Note

Antimicrobial Activity of Isothiocyanates (ITCs) Extracted from Horseradish (*Armoracia rusticana*) Root against Oral Microorganisms

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The antimicrobial activity of isothiocyanates (ITCs) extracted from horseradish root was investigated against oral microorganisms: 6 strains of facultative anaerobic bacteria, *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus casei*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Aggregatibacter actinomycetemcomitans*; one strain of yeast, *Candida albicans*, and 3 strains of anaerobic bacteria, *Fusobacterium nucleatum*, *Prevotella nigrescens*, and *Clostridium perfringens*. The ITCs extracted from horseradish root showed antimicrobial activity against all oral microorganisms by the paper disk method. The minimum bactericidal concentration (MBC) of the ITCs extracted from horseradish root ranged from 1.25 to 5.00 mg/ml against 6 strains of facultative anaerobic bacteria and one strain of yeast, and 4.17 to 16.67 mg/ml against 3 strains of anaerobic bacteria. The ITCs extracted from horseradish root showed the strongest antimicrobial activity, with a MBC of 1.25 mg/ml, against *C. albicans* among facultative microorganisms, and 4.17 mg/ml against *F. nucleatum* among anaerobic bacteria. These results suggest that the ITCs extracted from horseradish root may be a candidate for use as an antimicrobial agent against oral microorganisms.

Key words : Horseradish root extract/ Isothiocyanates (ITCs)/ Antimicrobial activity/ Oral microorganisms/ Minimum bactericidal concentration (MBC).

Tooth decay is one of the most common of all disorders, second only to the common cold. It usually occurs in children and young adults but can affect anyone. It is a common cause of tooth loss in younger people. Bacteria are normally present in the mouth, where they convert all foods, especially sugar and starch, into acids. Bacteria, acid, food debris, and saliva combine in the mouth to form a sticky substance called dental plaque that adheres to the teeth. It is most prominent on the molar teeth, just above the gum line on all teeth, and at the edges of fillings. Plaque that is not removed from the teeth mineralizes into dental calculus. The plaque and calculus irritate the gums, resulting in gingivitis and ultimately periodontitis.

The role of bacteria in dental root canal infection is already well known. Gomes et al. (2004) have reported that anaerobic bacteria are involved in infectious dental root canals and especially *Prevotella* sp., *Fusobacterium* sp., and *Peptostreptococcus* sp. are closely related to the inflammation and damage associated with dental root canals. When the symptoms of periapical infection are not relieved by mechanical instrumentation, antimicrobial agents including antibiotics are needed to remove microorganisms from the root canal. Sodium hypochlorite (NaOCl) is widely used for the cure of the root canal and is effective in killing *Enterococcus* sp., *Actinomyces* sp. and *Candida* sp. However, high concentrations of NaOCl cause side effects, such as the lysis of healthy tissue, and necrosis, pain and edema by inflammation. Chlorhexidine digluconate (CHX, Bukwang, Pharm, Co., LTD, Ansan, Korea) is

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also chemical agent for the disinfection of the root canal and effective against Gram-positive and Gram-negative bacteria. The long time use of CHX causes side effects, such as paraesthesia and glossodynia.

Over the years, extracts from plants believed to possess physiological activities have indeed been shown to contain several components showing antimicrobial activities. On the other hand, despite (and perhaps because of) the high diversity of old and new synthesized antibiotics available for therapeutic purposes, bacterial pathogens have developed a resistance mechanism, rendering their eradication highly difficult. Therefore, the search for antibacterial agents, such as plant extract compounds, has gained renewed impetus.

Plants and plant extracts have been used in traditional cures and herbal remedies for centuries worldwide. Recently, secondary plant metabolites have become the focus of renewed interest due to their potential preventive effects on chronic diseases such as cardiovascular disease and cancer (Rowland, 1999).

The horseradish (*Armoracia rusticana*) is a hardy perennial herb cultivated in temperate regions mainly for the culinary value of its roots. It is a pungent spice used in daily food, including Western dishes featuring beefsteak and frankfurters, and is a member of the *Cruciferae* family. Horseradish liberates isothiocyanates (ITCs) in a reaction with myrosinase (or thioglucoside glucohydrolase) (Kojima et al., 1973), and diethyl ether extracts of horseradish have been reported to demonstrate antimicrobial activity against food-poisoning bacteria (Isshiki and Tokuoka, 1993).

