

Article

Antibacterial Activity of Phytochemicals Isolated from *Atractylodes japonica* against Methicillin-Resistant *Staphylococcus aureus*

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) has been emerging worldwide as one of the most important problems in communities and hospitals. Therefore, new agents are needed to treat acute oral infections from MRSA. In this study, antibacterial compounds from the roots of *Atractylodes japonica* (*A. japonica*) were isolated and characterized. The compounds were isolated from the root extracts using HPLC-piloted activity-guided fractionations. Four *A. japonica* compounds were isolated and identified as atractylenolide III (**1**), atractylenolide I (**2**), diacetylatractylodiol [(6*E*,12*E*)-tetradeca-6,12-diene-8,10-diyne-1,3-diol diacetate, TDEYA, **3**], and (6*E*,12*E*)-tetradecadiene-8,10-diyne-1,3-diol (TDEA, **4**), which was obtained by hydrolysis of TDEYA. The minimum inhibitory concentrations (MICs) was determined in the setting of clinical MRSA isolates. Compound **4** showed anti-MRSA activity with a MIC value of 4-32 µg/mL. The overall results provide promising baseline information for the potential use of the extract of *A. japonica* as well as some of the isolated compounds in the treatment of bacterial infections.

Keywords: *Atractylodes japonica*; MRSA; MIC/MBC

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) accounts for a large proportion of hospital-acquired infections and is considered a serious problem because of its multi-drug resistant properties. Currently, vancomycin and its analog teicoplanin are the most effective antibiotics for MRSA infections, but their clinical use often results in unexpected side effects and the development of vancomycin-resistant *S. aureus* infections. The search for better drugs to combat MRSA infection is urgently needed.

Atractylodes japonica (Compositae) has traditionally been used for the treatment of water retention in the body. Administration of the aqueous extract of *Atractylodes japonica* to humans causes diuresis and its alcohol extract also shows a diuretic effect in mice [1,2]. *Atractylodes japonica* is known to be effective for the control of pain and treatment of arthritis. It was reported that the *Atractylodes* family possesses anti-inflammatory activity [3], and modulates the intestinal immune system [4]. Atractylon, a major component, and its derivatives isolated from the rhizome were shown to have antihepatic properties [5]. The sesquiterpenoid diacetyl atractylodiol and its derivatives were isolated from the non-polar fraction [2]. Recently, it was reported that *A. japonica* root extract protects osteoblastic MC3T3-E1 cells against hydrogen peroxide-induced inhibition of osteoblastic differentiation [6]. However, the antibacterial effects of *A. japonica* on MRSA has not been evaluated. In the course of our ongoing project on the detection of bioactive compound from medicinal plants, the CHCl₃-soluble extract of roots of *A. japonica* was found to exhibit distinctive antibacterial activity.

2. Results and Discussion

Today, with the emergence of antibiotic-resistant pathogens like MRSA, a new approach to natural products must be taken. These natural products are more and more in demand due to their benefits without side effects. Therefore, our ongoing efforts to find bioactive natural products have led us to study the antibacterial activity of *A. japonica*, which has been known to possess a variety of pharmacological properties against arthritis, bronchitis and respiratory infectious disease and to contain more than 50 phytochemicals, including atractylon and its derivatives [7,8], sesquiterpenoids [9] and diacetyl atractylodiol including its derivatives [10,11]. To elucidate the antibacterial effects of *A. japonica*, the methanol extracts of *A. japonica* rhizome were fractionized with *n*-hexane, CHCl₃, EtOAc, and *n*-BuOH. The fractions were tested for MIC determination using the microdilution broth method. The results were recorded as MIC values in Table 1. The CHCl₃ fraction of *A. japonica* roots showed good antibacterial effects against two strains of *S. aureus*. The MIC of CHCl₃ fraction shows equal efficacy to ampicillin at 32 µg/mL against ATCC 33591. This is an encouraging result in regards to the ability of MRSA to be resistant to most antibiotics. By further purifying the fractions, compounds **1**, **2** and **3** were isolated from the CHCl₃ fraction and were confirmed to be atractylenolide III (**1**), atractylenolide I (**2**), and (6*E*,12*E*)-tetradeca-6,12-diene-8,10-diyne-1,3-diol diacetate (diacetyl atractylodiol, TDEYA, **3**), respectively, by the comparison of their spectral data with those in

