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RESEARCH ARTICLE

Antibacterial Activities of Isothiocyanates Extracted from Horseradish (*Armoracia rusticana*) Root against Antibiotic-resistant Bacteria

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Abstract The antibacterial activities of isothiocyanates (ITCs) extracted from horseradish root was determined against 4 strains of antibiotic-resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), multidrug-resistant *Acinetobacter baumanii* (MRAB), and multidrug-resistant *Pseudomonas aeruginosa* (MRPA), and 3 strains of normal pathogenic bacteria, *S. aureus, A. baumanii,* and *P. aeruginosa*. The minimum bactericidal concentrations (MBC) of ITCs against MRSA, VRSA, MRAB, and MRPA were 666.7, 666.7, 333.3, and 208.3 μg/mL, respectively, and against *S. aureus*, *A. baumanii*, and *P. aeruginosa* were 833.3, 41.7, and 52.1 μg/mL, respectively. ITCs extracted from horseradish root showed the strongest antibacterial activity against *A. baumanii* with a MBC of 41.7 μg/mL. Among antibiotic-resistant bacteria, ITCs showed the strongest antibacterial activity against MRPA with a MBC of 208.3 μg/mL. MBC values of vancomycin against MRSA, VRSA, and *S. aureus* were 1,667.7, 2,000.0, and 1,333.3 μg/mL, levofloxacin against MRAB and *A. baumanii* were 833.3 and 1,333.3 μg/mL, respectively, norfloxacin against MRPA and *P. aeruginosa* were 666.7 and 7.8 μg/mL, respectively. ITCs showed stronger antibacterial activities than antibiotics against tested bacteria except *P. aeruginosa*. These results indicate that ITCs extracted from horseradish root should be candidates for antibacterial agent against antibiotic-resistant bacteria.

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Introduction

Horseradish (*Armoracia rusticana*) is grown mainly for the root to be used as a condiment and spice for foodstuffs, and formerly used medicinally, particularly as an antiscorbutic (1). When horseradish tissues are damaged by cutting, enzyme from the damaged horseradish plant cells breakdown sinigrin (a glucosinolate) to produce isothiocyantes (ITCs). The characteristic odor and taste of horseradish is due to ITCs formed by the action of myrosinase on glucosinolate when plant tissues are disrupted (1,2).

The major components for antimicrobial activity in horseradish are ITCs. Allyl isothiocyantes (AITC) (3), phenylethyl isothiocyanate (PITC), and other ITCs (4) are contained in ITCs extracted from horseradish root. AITC is known to possess a strong antimicrobial activity, capable of killing fungal and bacterial pathogens on plant seeds and fresh produce (5), bread (6), meat (7), and cheese (8), and to possess antimicrobial effects against *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Staphylococcus aureus* (9), *Staphylococcus* sp. (3), and lactic acid bacteria (10). Although applications of ITCs are limited due to a high volatility and poor water solubility (11,12), ITCs have strong antimicrobial activities that are capable of killing fungal and pathogenic bacteria (13). Therefore, it seems reasonable to explore the possibility of ITCs as an antibacterial agent. The antimicrobial activity of ITCs is believed to be due to the inactivation of extracellular enzymes through cleavage of disulfide bonds (14). Several mechanisms including the modulation of SH enzymes, inhibition of RNA synthesis, partial inhibition of

DNA synthesis, and inhibition of protein synthesis by an action of the ITCs moiety (-N=C=S), have been proposed for the antibacterial activity of ITCs (15).

Antibiotic-resistant bacteria in the medical field have been well recognized as a global nosocomial problem in recent years (5). Infections caused by multidrug-resistant bacteria challenge physicians and endanger the lives of patients (16). During the last decade, efforts to combat microorganisms have increased, and drug companies have developed several novel antimicrobial agents. Unfortunately, the growing problem of multidrug resistance in bacteria has not been paralleled by development of novel antimicrobials. A return to the pre-antibiotic era has become a reality in many parts of the world, indicating the poor effects of current antibacterial agents and a lack of newer active antibiotics (17).

