碩士學位論文

Antimicrobial Effects of Isothiocyanates from Cruciferous Vegetables against Oral Pathogens

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2016年 2月

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이 論文을 工學 碩士學位 論文으로 提出함

2016年 2月

高美玉의 工學 碩士學位 論文을 認准함

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濟州大學校 大學院

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Antimicrobial Effects of Isothiocyanates from Cruciferous Vegetables against Oral Pathogens

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Engineering

2016. 02.

This thesis has been examined and approved.

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Feb. 2016

Department of Food Science and Engineering
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Contents

Abstract

The potentials of 10 isothiocyanates (ITCs) present in cruciferous plants and radish root hydrolysate for inhibiting the growth of oral pathogens were investigated with an emphasis on any structure-function relationship. The inhibitory potential was assessed in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a broth microdilution technique, following the clinical and laboratory standard methods. The antimicrobial activity of ITCs was in the order of indole-3-carbinol (I3C) > benzyl ITC (BITC) > phenylethyl ITC (PEITC) > erucin > iberin > sulforaphene > sulforaphane > allyl ITC > phenyl ITC > hexyl ITC. I3C, BITC, and PEITC showed higher antimicrobial activity (lower MICs), 0.047-0.500 mg/mL, against *C. albicans*, *S. mutans*, and *L. casei*. The susceptibility of oral pathogens to ITCs was in the order of *S. mutans* > *C. albicans* > *L. casei* > *S. aureus* > *S. sobrinus* > *E. faecalis*. Antimicrobial activity (MIC) of radish root hydrolysate was 0.188 mg/mL against *S. mutans*, and 0.500 mg/mL against *L. casei* and *C. albicans*. The chemical structure of ITCs impacted their antimicrobial activities. The indolyl ITC was the most potent inhibitor of the growth of oral pathogens, followed by aromatic ITCs and aliphatic ITCs. The presence of a double bond and a thiol (-S-) group in the chemical structure of the ITCs, and ITCs with a short carbon chain showed increased antimicrobial activity. These results suggest that ITCs, ubiquitous in cruciferous vegetables, have strong antimicrobial activities and may be useful in the prevention and management of dental caries.

1. Introduction

Tooth decay is one of the most prevalent oral diseases. Bacteria are normally present in the mouth, and these bacteria convert sugar and starch into acids and cause calcified dental plaque [Kim et al., 2013]. Dental plaque control is an essential strategy for preventing dental caries. Tooth-brushing is the most accepted method for controlling plaque, but if this is not enough to remove dental plaque, antimicrobial agents are also needed to kill microorganisms [Park et al., 2012; Park et al., 2013]. Facultative anaerobic bacteria, such as *Streptococcus mutans*, *S. sobrinus*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Lactobacillus casei*, the anaerobic bacteria *Fusobacterium nucleatum* and *Prevotella nigrescens*, and a yeast, *Candida albicans*, are the major causative pathogens related to the damage and inflammation associated with tooth decay in humans [Lim et al., 2003; Park et al., 2012; Park et al., 2013; Balto et al., 2013].

Various chemicals have been used for killing microorganisms from root canals. Chlorhexidine digluconate (CHX) is used widely with regard to root canals and is effective in killing oral pathogens such as Gram-positive and -negative bacteria. However, long-term use of CHX may cause discoloration of teeth and pain in the tongue [Park et al., 2013]. Thus, in recent years, there has been increasing interest in the use of natural compounds of dietary origin for the management of oral infectious diseases.

Cruciferous vegetables, such as broccoli, cabbage, mustard, horseradish, and radishes, may be an interesting choice because they contain compounds with potent biological properties. Among these natural compounds are the isothiocyanates (ITCs; R-N=C=S). ITCs are a class of compounds derived from the enzymatic hydrolysis (myrosinase) of glucosinolates (GLs), which are sulfur-containing compounds present in cruciferous vegetables [Dias et al.,

2014]. While ITCs have been studied for their antioxidant and anticancer properties [Yuan et al, 2010; Pocasap et al., 2013], the antimicrobial properties of ITCs have received limited attention in the context of the prevention and treatment of dental caries.

