCC 25923)		0.125	0.031
S. aureus(ATCC 33591)	+	32	8
78	+	256	128
M11	+	256	256
21-8	+	256	64
6-2	+	256	128
7-3	+	256	64
8-4	+	256	256
9-5	+	256	64
13-7	+	128	64
27-9	+	128	32
47-10	+	512	16
105-13	+	256	64
106-14	+	128	64

a[+], mecA[a] positive; −, mecA[a] negative.

Table 2. MICs of the MeOH extract, $CHCl_3$ extract and continentalic acid (CA) and stigmasterol (SM) isolated from roots of A. continentalis against clinical isolates of 12 MRSA, standard MSSA and MRSA strains

Strains	MIC (μg/mL)				
	class	MeOH extract	$CHCl_3$ extract	CA	SM
S. aureus (ATCC 25923)		32	16	8	32
S. aureus (ATCC 33591)	+	32	16	8	32
MRSA 1	+	>512	128	16	64
MRSA 2	+	>512	512	16	32
MRSA 3	+	256	128	16	32
MRSA 6	+	64	64	16	32
MRSA 7	+	>512	128	16	64
MRSA 8	+	256	128	16	32
MRSA 9	+	512	256	16	32
MRSA 10	+	256	256	16	64
MRSA 11	+	32	32	16	32
MRSA 12	+	32	32	16	64
MRSA 13	+	>512	512	16	32
MRSA 14	+	64	128	8	32
MRSA 15	+	>512	512	8	32

Table 1 shows MICs of ampicillin and methicillin against S. aureus. Among 14 stains used in the present study, the standard strain of S. aureus ATCC 33591 and 12 strains were mecA positive and one standard strain of S. aureus ATCC 25923 was mecA negative. Each of the clinical isolated strains was highly resistant to ampicillin greater than methicillin and showed MICs equal to or greater than 16 μg/mL, indicating that MICs of ampicillin and methicillin against standard MSSA and MRSA were 0.125 and 0.031 μg/mL, respectively. MICs of the MeOH extract, $CHCl_3$ extract and the authentic compound CA against S. aureus are summarized in Table 2. As shown in Table 2, the chloroform extract of A. continentalis demonstrated a higher inhibitory activity (MIC: 16 μg/mL) against MSSA. Because of the bioactive constituents involved in the $CHCl_3$ extract, the antibacterial potency of the $CHCl_3$ extract was a remarkable improvement on levels nearly the same as those achieved by CA. Our findings indicate that this compound was uniformly active against all strains of MRSA. Lately, another structural isomer of isopimaric acid (IPA) was reported from the immature cones of Pinus nigra (Smith et al., 2005). IPA showed activity against a multidrug resistant (MDR) and a MRSA standard. The minimum inhibitory concentrations (MICs) were 32 64 μg/mL and compared with isolated CA, MICs of 8 16 μg/mL. The only difference between CA and IPA is the position of the double bond in the skeleton.

However, there is no previous report on the inhibitory effects on MRSA of continentalic acid in the literature. In addition, the present structure activity relationship of phytochemical diterpenic acid will provide an important indication to perform such studies, potentially leading to the development of anti MRSA agents in the future. Our findings suggest that CA is potentially useful both alone and as a combination agent in the phytotherapeutic strategy against MRSA infections.

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