Allyl isothiocyanate (AITC), which is a major pungent flavor compound in horseradish, mustard and wasabi, is generated from its precursor allyl glucosinolate or sinigrin (Kawakishi and Namiki, 1969; Masuda et al., 1996). Sinigrin, which is found widely in mustard and other *Brassica* cruciferous vegetable species such as cabbage and cauliflower, is broken down by the action of myrosinase when the plant tissue is disrupted to release ITCs (Chin and Lindsay, 1993; Shahidi, 1994). Diethyl ether extracts of horseradish demonstrated antimicrobial activity against food-poisoning bacteria including *Escherichia coli*, *Staphylococcus aureus* and *Vibrio parahaemolyticus* and also showed an inhibitory effect on the growth of mold and yeast (Isshiki and Tokuoka, 1993). ITCs extracted from horseradish showed bactericidal activity of against the anaerobic bacteria *Helicobacter pylori*, YS 27, and YS 50 (Shin et al., 2004). In addition, they exhibited antioxidant activity and superoxide scavenging potency (Kinae et al., 2000).

However, little research has been conducted concerning the effects of ITCs against oral microorganisms. Therefore, the purpose of this study was to investigate

the antimicrobial activity of ITCs extracted from horseradish root against oral microorganisms.

Horseradish powder was purchased from Biocoats Co. (Seoul, Korea). Standard AITC (>98% purity) was purchased from Fluka Co. (Haan, Germany). Hexane was purchased from Showa Co. (Tokyo, Japan). Isothiocyanates (ITCs) were extracted as follows: 200 g horseradish powder was mixed with 550 ml distilled water, and then incubated at 40°C in a water bath for 2 h for maximum production of ITCs from sinigrin. The reacted mixture was distilled and concentrated at 120°C in an oil bath for 120 min. The essential oil (ITCs solution) was separated from the concentrated solution by centrifuging at 5,000 × g for 20 min.

The Gram-positive bacteria *Streptococcus mutans* ATCC 25175, *Streptococcus sobrinus* ATCC 27607, *S. aureus* ATCC 25923, *Lactobacillus casei* ATCC 393 and *Enterococcus faecalis* ATCC 29212, the Gram-negative bacterium *Aggregatibacter actinomycetemcomitans* ATCC 29522, and the yeast *Candida albicans* ATCC 10231 were obtained from the American Type Culture Collection (ATCC). Brain heart infusion (BHI) broth (Becton, Dickinson Co., NJ., USA) was used for the culture of *S. mutans*, *S. sobrinus*, and *S. aureus* and the minimum bactericidal concentration (MBC) assay. Lactobacilli MRS broth (Becton, Dickinson Co.) was used for the culture of *L. casei* and the MBC assay. Trypticase soy broth (Becton, Dickinson Co.) was used for the culture of *E. faecalis* and *A. actinomycetemcomitans* and the MBC assay. Yeast malt broth (Becton, Dickinson Co.) was used for the culture of *C. albicans*, and the MIC and MBC assay. *S. mutans*, *S. sobrinus*, *S. aureus*, *L. casei*, and *E. faecalis* were incubated at 37°C, and *A. actinomycetemcomitans*, and *C. albicans* were incubated at 25°C under aerobic conditions. The anaerobic bacteria *Prevotella nigrescens* KCTC 5407 and *Fusobacterium nucleatum* KCTC 5103, which are often discovered when a root canal was infectious, were obtained from the Korean Collection for Type Cultures (KCTC). *Clostridium perfringens* KCTC 3269 was used as a representative antibiotic-resistant oral bacterium. Reinforced clostridial medium and reinforced clostridial agar containing 5% sheep blood were used for the anaerobic bacterial culture and the MBC assay, respectively. Anaerobic bacteria were incubated in an AnaeroPack (Mitsubishi Gas Chemical America, Inc., NY, USA) with AnaeroGen (Oxoid Ltd., Cambridge, UK) at 37°C for 48 h. Dry Anaerobic Indicator Strips (Becton Dickinson Co.) were used to confirm anaerobic conditions in the AnaeroPack.