The antimicrobial properties of ITCs have been studied previously, mainly in connection with food preservation and plant pathogen control [Dias et al., 2014; Dufour et al., 2015]. Benzyl ITC (BITC) and allyl ITC (AITC) showed stronger antibacterial activity against Gram-negative bacteria than Gram-positive bacteria, and against fungi than bacteria [Ahn et al., 1999]. AITC and 2-phenylethyl ITC (PEITC) inhibited various pathogenic microorganisms, such as *Salmonella Montevideo*, *Escherichia coli*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Bifidobacterium*, *Clostridium*, and *Lactobacillus* [Luciano & Holley, 2009; Wilson et al., 2013]. Diethyl ether extracts of horseradish demonstrated antimicrobial activity against food-poisoning bacteria [Isshiki et al., 1992]. The acetone fraction of radish root was effective against foodborne and resistant pathogens. Radish root extract was more active than the stem and leaf extracts in inhibiting bacterial growth [Beevi et al., 2009].

Recently, many studies have attempted to investigate the antibacterial activities of chemical compounds from plants against oral pathogens. The minimum inhibitory concentration (MIC) of leaf extracts from *Camellia sinensis* was 5 mg/mL on *Streptococcus mutans*, *S. sobrinus*, and nine clinical isolates of *S. mutans* [Lim et al., 2003]. The MIC of propolis for mutans streptococci isolated from oral cavity of Koreans was 35 μg/mL, and propolis had a bacteriostatic effect on *Streptococcus mutans* and bactericidal effects on *Streptococcus sobrinus* at >2MIC (70 μg/mL) [Kim et al., 2011]. *Salvadora persica* (tooth brush tree) root extracts, as well as commercial, synthetic BITC exhibited rapid and strong bactericidal effects against oral pathogens involved in periodontal disease as well as against other

Gram-negative bacteria (*Aggregatibacter actinomycetemcomi -tans*, *Porphyromonas gingivalis*, *Salmonella enterica*, *Typhimurium*, and *Pseudomonas aeruginosa*), while Gram-positive bacteria (*S. mutans*, *Lactobacillus acidophilus*, *S. pyogenes*, *S. aureus*, and *E. faecalis*) showed mainly growth inhibition or were unaffected [Softra et al., 2011]. The ITCs extracted from horseradish root showed the strongest antimicrobial activity, with a minimum bactericidal concentration (MBC) of 1.25 mg/mL against *C. albicans* among facultative microorganisms, and 4.17 mg/mL against *F. nucleatum* among anaerobic bacteria [Park et al., 2013]. Hexane extract of *Salvadora persica* (major ITC: BITC) exhibited maximum antimicrobial activity against *E. faecalis* and *C. albicans* [Balto et al., 2013].

There have also been several reports that the antimicrobial potential of ITCs depends on their chemical structure [Wilson et al., 2013; Dias et al., 2014]. ITCs are grouped into three main classes based on their chemical structures: aliphatic-, aromatic-, and indolyl- ITCs. ITC structures vary by side chains because they are derived from different amino acids. These differences confer different biological properties due to the specific side chain structure [Dufour et al., 2015]. Aromatic and indolyl groups show a higher antibacterial effect compared with aliphatic groups against plant pathogenic bacteria [Aires et al., 2009], foodborne pathogens and spoilage bacteria [Wilson et al., 2013], and methicillin-resistant *S. aureus* isolated from diabetic foot-ulcer patients [Dias et al., 2014].

To date, the antimicrobial activity of ITCs against oral pathogens has only been assessed using a limited number of ITCs, such as BITC and AITC, and the relationship between the structure and relative antimicrobial activity of various types of ITCs against oral pathogens has not yet been determined. Thus, the aim of the present study was to evaluate the potential of 10 ITCs and radish root hydrolysate to inhibit the growth of six oral pathogens, with an emphasis on a structure-function relationship.

2. Materials and methods

2.1. Chemicals and sample

Benzyl ITC (BITC), indole-3-carbinol (I3C), allyl ITC (AITC), phenyl ITC (PITC), hexyl ITC (HITC), and chlorhexidine digluconate (CHX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenylethyl ITC (PEITC), L-sulforaphene, R(-) iberin, and erucin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). DL-sulforaphane was purchased from Calbiochem (Merck Millipore, Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from Daejung Chemistry (Siheung, Korea).

