

Antimicrobial Activity of Brown Alga *Eisenia bicyclis* against Methicillin-resistant *Staphylococcus aureus*

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Abstract

We screened for antibacterial substances against methicillin-resistant *Staphylococcus aureus* (MRSA). Methanolic extract of *Eisenia bicyclis* exhibited anti-MRSA activity according to a disk diffusion assay. To identify the active compound(s), the methanolic extract was further fractionated using hexane, dichloromethane, ethyl acetate, and *n*-butanol. The ethyl acetate-soluble fraction showed both the greatest anti-MRSA activity and the highest polyphenol content. The minimum inhibitory concentrations of the ethyl acetate fraction ranged from 32 to 64 µg per mL against methicillin-susceptible *S. aureus* and MRSA strains. High-performance liquid chromatography analysis revealed that both the methanolic extract and the ethyl acetate soluble fraction contained sizeable quantities of dieckol, which is a known anti-MRSA compound. Thus, these data strongly suggest that the anti-MRSA activity of *E. bicyclis* may be mediated by phlorotannins such as dieckol.

Key words: Anti-MRSA activity, *Eisenia bicyclis*, Methicillin-resistant *Staphylococcus aureus*

Introduction

Since methicillin was introduced in 1959 to resolve infections caused by penicillin-resistant *Staphylococcus aureus*, the incidence of methicillin resistance in staphylococci has increased rapidly (Kaplan, 2005). As a result of widespread methicillin use, methicillin-resistant *S. aureus* (MRSA) has become a major problem globally (Lee et al., 2008). MRSA infections are difficult to treat because of the multidrug-resistance properties of MRSA, which is resistant to β-lactams as well as several other classes of antibiotics (Bramley et al., 1989; Hiramatsu et al., 1997). Vancomycin is commonly used for the treatment of MRSA-related infection. Vancomycin resistance was discovered first in enterococci and later in staphylococci (Isnansetyo and Kamei, 2009). The emergence of vancomycin-resistant *S. aureus* has recently been recog-

nized and leaves physicians with few options available to treat MRSA infections. Therefore, much attention has been given to the search for new antimicrobial agents (Lee et al., 2008).

In an effort to decrease usage of vancomycin and discover an alternative therapeutic agent for treating MRSA infection, we have screened marine algae for anti-MRSA compounds. *Eisenia bicyclis* is a common perennial phaeophyceae (brown alga) that generally inhabits the coast of Ulleung Island in the East Sea of Korea. This seaweed is used in various dishes, including appetizers, casseroles, muffins, pilafs, and soups (Eom et al., 2011; Kim et al., 2011). The antioxidant activity of *E. bicyclis* phlorotannins such as eckol (a trimer), phlorofucofuroeckol A (a pentamer), dieckol, and 8,8'-bieckol (hexamers) has been reported (Okada et al., 2004). This brown algae

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also exhibits activity against tumors (Noda et al., 1989), Alzheimer's disease (Jung et al., 2010), atherosclerosis (Kang et al., 2003), inflammatory diseases (Shibata et al., 2003), allergic disease, and cancer (Shibata et al., 2002). However, to our knowledge, no study has reported on the antimicrobial activity of *E. bicyclis*. Here, we examined the antibacterial activity of *E. bicyclis* against several pathogenic bacteria, including MRSA.

Materials and Methods

Raw materials and extraction

In late September 2010, the brown seaweed *E. bicyclis* was gathered from Ulleung Island, Korea. Dried *E. bicyclis* was ground and then finely powdered using a food mixer (HMF-1000A; Hanil Electronics, Seoul, Korea). The dried powder was stored at -20°C until required. Powdered *E. bicyclis* (1 kg) was extracted with methanol (3 times × 10 L) for 3 h. The combined filtrate was concentrated by rotary evaporation at 40°C. After suspending in water (1 L), the methanol extract was partitioned with *n*-hexane (hexane), dichloromethane (DCM), ethyl acetate (EtOAc), and *n*-butanol (BuOH), in sequence.