The antibacterial activity of ITCs extracted from horseradish root was evaluated by the paper disk method. Bacterial culture was adjusted to 5.0×10^7 CFU/ml with sterile culture medium, and 100 µL of each

bacterial culture was spread on the surface of BHI agar (Difco Co., Detroit, USA) with a sterile glass rod. After absorption of ITCs (5,000 mg/L) into an 8-mm filter paper disk (Whatman No. 2), the disk was placed on the surface of the agar and incubated at 37°C for 48 h. Antibacterial activity was expressed with diameter (mm) of clear zone (Circumference of filter paper disk).

Various concentrations of the ITCs extracted from horseradish root and the standard AIT were initially dissolved in ethyl alcohol and then serially diluted in a two-fold series with sterilized culture medium. Each bacterium was adjusted to a concentration of 1.0×10^7 CFU/ml with culture medium, and 5 μ l adjusted culture was inoculated into each well of a 96-well flat-bottom microplate (Nunc Ltd., Roskilde, Denmark), which had been previously filled with 100 μ l medium containing 50 μ l of various the ITCs concentrations. The plates were incubated under the conditions described above. The growth of each bacterium was judged by the absorbance measured with a microplate reader (EL800, Bio-Tek Instrument Inc., California, USA) at 660 nm. Minimum inhibitory concentration (MIC) was defined as the lowest concentration that showed visible absorbance. Standard AIT purchased from Wako Pure Chemical Industries (Osaka, Japan) was used as a positive control. MBC was assayed by the method of Bamba et al. (1997). A loopful of each bacterial culture in each 96-well flat-bottom microplate was inoculated onto the culture medium agar plate, and incubated under the same conditions described above. MBC was

defined as the lowest concentration that showed no bacterial colony formation on agar plates.

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

ITCs (5-10 mg/ml) extracted from horseradish root showed antimicrobial activity against *S. mutans*, *L. casei*, and *C. albicans*. The MICs and MBCs of ITCs extracted from horseradish root against facultative anaerobic oral microorganisms are shown in Table 1 and Table 2, respectively. The ITCs extracted from horseradish root showed the strongest antimicrobial activity against the yeast, *C. albicans* with a MIC of 0.52 ± 0.18 mg/ml (Table 1) and MBC of 1.25 ± 0.00 mg/ml (Table 2). Among tested bacteria, ITCs extracted from horseradish root showed the strongest antibacterial activity against *L. casei*, a gram-positive bacteria, with a MIC of 0.84 ± 0.36 mg/ml (Table 1) and MBC of 1.67 ± 0.72 mg/ml (Table 2).

The ITCs extracted from horseradish root seem to

TABLE 1. MIC of ITCs extracted from horseradish, purchased AIT and CHX against facultative anaerobic oral microorganisms

Tested strain	MIC ^a (mg/ml)		
	ITCs extracted from horseradish root	Purchased AIT	CHX
<i>S. mutans</i> ATCC 25175	1.04 ± 0.36	2.50 ± 0.00	0.11 ± 0.05
<i>S. sobrinus</i> ATCC 27607	2.08 ± 0.72	4.17 ± 1.44	0.84 ± 0.36
<i>S. aureus</i> ATCC 25923	1.67 ± 0.72	3.33 ± 1.44	0.42 ± 0.18
<i>L. casei</i> ATCC 393	0.84 ± 0.36	2.50 ± 0.00	0.31 ± 0.00
<i>E. faecalis</i> ATCC 29212	2.08 ± 0.72	4.17 ± 1.44	0.21 ± 0.09
<i>A. actinomycetemcomitans</i> ATCC 9522	2.50 ± 0.00	6.67 ± 2.89	0.42 ± 0.18
<i>C. albicans</i> ATCC 10231	0.52 ± 0.18	0.84 ± 0.36	0.21 ± 0.09

^aMean value $\pm$ standard deviation of the triplicated experiments.