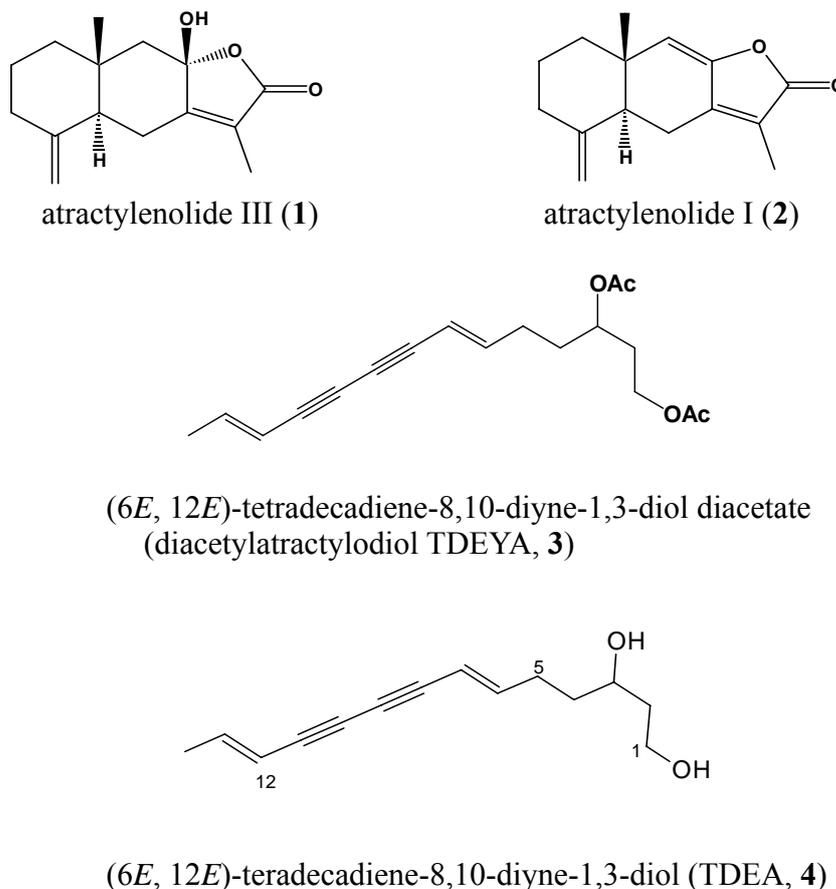
the references [12]. Also, (6*E*,12*E*)-tetradecadiene-8,10-diyne-1,3-diol (TDEA, **4**) [11] was obtained by hydrolysis of TDEYA (**3**) (Figure 1).

Table 1. Antimicrobial activity of *A. japonica* root MeOH extract and *n*-hexane, CHCl₃, EtOAc, and *n*-BuOH fractions against *S. aureus* (ATCC 33591, ATCC 25923) strains.

<i>S. aureus</i> strain	MIC (μg/mL)					
	MeOH	Hexane	CHCl ₃	EtOAc	BuOH	Ampicillin
ATCC 33591	64	128	32	128	128	32
ATCC 25923	64	64	32	64	128	0.125

The isolated compounds after were tested against different strains of *S. aureus* as shown in Table 2. Among the obtained isolates, TDEA (**4**) showed antibacterial activity with MICs ranging from 4 to 32 μg/mL independently.

Figure 1. Chemical structures of isolated compounds from roots of *A. japonica*: atractylenolide III (**1**); atractylenolide I (**2**); (6*E*, 12*E*)-tetradecadiene-8,10-diyne-1,3-diol diacetate (diacetylatractylodiol TDEYA, **3**); (6*E*, 12*E*)-tetradecadiene-8,10-diyne-1,3-diol (TDEA, **4**).