Radish (*Raphanus sativus* L.) roots were purchased at a local market, and washed, sliced, and freeze-dried. Radish root powders were stored at -20°C until needed.

2.2. Preparation of isothiocyanate solutions

Ten ITCs were used in this study (Table 1). A fresh stock solution of each ITC was prepared in 100% dimethyl sulfoxide and stored at -20°C. Dilutions of the stock were made in the respective growth medium for each strain. The final concentration of DMSO was adjusted to 1% (v/v), which was shown to have no discernible effect on the growth of the test strains. Tested concentrations of each ITC were as follows: 0.031-4.000 mg/mL (BITC, AITC, HITC, and PITC), 0.007-1.000 mg/mL (sulforaphane, sulforaphene, erucin, iberin, PEITC, and I3C), 0.007-1.000 mg/mL (CHX), and 0.003-0.500 mg/mL (radish root hydrolysate).

Table 1. Chemical structure of isothiocyanates used in this study

Common name	Side chain name	Side chain structure	MW	Main dietary source
Aliphatic ITC[1)]				
Sulforaphane	4-methylsulfinylbutyl	CH_3-SO-$(CH_2)_4$-	177	Broccoli
Sulforaphene	4-methylsulfinyl-3-butenyl	CH_3-SO-CH=CH-$(CH_2)_2$-	175	Radish
Iberin	3-methylsulfinylpropyl	CH_3-SO-$(CH_2)_3$-	163	Broccoli and cabbage
Erucin	4-methylthiobutyl	CH_3-S-$(CH_2)_4$-	161	Turnip and kohlrabi
Allyl ITC	2-propenyl	CH_2=CH-CH_2-	99	Horseradish and cabbage
Hexyl ITC	hexyl	$CH_3(CH_2)_5$-	143	
Aromatic ITC				
Phenylethyl ITC	2-phenylethyl	C_6H_5-$(CH_2)_2$-	163	Water cress
Benzyl ITC	benzyl	C_6H_5-CH_2-	149	Wasabi and mustard
Phenyl ITC	phenyl	C_6H_5-	135	
Indolyl ITC				
Indole-3-carbinol	H-indol-3-yl methanol	C_8H_6N-CH_2OH	147	All vegetables

[1)] Isothiocyanate

2.3. Preparation and GC/MS analysis of radish root hydrolysate

Radish root hydrolysate was prepared as follows [Kim et al., 2015]. Distilled water (8 mL) was added gently to 0.5 g of freeze-dried radish root powders in a 50-mL centrifuge tube. An endogenous enzymatic hydrolysis was performed at 25°C for 10 min without shaking. Dichloromethane was then added and the mixture was further hydrolyzed with shaking in a water bath at 25°C for 15 min. The hydrolysate was extracted with dichloromethane three times and the combined extracts were filtered through a No. 5A filter paper (Advantec, Kashiwa, Japan) with 2 g of anhydrous sodium sulfate. The filtrate was evaporated to remove the solvent using a rotary vacuum evaporator (Rotavapor R-124, Büchi Labortechnik AG, Flawil, Switzerland) at 25°C. The dried residue was dissolved in DMSO for antimicrobial tests and in dichloromethane for GC/MS analysis. ITCs in the hydrolysis product of radish root were analyzed to clarify the main active components involved in the antimicrobial activity with an Agilent 6890N GC/5973 MSD (Agilent Technologies, Santa Clara, CA, USA) according to Kim et al. [2015].

2.4. Microorganisms and culture media

The Gram-positive bacteria *Streptococcus mutans* KCOM 1054, *S. sobrinus* KCOM 1157, *Staphylococcus aureus* KCOM 1492, *Enterococcus faecalis* KCOM 1083, *Lactobacillus casei* KCTC 3109, and the yeast *Candida albicans* KCTC 7965 were obtained from the Korean Collection for Oral Microbiology (KCOM, Gwangju, Korea) and the Korean Collection for Type Cultures (KCTC, Daejeon, Korea).