TABLE 2. MBC of ITCs extracted from horseradish, purchased AIT and CHX against facultative anaerobic oral microorganisms

Tested strain	MBC ^a (mg/ml)		
	ITCs extracted from horseradish root	Purchased AIT	CHX
<i>S. mutans</i> ATCC 25175	2.50 ± 0.00	2.50 ± 0.00	0.21 ± 0.09
<i>S. sobrinus</i> ATCC 27607	4.17 ± 1.44	4.17 ± 1.44	2.08 ± 0.72
<i>S. aureus</i> ATCC 25923	4.17 ± 1.44	3.33 ± 1.44	1.04 ± 0.36
<i>L. casei</i> ATCC 393	1.67 ± 0.72	2.50 ± 0.00	1.38 ± 0.22
<i>E. faecalis</i> ATCC 29212	4.17 ± 1.44	4.17 ± 1.44	0.52 ± 0.18
<i>A. actinomycetemcomitans</i> ATCC 9522	5.00 ± 0.00	6.67 ± 2.89	1.04 ± 0.36
<i>C. albicans</i> ATCC 10231	1.25 ± 0.00	0.84 ± 0.36	0.52 ± 0.18

^aMean value $\pm$ standard deviation of the triplicated experiments.

have multi-targeted mechanisms of action in metabolic pathways, membrane integrity, cellular structure and statistically significant higher release of the cell compounds of microorganisms (Turgis et al., 2009). Ahn et al. (1999), and Shin and Lee (1998) have reported that ITCs showed stronger antimicrobial activity against fungi than bacteria, and showed stronger antibacterial activity against gram-negative bacteria than gram-positive bacteria. In the present study, the ITCs extracted from horseradish root showed stronger antimicrobial activity against fungi than facultative anaerobic bacteria, and showed stronger antibacterial activity against gram-positive bacteria (*S. mutans* and *L. casei*) than gram-negative bacteria (*A. actinomycetemcomitans*). Few reports are available concerning the antibacterial activity of ITCs against oral bacteria. Yu et al. (2006) reported that the MBCs of ITCs extracted from horseradish root extract against the same bacteria ranged from 0.13–0.50 mg/ml, which is lower than those determined in this study. These differences in MBCs against the same bacteria may be due to the use of different strains and minute mutations.

Root canal infection is a dynamic process and the dominant bacterial species differ at different stages of the process. However, a number of studies have shown various species according to the identification method, intra-canal ecological environment, area of residence, etc (Kim et al., 2003). Black-pigmentation bacteria (BPB) are implicated in apical abscess formation (Siqueria et al., 2001). Generally most often *Prevotella* sp. and *Porphyromonas* sp. are mentioned when discussing the participation of BPB in the pathogenesis of root canal infection. Baumgartener et al. (1999) have reported that *Prevotella* sp. was frequently isolated in infected root canals and *P. nigrescens* was more commonly found than *P. intermedia*. In our previous study (Jang et al., 2010), *F. nucleatum* was mostly isolated

from infected root canals. That is why we selected *P. nigrescens* and *F. nucleatum* as representative anaerobic oral bacteria. ITCs extracted from horseradish root showed antimicrobial activity against *P. nigrescens* and *F. nucleatum*. The MICs and MBCs of the ITCs extracted from horseradish root against anaerobic oral bacteria are shown in Table 3 and Table 4, respectively. ITCs extracted from horseradish root showed strong antimicrobial activity with MICs of 1.67–6.67 mg/ml (Table 3) and MBCs of 4.17–16.67 mg/ml (Table 4) against 3 tested anaerobic bacteria but were weaker than those of CHX as used the positive control. The ITCs showed the strongest antimicrobial activity against *F. nucleatum* with a MIC of 1.67 ± 0.72 mg/ml (Table 3) and a MBC of 4.17 ± 1.44 mg/ml (Table 4). *C. perfringens*, used as a representative antibiotics-resistant oral bacterium, was the most resistant strain to ITCs, with a MBC of 16.67 ± 5.77 mg/ml (Table 4).

The antibacterial activity of the ITCs reported here is difficult to compare with other results because few studies of the antibacterial activity of ITCs against anaerobes have been published. We found that our own results indicate that the ITCs extracted from horseradish root had stronger antibacterial activity against facultative anaerobic bacteria than anaerobic bacteria. CHX showed the MBC of 0.13–0.21 mg/ml against 3 tested anaerobic bacteria (Table 4). Ferreira et al. (2002) reported that the MBCs of CHX against the same bacteria used in this study were 0.06–0.20 mg/ml.