Microorganisms and culture

Standard bacterial strains were obtained from the Korean Collection of Type Cultures (KCTC; Daejeon, Korea) and the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). The bacterial strains used were methicillin-susceptible *S. aureus* (MSSA; KCTC 1927), two MRSA strains (MRSA; KCCM 40510 and KCCM 40511), *Bacillus cereus* (KCTC 3624), *B. subtilis* (KCTC 1028), *Enterococcus faecalis* (KCTC 3206), *Escherichia coli* (KCTC 1682), *Listeria monocytogenes* (KCTC 3710), *Salmonella typhimurium* (KCTC 1925), and *Vibrio parahaemolyticus* (KCTC 2729). All strains were grown aerobically at 37°C in Mueller-Hinton broth (MHB; Difco, Detroit, MI, USA) or tryptic soy broth (TSB; Difco) for determination of the minimum inhibitory concentration (MIC) and in Mueller-Hinton agar (MHA; Difco) for disk diffusion and minimum bactericidal concentration (MBC) assays.

Disk diffusion assays

Antibacterial activity was evaluated by a disk diffusion assay, as described by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 2004). In brief, bacterial strains were cultured in TSB at 37°C until an OD₆₀₀ of 0.5. Bacterial culture (1 mL), containing approximately 10⁴ CFU, was spread on a MHA plate, and a paper disk (6 mm diameter) containing 1

mg extract was then placed on the agar surface. After incubation for 24 h at 37°C, the diameter of the inhibition zone was measured. The determination was performed three times and the mean values presented.

Minimum inhibitory concentrations (MIC)

The MIC is the lowest concentration of antimicrobials that will inhibit the visible growth of microorganisms after overnight incubation (Grierson and Afolayan, 1999). MICs of the extracts and vancomycin were determined by the twofold serial dilution method in MHB (NCCLS, 2003). MIC was defined as the lowest concentration of crude extract that inhibited visual growth after incubation at 37°C for 20-24 h, and was performed in triplicate (Grierson and Afolayan, 1999).

Minimum bactericidal concentrations (MBC)

The MBC value was defined as the lowest concentration of *E. bicyclis* extracts required for a 99.9% reduction in the viable cell population (Shen et al., 2002; NCCLS, 2003). For determining MBC values, an aliquot (0.1 mL) of MIC mixtures that showed no growth was inoculated onto MHA plates and incubated at 35°C for 48 h (Syu et al., 2004).

High-performance liquid chromatography

To identify the active compound(s) in *E. bicyclis* extracts, high-performance liquid chromatography (HPLC) was performed using a Hitachi 2000 series HPLC system (Hitachi Tech, Tokyo, Japan) equipped with Shiseido C₁₈ reverse-phase column (250 mm × 4.6 mm, I.D. 5 μm; Shiseido Co., Tokyo, Japan). For detection of the bioactive substance, a linear gradient elution of 90% water with 10 to 100% (v/v) methanol was used at a flow rate of 1.0 mL per min for 45 min. Eluates were monitored at 230 nm.

Standard phlorotannins (dieckol, eckol, and eckstolonol) were used to allow quantification. Standard compound concentrations were calculated in the range of 0.01 to 0.1 mg by linear regression from the respective calibration curves. Eckol (Y = 12.973X - 0.4261; -r² = 0.9991), dieckol (Y = 17.065X + 1.3499; -r² = 0.9993), and eckstolonol (Y = 10.192X + 0.7048; -r² = 0.9963) standard curves were obtained (data not shown).