Methicillin-resistant *S. aureus* (MRSA) has been identified as an important gram-positive bacterium involved in both hospital-acquired and community-acquired infections. MRSA has become a worldwide concern because it is highly prevalent, capable of developing new clones, resistant to almost all currently available antibiotics except vancomycin and teicoplanin, and can cause death. Clinically-used linezolid and teicoplanin produce undesirable side effects, such as anaphylactoid symptoms, acute renal failure and disruption of liver function (18). Recently, the susceptibility of MRSA to vancomycin has decreased. Thus, an increase in vancomycin-resistant *S. aureus* (VRSA) cases has been reported in several countries (18,19). Vancomycin is the last resort anti-MRSA antibiotic (18).

Acinetobacter baumanii, members of the genus *Acinetobacter,* are gram-negative, aerobic coccobacilli (family Moraxellaceae). Several species are widely distributed in soil and water and may occasionally become part of the normal microbiota of the skin, throat and rectum (20). *A. baumanii* causes nosocomial infections such as bloodstream infections, ventilator-associated pneumonia and wound infections, particularly in critical patients admitted to the intensive care unit (ICU). *A. baumanii* is characterized by a tendency to acquire resistance to multiple classes of antimicrobial agents (21,22). Most reported *A. baumanii* outbreaks are due to multidrug-resistant isolates (16,22,23). Resistance to carbapenems has been observed worldwide in the past decade (24) and resistance to fluoroquinolones, aminoglycosides, sulphonamides, and third-generation cephalosporins (16,25,26) has increased.

Pseudomonas aeruginosa is one of the leading causes of bacteremia and pneumonia in patients hospitalized in ICUs. In addition to being intrinsically resistant to several antimicrobial agents, *P. aeruginosa* can acquire resistance to conventional antipseudomonal antibiotics including antipseudomonal penicillins, ceftazidime, carbapenems, aminoglycosides, and fluoroquinolones (27). Resistance to anti-pseudomonal beta-lactams, advanced generation cephalosporins, monobactams, and carbapenems is also an increasing clinical problem (28). *P. aeruginosa* is one of the most important opportunistic pathogens responsible for various types of infections, especially in patients in intensive care. This organism is remarkably versatile in combining different intrinsic and acquired resistance mechanisms (29), which underlines resistance to multiple antimicrobial agents, a situation that is commonly encountered among hospital *P. aeruginosa* strains. Together with *A. baumannii* and *Klebsiella pneumoniae*, *P. aeruginosa* is among microorganisms with the greatest mismatch between an unmet medical need for new effective antimicrobials and the current antimicrobial research and development pipeline (30). Therefore, there is an urgent and growing need for development of new antibiotics with novel modes of action to help overcome these problems (18).

In this study, the antibacterial activity of ITCs extracted from horseradish (*A. rusticana*) root for development of natural antibacterial agents against antibiotic-resistant bacteria, MRSA, VRSA, multidrug-resistant *Acinetobacter baumanii* (MRAB), and multidrug-resistant *Pseudomonas aeruginosa* (MRPA) were investigated.

Materials and Methods

Preparation of extracts To extract ITCs, 1,000 g of horseradish (*A. rusticana*) root powder purchased from Biocoats Co. (Seoul, Korea) was mixed with 2,750 mL of distilled water and allowed to react at 40°C in a water bath for 2 h to produce ITCs from sinigrin in the horseradish. Subsequently, the reacted mixture was distilled and concentrated by rotary evaporator (Rotavapor R-200; BUCHI Co., new Castle, DE, USA) at 120°C of oil bath for 120 min. The essential oil (ITCs) was separated from the concentrated solution by centrifugation (Mega 17R; Hanil Science Industrial Co., Seoul, Korea) at 5,000×*g* for 20 min.

Microorganisms and media Four strains of antibiotic-resistant bacteria were used for an antibacterial activity assay of ITCs extracted from horseradish (*A. rusticana*) root. VRSA ATCC 12692 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MRSA CCARM 3108, MRAB CCARM 12007, and MRPA CCARM 2046 were obtained from the Culture Collection of Antimicrobial Resistant Microbes, Department of Biology, Seoul Women's University, Seoul, Korea. These antibiotic-resistant bacteria were grown in Brain heart infusion (BHI) medium (Difco Co., Detroit, MI, USA) at 37°C for 24 h. The 3 normal pathogenic bacteria, *S. aureus* ATCC 25923, *A. baumanii* KCCM 35453, and *P. aeruginosa* KCCM 11328 obtained from the Korean

Culture Center of Microorganisms (KCCM, Seoul, Korea) were grown in BHI medium at 37°C for 24 h.