Also, we investigated phytochemical profiles in *A. japonica* after separation using the developed Smart HPLC method. Typical chromatograms of the MeOH extract of *A. japonica* roots are shown in

Table 2. The MICs/MBCs of *A. japonica* CHCl₃ fraction (AJCH), atractylenolide III (**1**), atractylenolide I (**2**), (6*E*,12*E*)-tetradecadiene-8,10-diyne-1,3-diol diacetate (**3**) and (6*E*,12*E*)-tetradecadiene-8,10-diyne-1,3-diol (**4**), and ampicillin (AM) against *S. aureus* strains.

<i>S. aureus</i> strain	Class	<i>mecA</i> gene	MIC(μg/mL)					
			AJCH	1	2	3	4	AM
ATCC 25923	MSSA	-	32	64	64	32	16	0.12
ATCC 33591	MRSA	+	32	128	256	64	16	32
<i>Clinical isolates</i>								
<i>S. aureus</i> 78	MRSA	+	64	64	64	64	8	256
<i>S. aureus</i> M11	MRSA	+	64	64	64	16	8	256
<i>S. aureus</i> 21-8	MRSA	+	64	32	32	128	4	256
<i>S. aureus</i> 6-2	MRSA	+	128	64	64	64	8	256
<i>S. aureus</i> 7-3	MRSA	+	64	64	64	64	32	256
<i>S. aureus</i> 8-4	MRSA	+	64	128	128	64	16	256
<i>S. aureus</i> 9-5	MRSA	+	256	128	128	32	16	256
<i>S. aureus</i> 13-7	MRSA	+	32	32	32	32	4	128
<i>S. aureus</i> 27-9	MRSA	+	64	32	128	64	8	128
<i>S. aureus</i> 47-10	MRSA	+	32	32	32	64	16	512
<i>S. aureus</i> 105-13	MRSA	+	32	8	8	64	4	256
<i>S. aureus</i> 106-14	MRSA	+	32	16	16	32	4	128

(+) positive, (-) negative

3. Experimental

3.1. Plant material

The fresh roots of *Atractylodes japonica* Koidz (Family Compositae) were collected in July 2009 at Jinan, Jeonbuk province, Korea. The plant was authenticated by Prof. Hong-Jun Kim, College of Oriental Medicine, Woosuk University. A voucher specimen (No. JSI 62) was deposited in the Herbarium of College of Oriental Medicine, Woosuk University.

3.2. Extraction and isolation of test material

Air-dried and powdered roots of *A. japonica* (3.0 kg) were extracted with two portions of MeOH (10 L) for one week at room temperature. To give 319g of dark-brown extract, which was suspended in water. The suspension was extracted with hexane, CHCl₃, EtOAc and *n*-BuOH, respectively, and the organic layer was concentrated to dryness *in vacuo* to give the hexane (48 g), CHCl₃ (62 g), EtOAc (23g), and *n*-BuOH fractions (61 g). The CHCl₃ soluble portion (42 g) was chromatographed on silica gel (6 cm i.d. × 50 cm, 1.5 kg) eluted stepwise with a mixture of EtOAc and *n*-hexane (16:1 → 8:1 → 4:1 → 2:1 → 0:1, each 800 mL), yielding seven fractions (A1-A7). Fraction A1 was purified by prep-LC JAI gel W252 column; 100% MeOH; flow rate 3.5 mL/min; UV detection at 254 nm) to give **1** (15 mg). Fraction A3 was also applied to a prep-LC (JAI gel W252 column; 100% MeOH; flow rate 3.5 mL/min; UV detection at 254 nm) to give **2** (30 mg) and **3** (12 mg). These compounds were identified as atractylenolide III (**1**), atractylenolide I (**2**), and (6*E*,12*E*)-tetradeca-6,12-diene-8,10-diyne-1,3-diol diacetate (diacetyl-atractylodiol, TDEYA, **3**) [12]. Compound **4** ((6*E*,12*E*)-tetradecadiene-8,10-diyne-1,3-diol, TDEA) [11] was obtained by hydrolysis of **3** (see Figure 1).