Results and Discussion

Anti-MRSA activity of *E. bicyclis* extract

E. bicyclis methanolic extract exhibited anti-MRSA activity, suggesting the presence of an antibacterial substance (Table 1). Also, the extract exhibited similar activity against MSSA (Table 1). To identify the antimicrobial substance, the extract was further fractionated using organic solvents. Lyophilized

E. bicyclis extract (1.0 kg) was percolated in methanol (3 times \times 1,000 mL), followed by fractionation with organic solvents to yield hexane- (0.05 g), DCM- (0.05 g), EtOAc- (0.11 g), BuOH ion- (0.39 g), and water-soluble (0.57 g) fractions. The anti-MRSA activity of the hexane, DCM, EtOAc, BuOH, and water-soluble fractions was evaluated by measuring the inhibition zones. Of these, the EtOAc-soluble fraction showed the strongest anti-MRSA activity, followed by DCM, BuOH, and hexane, in that order (Table 1). No anti-MRSA activity was detected in the water-soluble fraction. These results were consistent with the reports of Lee et al. (2008) and Choi et al. (2010) stating that the EtOAc-soluble fraction of *Ecklonia stolonifera* and *Ecklonia cava* exhibited the strongest anti-MRSA activity. The antibacterial substance was subsequently identified.

Determination of the MIC and MBC of *E. bicyclis* extract

The current study focused on the antibacterial activity of *E. bicyclis* extracts against MRSA. To quantitatively evaluate this antibacterial activity, we investigated the MIC and MBC values of the extract (Table 2). The highest anti-MRSA and MSSA activities were present in the EtOAc-soluble fraction at 32-64 μ g/mL. These results were consistent with those of the disk diffusion assay. The MICs and MBCs of the methanolic extract against MSSA and MRSA ranged from 64 to 256 μ g/mL; those of the other fractions (hexane, DCM and BuOH) were between 32 and 64 μ g/mL for MICs and 64 and 256 μ g/mL for MBCs. However, no antibacterial activity was detected in the water-soluble fraction (Table 2). These results strongly suggest the presence of an anti-MRSA compound in the EtOAc-soluble fraction of the *E. bicyclis* methanolic extract.

Kim et al. (2002) reported that the EtOAc extract of this seaweed showed the highest antibacterial activity against the cavity-causing Gram-positive bacterium *Streptococcus mutans*. Therefore, we investigated the antibacterial activity of *E.*

Table 1. Growth inhibitory of *Eisenia bicyclis* extracts against methicillin-resistant *Staphylococcus aureus* (MRSA) and other strains

Strains	Concentration	Zone of inhibition (mm)					
		MeOH	Hexane	DCM	EtOAc	BuOH	H ₂ O
<i>Staphylococcus aureus</i> (KCTC 1927)	1 mg/disk	10.0*	10.5	10.0	15.0	9.5	-
MRSA (KCCM40510)	1 mg/disk	13.5	10.0	15.5	17.0	10.0	-
MRSA (KCCM40511)	1 mg/disk	11.0	9.0	11.0	16.0	10.0	-

Each extract of *Eisenia bicyclis* was loaded onto a disk (6 mm in diameter).

MeOH, methanolic extract; DCM, dichloromethane soluble fraction; EtOAc, ethyl acetate soluble fraction; BuOH, butanol soluble fraction; H₂O, water soluble fraction; -, no growth inhibition.

*Data are the averages of triplicate experiments.

Table 2. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of *Eisenia bicyclis* extracts against methicillin-resistant *Staphylococcus aureus* (MRSA) and other strains