Paper disk diffusion assay Antibacterial activities of ITCs extracted from horseradish were measured using a paper disk diffusion assay. Bacterial cultures were adjusted to 5.0×10^7 CFU/mL using sterile BHI medium, and 100 μL of each bacterial culture was spread on the surface of BHI agar (Difco Co.) using a sterile glass rod. After absorption of ITCs (5,000 mg/L, 50 μL) into an 8-mm Whatman No. 2 filter paper disk (Sigma-Aldrich, St. Louis, MO, USA), the disk was placed on the agar surface and incubated at 37°C for 48 h. Antibacterial activity was expressed with diameter (mm) of clear zone (Circumference of filter paper disk).

Minimum inhibitory concentration (MIC) assay MIC values of ITCs against antibiotic resistant bacteria were determined following the broth microdilution method as described by the Clinical Laboratory Standards Institute (31). ITCs (2,000 μg/mL) extracted from horseradish root were initially dissolved in ethyl alcohol, then serially diluted in a two-fold series with sterilized BHI medium. Each bacterium was adjusted to a concentration of 1.0×10^7 CFU/mL using the culture medium, then a 5 μL culture was inoculated into each well of a 96-well flat-bottom microplate (Nunc Ltd., Roskilde, Denmark), that had been previously filled with 100 μL of medium containing 50 μL of different ITCs concentrations. Plates were incubated at 37°C for 48 h. Growth of each bacterium was determined based on the absorbance value measured using a microplate reader (EL800; Bio-Tek Instrument Inc., Winooski, VT, USA) at 660 nm. The MIC was defined as the lowest concentration that showed the lowest absorbance. Positive control against MRSA and VRSA was 2,000 μg/mL of vancomycin, and the positive controls against MRAB, and MRPA were 2,000 μg/mL of levofloxacin and 2,000 μg/mL of norfloxacin, respectively. These antibiotics are well known to have antibacterial activities against the antibiotic resistant bacteria used here. The negative control was a mixture of 100 μL of sterilized BHI medium and 5 μL of each strain inoculum. Sterilized BHI medium was used as a blank.

Minimum bactericidal concentration (MBC) assay MBC values of ITCs against antibiotic resistant bacteria were determined following the method of Bamba *et al*. (32). A loopful of each bacterial culture lacking visible growth in each 96-well flat-bottom microplate was inoculated onto the culture medium agar plate, and incubated at 37°C for 24 h. The MBC value was defined as the lowest concentration that showed no bacterial colony formation on BHI agar plates.

GC-MS analysis of ITCs extracted from horseradish root ITCs extracted from horseradish root were analyzed to clarify the main active components involved in the antibacterial activity using an Agilent 7890A GC system equipped with an Agilent 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). ITCs were prepared using dissolution in n-hexane at a 1:1 ratio. The hexane fraction was separated using an HP-5 column (30 m×0.25 mm I.D., 0.25 μm film thickness; Agilent Technologies) at a column temperature held at 50°C for 1 min, then increased to 250°C for 3 min. The inlet temperature was 250°C for 3 min. Helium was used as a carrier gas at flow rate of 1.0 mL/min.

Results and Discussion

Antibacterial activities of ITCs extracted from horseradish root The antibacterial activities of ITCs extracted from horseradish root and positive control antibiotics are shown in Table 1. ITCs at concentrations of more than 2,000 μg/mL showed inhibition effects against MRSA, VRSA, MRAB, and MRPA, and 3 strains of normal bacteria. ITCs (10,000 μg/mL) showed inhibition zones of 85, 10, 18, and 11 mm against MRSA, VRSA, MRAB, and MRPA, respectively. Inhibition zone sizes were 55, 32, and 85 mm against *S. aureus, A. baumanii* and *P. aeruginosa*, respectively (Table 1). The positive control vancomycin showed inhibition zones of 35 and 0 mm against MRSA and VRSA, respectively, while vancomycin showed an inhibition zone of 12 mm against *S. aureus*. Levofloxacin and norfloxacin showed inhibition zones of 21 and 23 mm against MRAB and MRPA, respectively, while Levofloxacin showed an inhibition zone of 29 mm against *A. baumanii* and Norfloxacin showed an inhibition zone of 55 mm against *P. aeruginosa.*