ITCs extracted from horseradish root were analyzed by GC-MS to clarify the main components involved in the antibacterial activity (Fig. 1). The ITCs extracted from horseradish root contained 2 major isothiocyanate derivatives, 59.995% of AITC and 35.819% of Phenylethyl ITC (PITC), and one minor isothiocyanate derivative, 1.532% of 3-Butenyl ITC (BITC). The concentration of AITC and PITC in ITCs extracted from

TABLE 3. MIC of ITCs extracted from horseradish, purchased AIT and CHX against anaerobic oral bacteria

Tested strain	MIC ^a (mg/ml)		
	ITCs extracted from horseradish root	Purchased AIT	CHX
<i>C. perfringens</i> KCTC 3269	6.67 ± 2.89	8.33 ± 2.89	0.13 ± 0.05
<i>P. nigrescens</i> KCTC 5407	4.17 ± 1.44	6.67 ± 2.89	0.13 ± 0.05
<i>F. nucleatum</i> KCTC 5103	1.67 ± 0.72	3.33 ± 1.44	0.11 ± 0.05

^aMean value $\pm$ standard deviation of the triplicated experiments.

TABLE 4. MBC of ITCs extracted from horseradish, purchased AIT and CHX against anaerobic oral bacteria

Tested strain	MBC ^a (mg/ml)		
	ITCs extracted from horseradish root	Purchased AIT	CHX
<i>C. perfringens</i> KCTC 3269	16.67 ± 5.77	8.33 ± 2.89	0.21 ± 0.09
<i>P. nigrescens</i> KCTC 5407	8.33 ± 2.89	6.67 ± 2.89	0.13 ± 0.16
<i>F. nucleatum</i> KCTC 5103	4.17 ± 1.44	3.33 ± 1.44	0.21 ± 0.09

^aMean value $\pm$ standard deviation of the triplicated experiments.

FIG. 1. Analysis of ITCs from horseradish root by GC-MS.

horseradish root were calculated. The horseradish extract contained 208,766 $\mu\text{g/ml}$ of AIT and 72,778 $\mu\text{g/ml}$ of PITC from the standard curve. The active components in ITCs extracted from horseradish root are different from the active components in ITCs extracted from wasabi (*Wasabia japonica*) which contains various ITCs but no PITC in the root, stem and leaf (Etoh et al., 1990).

In this study, we verified the antimicrobial activity of the ITCs extracted from horseradish root, which may be a useful intraoral medicament (for example, as a mouthwash, root canal irrigant, or intraoral ointment). Some studies have demonstrated conclusively that mechanical instrumentation cannot sufficiently disinfect the root canals of teeth (Byström and Sundqvist, 1981; Dalton et al., 1998). Intracanal irrigants are required to eradicate microorganisms, and over time a variety of chemicals have been promoted for this purpose. The ideal intracanal irrigant kills bacteria, dissolves necrotic tissue, lubricates the root canal, removes the smear layer, and does not irritate healthy tissue. The ITCs extracted from horseradish root are volatile compounds. Volatility is particularly important in root canal irrigants, because a vapor is able to kill microorganisms in the accessory canals that are not disinfected sufficiently by mechanical instrumentation. Formocresol, camphorated phenol, iodine-potassium iodide and other volatile compounds with antimicrobial activity have been used as intracanal irrigants (Lim, 1999), but they are no longer recommended due to issues of tissue irritability.

As natural extracts, ITCs have a good degree of tissue compatibility and may be applied in the future as a primary component of tooth canal irrigants.

The results of this study suggest that ITCs extracted from horseradish root have strong antibacterial activity and may be a candidate for use as a natural disinfectant against oral microorganisms. Further testing of the antibacterial activity of the ITCs extracted from horseradish root against additional oral bacteria, including *Actinobacteria* sp., *Spirochaetes*, and *Proteobacteria* sp., is needed to better study in their potential use in the treatment of multi-bacterial root canal infection.

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