Brain heart infusion (BHI) broth (Becton, Dickinson and Company, MD, USA) was used for the culture of *S. mutans*, *S. sobrinus*, and *S. aureus*. Tryptic soy broth (TSB; Becton, Dickinson and Company) was used for the culture of *E. faecalis*. Lactobacilli MRS broth (Becton, Dickinson and Company) was used for the culture of *L. casei*. Yeast malt broth (YM; Becton, Dickinson and Company) was used for the culture of *C. albicans*. *S. mutans*, *S. sobrinus*, *S. aureus*, *L. casei*, and *E. faecalis* were incubated at 37°C, and *C. albicans* was incubated at 25°C (Table 2).

Table 2. List of oral pathogens and incubation condition used in this experiment

	Strains	Broth Medium	Incubation condition
Facultative anaerobic bacteria	*Streptococcus mutans* KCOM[1)]1054	BHI[3)]	37°C, 48 hr
	Streptococcus sobrinus KCOM 1157	BHI	37°C, 48 hr
	Staphylococcus aureus KCOM 1492	BHI	37°C, 15 hr
	Enterococcus faecalis KCOM 1083	TSB[4)]	37°C, 15 hr
	Lactobacillus casei KCTC[2)] 3109	MRS[5)]	37°C, 48 hr
Yeast	*Candida albicans* KCTC 7965	YM[6)]	25°C, 24 hr

[1)] KCOM : Korean Collection for Oral Microbiology (Gwangju, Korea)

[2)] KCTC : Korean Collection for Type Cultures (Deajeon, Korea)

[3)] BHI : Brain Heart Infusion; Becton, Dickinson and Company, MD, USA

[4)] TSB : Tryptic Soy Broth; Becton, Dickinson and Company, MD, USA

[5)] MRS : Lactobacilli MRS broth; Becton, Dickinson and Company, MD, USA

[6)] YM : Becton, Dickinson and Company, MD, USA

2.5. Antimicrobial activity test

The inhibitory potential of the 10 ITCs and radish root hydrolysate against growth of six test strains was assessed in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using broth microdilution techniques following clinical and laboratory standard methods [CLSI, 2012]. Stock culture suspensions were prepared by a direct colony suspension method and were adjusted to 0.5 McFarland standard solution turbidity (1×10^8 CFU/mL). A stock culture was diluted 1:20 to obtain a working culture containing ~5×10^6 CFU/mL.

Next, 5 μL of each ITC stock solution was mixed with 495 μL sterile culture medium (working solution) and 100 μL working solution was added to each well of a 96-well round-bottom microplate (Nunc Ltd., Roskilde, Denmark). Then, 5 μL working culture was inoculated into each well. The 1% DMSO in the sterile culture medium was used as a negative control and CHX was used as a positive control. The plates were incubated under the conditions described above. The MIC was defined as the lowest concentration that completely inhibited growth of the organism in the wells, as detected by the unaided eye after incubation for 24 h.

The MBC was also measured. A loop of each bacterial culture in each 96-well round-bottom microplate at the MIC value determined above was inoculated onto a culture medium agar plate, and incubated under the same conditions described above. The MBC was defined as the lowest concentration at which no microorganism growth was detected on the agar plate.

2.6. Statistical analysis

All experiments were performed in quadruplicate. Data are presented as means ± standard deviation (S.D.). Statistical analysis were performed using the SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA) and significant differences ($p<0.05$) among the treatment means were determined by Duncan's multiple range test.

3. Results

3.1. Minimum inhibitory concentration and minimum bactericidal concentration of 10 ITCs

The antimicrobial effects of 10 ITCs on oral pathogenic microorganisms were investigated and are reported as MICs (Table 3) and MBCs (Table 4). A selection of 10 ITCs from the three major groups, aliphatic ITCs (n=6), aromatic ITCs (n=3), and indolyl ITC (n=1), was used. CHX was used as a positive control; it is used widely for root canal treatment and is effective in killing oral pathogens [Park et al., 2013].