MIC and MBC ITCs extracted from horseradish root showed potent antibacterial activities based on MIC and MBC values against all tested antibiotic-resistant bacteria and normal bacteria (Table 2). The MBC values of ITCs against the antibiotic resistant bacteria, MRSA, VRSA, MRAB, and MRPA were 666.7, 666.7, 333.3, and 208.3 μg/mL, respectively. The MBC values of ITCs against *S. aureus, A. baumanii* and *P. aeruginosa* were 833.3, 41.7, and 52.1 μg/mL, respectively. ITCs extracted from horseradish root showed the strongest antibacterial activity against *A. baumanii* with an MBC value of 41.7 μg/mL. Among antibiotic-resistant bacteria, ITCs showed the strongest antibacterial activity against MRPA with an MBC value of 208.3 μg/mL. The MBC values of vancomycin against MRSA, VRSA, and *S. aureus* were

Table 1. Diameter of inhibition zones of ITCs extracted from horseradish (*A. rusticana*) root against 7 strains of pathogenic bacteria

Bacterial strain	Inhibition zone (mm)								
	Negative control[1)]	Positive control			ITCs (μg/mL)				
		Vancomycin (100 μg/mL)	Levofloxacin (100 μg/mL)	Norfloxacin (500 μg/mL)	10,000	5,000	2,000	1,000	500
MRSA	0	35	-	-	85	20	9	0	0
VRSA	0	0	-	-	10	10	10	0	0
MRAB	0	-[2)]	21	-	18	12	11	0	0
MRPA	0	-	-	23	11	10	10	10	8
S. aureus	0	12	-	-	55	17	11	0	0
A. baumanii	0	-	29	-	32	10	10	0	0
P. aeruginosa	0	-	-	55	85	11	11	0	0

[1)]Sterile distilled water
[2)]Not tested

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of ITCs extracted from horseradish (*A. rusticana*) root against 7 strains of pathogenic bacteria

Bacterial strain	Vancomycin (μg/mL)		Levofloxacin (μg/mL)		Norfloxacin (μg/mL)		ITCs (μg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MRSA	13.2±4.6[1)]	1,666.7±577.4	-	-	-	-	666.7±2888.7	666.7±288.7
VRSA	1,666.7±577.4	2,000.0±0.0	-	-	-	-	83.3±36.1	666.7±288.7
MRAB	-[2)]	-	6.5±2.3	833.3±288.7	-	-	166.7±72.2	333.3±144.3
MRPA	-	-	-	-	26.1±9.1	666.7±288.7	166.7±72.2	208.3±72.2
S. aureus	26.1±9.1	1,333.3±577.4	-	-	-	-	666.7±288.7	833.3±288.7
A. baumanii	-	-	416.7±144.3	1,333.3±577.4	-	-	41.7±18.0	41.7±18.0
P. aeruginosa	-	-	-	-	3.3±1.1	7.8±0.0	41.7±18.0	52.1±18.0

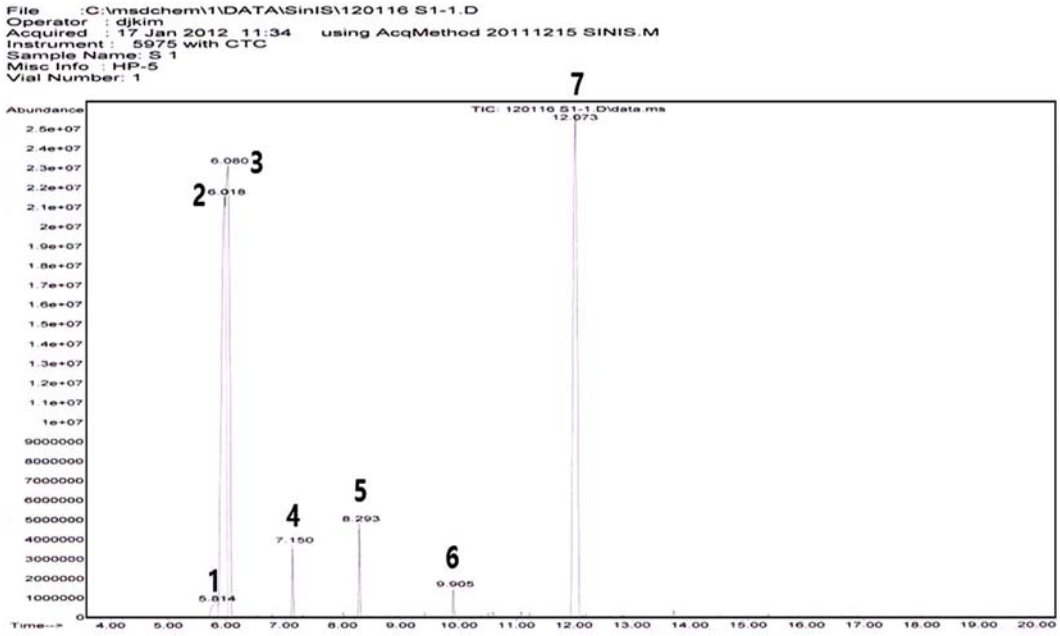
[1)]Mean value±standard deviation of triplicate experiments.
[2)]Not tested