Strains	MeOH		Hexane		DCM		EtOAc		BuOH		H ₂ O		Vancomycin	
	MIC*	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive bacteria														
MRSA (KCCM 40510)	64	64	64	64	128	128	32	64	128	256	>512	>512	2	4
MRSA (KCCM 40511)	128	128	128	128	128	128	64	64	128	128	>512	>512	2	4
<i>Staphylococcus aureus</i> (KCTC 1927)	128	256	128	128	64	128	32	32	128	128	>512	>512	0.5	1
<i>Bacillus cereus</i> (ATCC 14579)	256	256	32	64	32	64	32	32	64	64	512	>512	1	2
<i>Bacillus subtilis</i> (KCTC 1028)	128	128	64	64	64	64	32	32	128	128	256	512	0.5	1
<i>Listeria monocytogenes</i> (KCTC 3710)	256	256	256	256	128	128	64	64	256	256	>512	>512	1	4
<i>Enterococcus faecalis</i> (KCTC 3206)	512	512	512	512	256	512	64	128	512	>512	>512	>512	1	4
Gram-negative bacteria														
<i>Escherichia coli</i> (KCTC 1682)	256	256	512	512	256	256	128	256	512	>512	>512	>512	512	>512
<i>Salmonella typhimurium</i> (KCTC 1925)	256	512	512	512	64	128	32	64	256	512	>512	>512	512	>512
<i>Vibrio parahaemolyticus</i> (KCTC 2729)	128	128	128	128	32	32	32	32	128	128	>512	>512	256	>512

Values are presented as μ g/mL. MeOH, methanolic extract; DCM, dichloromethane soluble fraction.; EtOAc, ethyl acetate soluble fraction; BuOH, butanol soluble fraction; H₂O, water soluble fraction.

*Data are the averages of triplicate experiments.

bicyclis extract against several pathogenic and spoilage bacteria (Table 2). The EtOAc fraction showed the highest antibacterial activity against these bacterial strains. The MICs and MBCs of the EtOAc fraction were 32-256 µg/mL.

Vancomycin, a tricyclic glycopeptide antibiotic, is used to treat MRSA infections (Lee et al., 2008). Vancomycin interferes with bacterial cell wall synthesis, as does penicillin, eventually leading to cell lysis (Barna and Williams, 1984). Most Gram-negative bacteria are less sensitive to vancomycin than Gram-positives (Totsuka et al., 1999; Lee et al., 2008). As expected, the MICs of vancomycin against Gram-negative bacteria were over 512 µg/mL compared to 0.5-4 µg/mL against Gram-positives (Table 2). Unlike vancomycin, the EtOAc fraction exhibited strong antibacterial activity (MICs 32-128 µg/mL; MBCs 32-256 µg/mL) against Gram-negative bacteria. These data suggest the existence of different mechanisms of inhibiting cell growth and bacterial cell wall synthesis.

Identification of an anti-MRSA substance from *E. bicyclis*

The biological activities of plant materials are related to their phenolic compound content (Kim et al., 2006; Lin et al., 2008). Such activity is based on the physiological functions of polyphenol polymers (McDougall et al., 2005). Seaweed polyphenol (phlorotannin) is the predominant EtOAc-soluble compound in brown algae (Lee et al., 2008; Choi et al., 2010). Phloroglucinols, such as eckol, phlorofucofuroeckol-A, diek-

col, and 8,8'-bieckol, also exhibit antibacterial activity (Isnansetyo et al., 2001; Nagayama et al., 2002).

We detected anti-MRSA activity in *E. bicyclis* methanolic extract. Additionally, the ethyl acetate-soluble fraction exhibited the highest antibacterial activity against other pathogenic bacteria, suggesting that the antibacterial compound is abundant in the EtOAc fraction (Table 2). To identify the active compound, we conducted HPLC analysis (Figs. 1 and 2). Purified phloroglucinol compounds (dieckol, eckol, and eckstolonol) were used as controls. Identification of unknown phloroglucinol compounds was achieved by comparing retention time with those of control compounds. The retention times of dieckol, eckol, and eckstolonol were 12.98 ± 0.30, 10.87 ± 0.29, and 21.52 ± 0.45 min, respectively (Fig. 1). HPLC analysis showed that only the methanolic extract and the EtOAc-

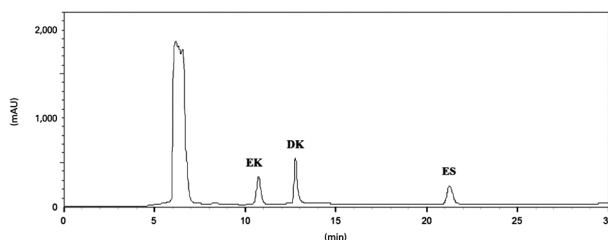


Fig. 1. High-performance liquid chromatography (HPLC) profile of standard phlorotannins. HPLC analysis was performed as described in Materials and Methods. EK, 100 µg per mL of eckol; DK, 100 µg per mL of dieckol; ES, 100 µg per mL of eckstolonol.