3.3. Hydrolysis of TDEYA(3)

A solution of **3** (20 mg) in 3% K₂CO₃(cat.)/MeOH (7 mL) was heated at 60 °C for 10 min. After cooling, the solution was neutralized with 1 N HCl, and MeOH was extracted with Et₂O and then washed with deionized water. The organic layer was dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to obtain compound **4** as a pale yellow amorphous powder (8 mg).

3.4. Chromatographic fingerprint conditions

A LC800 series (Smart LC, GL sciences, Tokyo, Japan) system equipped with binary solvent delivery pump, auto sampler, degasser, system oven and UV-visible detector was used to achieve Smart LC fingerprints. The chromatographic separation was carried out on a 2.1 mm × 50 mm, 2 μm particle, Innertsil ODS-4 C18 column (GL sciences, Japan) maintained at 40 °C. The mobile phase consisting a mixture 0.05% aqueous phosphoric acid and acetonitrile in the ratio of 34:66 (v/v) with flow rate of 0.5 mL/min was employed. The detector wavelength was monitored at 254 nm. All injection volumes of sample and standards were 1.5 μL.

3.5. Antibiotics

All antibiotics, including ampicillin was purchased from Sigma Chemical Co. (St Louis, MO, USA).

3.6. Bacterial strains

The 12 MRSA isolates used in this study were clinical isolates from Wonkwang University Hospital, Iksan, Jeonbuk, Korea. The MRSA strains were defined on the basis of the occurrence of the *mecA* gene and of their resistance to ampicillin and oxacillin, according to the 2009 guidelines of the Clinical and Laboratory Standards Institute (CLSI) [16]. The standard strains of MRSA ATCC 33591 and *S. aureus* ATCC 25923, which is a methicillin susceptible *Staphylococcus aureus* (MSSA), were used as control strains. After culturing all stains on Mueller-Hinton agar (MHA), all bacteria were resuspended in Mueller-Hinton broth (MHB) to give 10⁸ colony forming units/mL; the resuspended cells were then incubated.

3.7. Detection of *mecA* gene

Detection of the *mecA* gene in the MRSA strains was performed by polymerase chain reaction (PCR) amplification. For rapid extraction, one to five bacterial colonies were suspended in 300 L of cell lysis buffer and heated at 100 °C for 20 min. After centrifugation at 12,000 rpm for 10 min, 2 L of the supernatant was used for the DNA extraction. PCR reactions were performed using a MRSA Primer Mix Kit (Genotex Co., Korea). The PCR amplification consisted of 30 cycles (94 °C, 60 seconds; 55 °C, 60 seconds; 72 °C, 60 seconds). The primers used in this study were as follows: *mecA* forward primer, 5'-ATGAGATTAGGCATCGTTTC-3'; reverse primer, 5'-TGGATGACAGTACCTGAGCC-3' [14]. The final PCR products were separated 0.2% agarose gel.

3.8. Minimum Inhibitory Concentration (MIC)

The standard agar dilution method was used to determine the MIC of the antibiotics alone in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. To determine the MIC of *A. japonica* extract and isolated compounds, the specimen was initially dissolved in minimal amount of dimethyl sulphoxide followed by water to give a range of concentrations from 0.325% to 5% with a volume of 2 mL. Each samples were diluted to give a final concentrations from 0.0625% to 1% in each agar plate. Cell suspensions (1×10^4 colony-forming units/mL) of 12 MRSA isolates and standard strain (ATCC 25923 and ATCC 33591) were inoculated onto agar plates and then incubated at 35 °C for 18 h. The MICs were defined as the lowest concentration of a test compound that completely inhibited cell growth in visible.

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Sample Availability: Samples of the compounds atractylenolide I, atractylenolide III, and diacetyl-atractylodiol (TDEYA) are available from the authors.

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