1,667.7, 2,000.0, and 1,333.3 μg/mL, respectively. The MBC values of levofloxacin against MRAB and *A. baumanii* were 833.3 and 1,333.3 μg/mL, respectively. The MBC of norfloxacin against MRPA and *P. aeruginosa* were 666.7 and 7.8 μg/mL, respectively. Based on MBC values, ITCs showed stronger antibacterial activities than antibiotics against 7 tested bacteria. The exception was the MBC value against *P. aeruginosa*.

Akinjogunla *et al*. (33) reported that MIC and MBC values of leaf extracts from *Nymphaea lotus* against MRSA and VRSA were in the range of 5,000-10,000 μg/mL and 10,000-30,000 μg/mL, respectively. Yuvaraj *et al.* (34) reported that the MIC value of a crude extract from the seaweed *Clodophora glomerata* against MRAB was 100 μg/mL. Nascimento *et al.* (35) reported that MIC values of Jambolan and clove extracts against MRPA were 50,000 μg/mL. Thus, ITCs extracted from horseradish root had stronger antibacterial activities than extracts from *N. lotus*, Jambolan and cloves against MRSA, VRSA, and MRPA. However, an extract from the seaweed, *C. glomerata,* had as stronger antibacterial activity than ITCs against MRAB.

Active components in ITCs extracted from horseradish (*A. rusticana*) root for antibacterial activities ITCs extracted from horseradish root were analyzed using GC-MS to identify the main components for antibacterial activities (Fig. 1). ITCs extracted from horseradish root contained the 2 major isothiocyanate derivatives, AITC (60.857%) and PITC (35.268%), and the minor isothiocyanate derivative, 3-butenyl isothiocyanate (BITC, 1.532%). From the standard curve (data not shown), AITC and PITC concentration in ITCs extracted from horseradish root were calculated. The horseradish extract contained 208,766 μg/mL of AITC and 72,778 μg/mL of PITC. The active components in ITCs extracted from horseradish root is different from active components in ITCs extracted from wasabi (*Wasabia japonica*), which contained various ITCs but no PITC in roots, stems, and leaves (36).

In conclusion, ITCs extracted from horseradish (*A. rusticana*) root showed potent antibacterial activities against all tested antibiotic-resistant bacteria. ITCs were most effective against MRPA with an MBC value of 208.3 μg/mL, followed MRAB with an MBC value of 333.3 μg/



Peak No.	Retention time (min)	Compound	Peak area (%)
1,2,3	5.814/6.018/6.080	Allyl ITC (AITC)	60.857
4	7.150	3-Butenyl ITC (BITC)	0.969
5	8.293	Triethyl-2-propenyl	2.167
6	9.905	Benzene propanenitrile	0.739
7	12.073	Phenylethyl ITC (PITC)	35.268

Fig. 1. Analysis of ITCs from horseradish root using GC-MS.

mL. The 2 main components in ITCs extracted from horseradish root were AITC (60.857%) and PITC (35.268). Thus, the active components in ITCs extracted from horseradish root for antibacterial activity were AITC and PITC. These results indicate that ITCs extracted from horseradish (*A. rusticana*) root should be candidate for natural antibacterial agents against antibiotic-resistant bacteria.

Disclosure The authors declare no conflict of interest.

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