The antimicrobial activity of the ITCs was in the order of I3C > BITC > PEITC > erucin > iberin > sulforaphene > sulforaphane > AITC > PITC > HITC. Wide variations in the susceptibility (mean MIC ± SD) to ITCs were observed among the test strains. *S. mutans* showed highest susceptibility to the ITCs tested, with a mean MIC of 0.597±0.749 mg/mL, versus *S. sobrinus* (1.682 ± 1.517 mg/mL), *S. aureus* (1.489 ± 1.447 mg/mL), *E. faecalis* (1.900 ± 1.449 mg/mL), *L. casei* (0.975 ± 1.139 mg/mL), and *C. albicans* (0.724 ± 1.204 mg/mL). The order of ITC susceptibility of the test strains was *S. mutans* > *C. albicans* > *L. casei* > *S. aureus* > *S. sobrinus* > *E. faecalis*.

I3C, BITC, and PEITC showed remarkable antimicrobial activities against *S. mutans*, *C. albicans*, and *L. casei*, with MICs ranging from 0.047 to 0.500 mg/mL. I3C, derived from glucobrassicin, a major glucosinolate in all cruciferous vegetables, showed the strongest antimicrobial activity (MIC 0.125 mg/mL) against *S. mutans* and *C. albicans*. It also exhibited strong antimicrobial effects against *S. sobrinus* and *S. aureus* (0.250 mg/mL), and

against *L. casei* (0.500 mg/mL). However, the antimicrobial activity against *E. faecalis* was low (high MIC >1,000 mg/mL) compared with the other strains. The MBC for I3C was either the same or 2×MIC. This difference between MIC and MBC values reflects that I3C was bactericidal in its activity against the test strains.

BITC, derived from glucotropaeolin as a glucosinolate and found abundantly in wasabi and mustard, showed remarkable antimicrobial activities, especially against *C. albicans* and *S. mutans*. The MIC values of BITC were 0.047, 0.109, 0.250, and 0.500 mg/mL against *C. albicans*, *S. mutans*, *L. casei*, and *S. aureus*, respectively. The MBC for BITC was less than 2×MIC and BITC was bactericidal. PEITC, derived from gluconasturtin, a major glucosinolate of water cress, showed the strongest antimicrobial activity against *S. mutans*. The MIC values of PEITC were 0.156, 0.250, 0.500, and 0.750 mg/mL against *S. mutans*, *S. aureus*, *L. casei*, and *C. albicans*, respectively. The MBC for PEITC was either the same or 2×MIC and PEITC was bactericidal.

The MIC values of erucin, derived from glucoerucin, rich in turnip and kohlrabi, were 0.055, 0.063, and 0.625 mg/mL against *S. mutans*, *C. albicans*, and *S. aureus*, respectively. Erucin showed strong antimicrobial activities against *S. mutans* and *C. albicans*. The MBC for erucin was either the same or 2×MIC and erucin was bactericidal in its activity against the test strains. Iberin, derived from glucoiberin, a major glucosinolate of broccoli and cabbage, showed strong antimicrobial activity (0.188 mg/mL) against *C. albicans*. The MIC values of iberin were 0.250, and 0.375 mg/mL against *L. casei* and *S. mutans*, respectively. The MBC for iberin was less than 2×MIC and iberin was bactericidal.

The MIC values of sulforaphene, from glucoraphenin, the second most common ITC in radish root, were 0.250 mg/mL against *S. mutans* and *L. casei*, and 0.344 mg/mL against *C. albicans*. The MBC for sulforaphene was less than 4×MIC and sulforaphene was bacteriostatic. The MIC values of sulforaphane, which is similar in structure to sulforaphene and a major ITC in broccoli, were 0.250 and 0.500 mg/mL against *S. mutans* and *L. casei*, respectively. The MBC for sulforaphane was less than 4×MIC and sulforaphane was bacteriostatic.

AITC, derived from sinigrin, a major glucosinolate of cabbage and horseradish, showed high antimicrobial activity (MIC value: 0.219 mg/mL) only against *C. albicans*, and lower activities (>1,000 mg/mL) against the other oral pathogens tested.