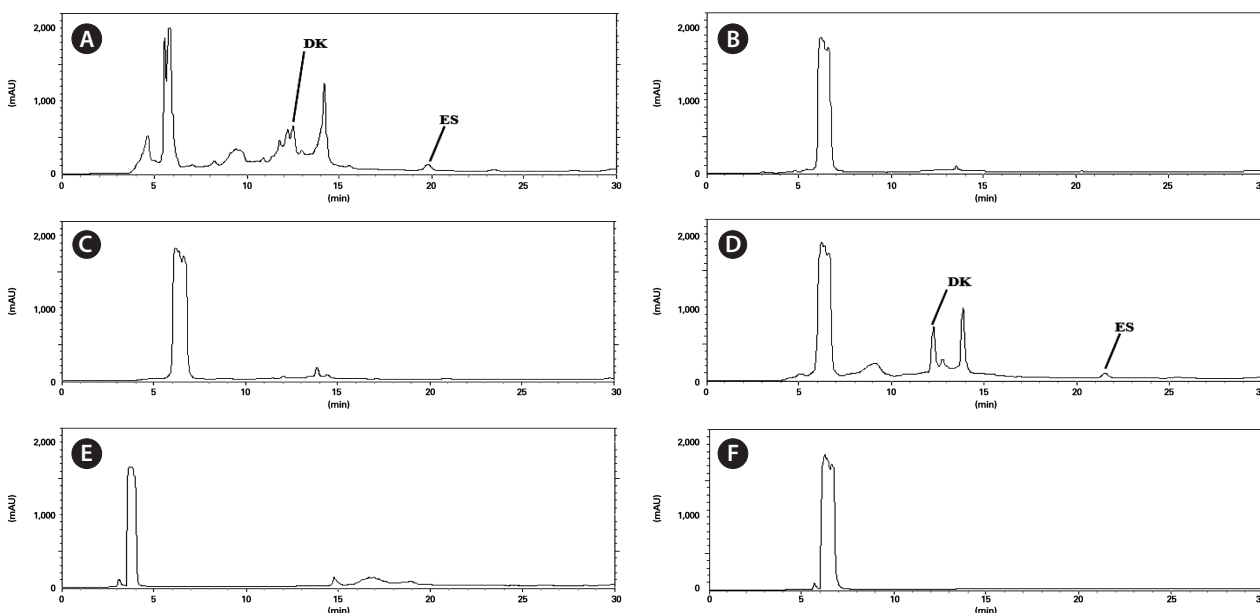


Fig. 2. HPLC profiles of *Eisenia bicyclis* extracts after 5 days of fermentation. A, B, C, D, E and F are the HPLC profiles for methanol extract, *n*-hexane-soluble extract, dichloromethane-soluble extract, ethyl acetate-soluble extract, *n*-butanol-soluble extract, water-soluble extract. The injection amount was 1,000 ppm.

soluble fraction contained sizeable quantities of dieckol (76.9 mg/g in the EtOAc-soluble fraction) (Table 2).

Dieckol is a known antibacterial substance with activity against MRSA (Lee et al., 2008). Of the soluble fractions, dieckol was present only in the EtOAc fraction, which exhibited the highest anti-MRSA activity. Our previous report showed that of the phlorotannins tested, dieckol showed the highest anti-MRSA activity (Lee et al., 2008). Considering these data, we suggest that the anti-MRSA activity of *E. bicyclis* is likely mediated by phlorotannins such as dieckol.

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