The MIC values of CHX, as the positive control, were 0.003, 0.005, 0.006, 0.009, 0.013, and 0.022 mg/mL against *S. mutans*, *S. sobrinus*, *S. aureus*, *E. faecalis*, *L. casei*, and *C. albicans*, respectively. Thus, CHX exhibited more potent activity than the 10 ITCs tested.

Table 3. Minimum inhibitory concentration (mg/mL) of pure isothiocyanates and radish root hydrolysate against oral pathogenic microorganisms

Isothiocyanates	Oral pathogens					
	S. mutans	*S. sobrinus*	*S. aureus*	*E. faecalis*	*L. casei*	*C. albicans*
Aliphatic ITC[1)]						
Sulforaphane	0.250±0.177^{c}[3)]	1.000±0.000^{b}	1.000±0.000^{b}	1.000±0.000^{a}	0.500±0.000^{c}	1.000±0.000^{a}
Sulforaphene	0.250±0.000^{c}	1.000±0.000^{b}	1.000±0.000^{b}	1.000±0.000^{a}	0.250±0.000^{cd}	0.344±0.188^{c}
Iberin	0.375±0.144^{c}	1.000±0.000^{b}	1.000±0.000^{b}	1.000±0.000^{a}	0.250±0.000^{cd}	0.188±0.072^{de}
Erucin	0.055±0.016^{c}	1.000±0.000^{b}	0.625±0.250^{bc}	>1.000	1.000±0.000^{b}	0.063±0.000^{f}
Allyl ITC	1.375±0.750^{b}	4.000±0.000^{a}	3.000±1.155^{a}	>4.000	1.000±0.000^{b}	0.219±0.063^{de}
Hexyl ITC	2.250±1.258^{a}	>4.000	>4.000	>4.000	>4.000	>4.000
Aromatic ITC						
Phenylethyl ITC	0.156±0.063^{c}	1.000±0.000^{b}	0.750±0.289^{bc}	>1.000	0.500±0.000^{c}	0.250±0.000^{cd}
Benzyl ITC	0.109±0.031^{c}	1.000±0.000^{b}	0.500±0.000^{bc}	1.000±0.000^{a}	0.250±0.000^{cd}	0.047±0.018^{f}
Phenyl ITC	1.500±0.577^{ab}	4.000±0.000^{a}	>4.000	>4.000	1.500±0.577^{a}	1.000±0.000^{a}
Indolyl ITC						
Indole-3-carbinol	0.125±0.000^{c}	0.250±0.000^{c}	0.250±0.000^{bc}	>1.000	0.500±0.000^{c}	0.125±0.000^{ef}
Hydrolysate of radish root	0.188±0.072^{c}	>0.500	>0.500	>0.500	0.500±0.000^{c}	0.500±0.000^{b}
Chlorhexidine digluconate[2)]	0.003±0.000^{c}	0.005±0.002^{d}	0.006±0.000^{c}	0.009±0.003^{b}	0.013±0.000^{d}	0.022±0.006^{f}

[1)] Isothiocyanate

[2)] Chlorhexidine digluconate: positive control

[3)] Values are expressed as means ± SD (n=4). Means with different superscripts in the same column are significantly different by Duncan's multiple range test (p<0.05)

Table 4. Minimum bactericidal concentration (mg/mL) of pure isothiocyanates and radish root hydrolysate against oral pathogenic microorganisms

Isothiocyanates	Oral pathogens					
	S. mutans	*S. sobrinus*	*S. aureus*	*E. faecalis*	*L. casei*	*C. albicans*
Aliphatic ITC[1)]						
Sulforaphane	0.250±0.000[b3)]	>1.000	>1.000	>1.000	>1.000	1.000±0.000[b]
Sulforaphene	0.250±0.000[b]	1.000±0.000[a]	>1.000	>1.000	>1.000	0.375±0.144[cd]
Iberin	0.375±0.144[b]	1.000±0.000[a]	>1.000	>1.000	>1.000	0.250±0.000[cde]
Erucin	>0.125	>1.000	>1.000	>1.000	>1.000	0.094±0.036[de]
Allyl ITC	4.000±0.000[a]	>4.000	>4.000	>4.000	>4.000	>0.500
Hexyl ITC	>4.000	>4.000	>4.000	>4.000	>4.000	>4.000
Aromatic ITC						
Phenylethyl ITC	0.313±0.125[b]	1.000±0.000[a]	>1.000	>1.000	>1.000	0.313±0.125[cde]
Benzyl ITC	0.250±0.000[b]	1.000±0.000[a]	>1.000	2.000±0.000	>0.500	0.125±0.000[de]
Phenyl ITC	>4.000	>4.000	>4.000	>4.000	>4.000	1.750±0.500[a]
Indolyl ITC						
Indole-3-carbinol	0.250±0.000[b]	0.313±0.125[b]	0.500±0.000	>1.000	>1.000	0.125±0.000[de]
Hydrolysate of radish root	0.313±0.125[b]	>0.500	>0.500	>0.500	>0.500	0.500±0.000[c]
Chlorhexidine digluconate[2)]	0.003±0.000[c]	0.005±0.002[c]	0.006±0.000	>0.012	>0.025	0.025±0.000[e]

[1)] Isothiocyanate.

[2)] Chlorhexidine digluconate: positive control.

[3)] Values are expressed as means ± SD (n=4). Means with different superscripts in the same column are significantly different by Duncan's multiple range test ($p<0.05$).

3.2. Minimum inhibitory concentration and minimum bactericidal concentration of radish root hydrolysate

The antimicrobial effects of radish root hydrolysate on oral pathogenic microorganisms were also investigated and reported in terms of MIC (Table 3) and MBC (Table 4). The MIC values of radish root hydrolysate were 0.188 mg/mL against *S. mutans*, 0.500 mg/mL against *C. albicans* and *L. casei*, and >0.500 mg/mL against *S. sobrinus*, *S. aureus*, and *E. faecalis* in terms of the concentration of raphasatin. The MBC for radish root hydrolysate was either the same or less than 2×MIC and radish root hydrolysate was bactericidal in its activity against the test strains.

The main components of radish root hydrolysate for the antimicrobial activity were identified as raphasatin and sulforaphene as shown in Fig. 1. The ratio of raphasatin and sulforaphene was 1:0.16. Raphasatin is similar to erucin in chemical structure and is not available commercially due to its instability.

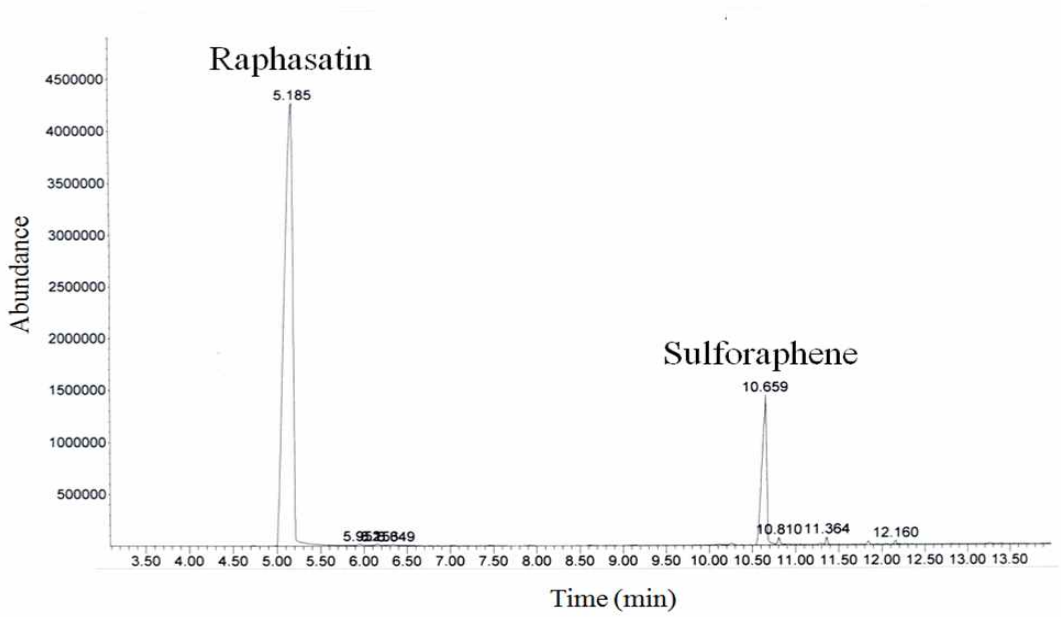

Fig 1. GC/MS chromatogram of the radish root hydrolysate

3.3. Relationship between ITCs structure and antimicrobial activity

ITCs were grouped into aliphatic-, aromatic-, and indolyl-ITCs, based on their chemical structures, and their antimicrobial activity was evaluated based on the ITC structure. Indolyl ITC was the most potent inhibitor of the growth of oral pathogens, followed by aromatic ITCs and aliphatic ITCs. For example, I3C (an indolyl ITC containing an indole group) showed high activity against five of the six oral pathogens tested (*S. mutans*, *C. albicans*, *S. aureus*, *S. sobrinus*, and *L. casei*). BITC and PEITC (aromatic ITCs containing a benzene ring) showed potent activity against four of the six oral pathogens tested (*S. mutans*, *C. albicans*, *S. aureus*, and *L. casei*). However, sulforaphane, sulforaphene, iberin, and erucin, all aliphatic compounds, were less effective and showed high antimicrobial activities against only three of the six oral pathogens (*S. mutans*, *C. albicans*, and *L. casei*).

The presence of a double bond in the chemical structure of the ITCs seemed to increase the antimicrobial activity. For example, sulforaphene (CH_3-SO-CH=CH-CH_2-CH_2-), which is similar in structure, but has one double bond, showed higher antimicrobial activity (0.250 and 0.344 mg/mL) against *L. casei* and *C. albicans* than sulforaphane (CH_3-SO-CH_2-CH_2-CH_2-CH_2-) (0.500 and 1.000 mg/mL).

Thiol (-S-) or sulfinyl (-SO-) groups in the chemical structure also seemed to make a difference to antimicrobial activity. Erucin (CH_3-S-CH_2-CH_2-CH_2-CH_2-), which is similar in structure, but has a thiol group, showed higher antimicrobial activities (0.055, 0.625, and 0.625 mg/mL) against *S. mutans*, *S. aureus*, and *C. albicans* than sulforaphane (CH_3-SO-CH_2-CH_2-CH_2-CH_2-), which has a sulfinyl group (0.250, 1.000, 1.000 mg/mL, respectively).

The antimicrobial activity was also dependent on the length of the hydrocarbon chain. BITC (C_6H_5-CH_2-), with a short chain, exhibited higher antimicrobial potentials (0.047, 0.109, 0.250, and 0.500 mg/mL) against *C. albicans*, *S. mutans*, *L. casei*, and *S. aureus* than PEITC (C_6H_5-CH_2-CH_2-) with a longer chain (0.250, 0.156, 0.500, and 0.750 mg/mL). A similar trend was observed for iberin and sulforaphane. Iberin (CH_3-SO-CH_2-CH_2-CH_2-) showed higher activities (0.188 and 0.250 mg/mL) against *C. albicans* and *L. casei* than sulforaphane (CH_3-SO-CH_2-CH_2-CH_2-CH_2-) (1.000 and 0.500 mg/mL).

4. Discussion

Tooth decay is a chronic infectious disease mediated by pathogens in the mouth. Various chemicals, such as chlorhexidine and antibiotics, have been used to kill microorganisms from root canals. 'Ideal' chemicals would kill bacteria, dissolve necrotic tissue, lubricate the root canal, remove the smear layer, and not irritate healthy tissue. However, the long-term use of chemicals may cause discoloration of teeth and the acquisition of antibiotic resistance in oral pathogens [Kim et al., 2013; Park et al., 2013]. Thus, there is increasing interest in natural alternative therapeutic agents for the prevention and/or management of dental caries. Here, we have described the antimicrobial potentials of ITCs, rich in cruciferous vegetables, against key oral pathogens, which may be helpful in the prevention and possibly the management of dental caries. Of the 10 ITCs tested, 8 showed mostly effective inhibition of growth against five strains, but not *E. faecalis*. *E. faecalis* strains are known to survive under harsh environments, and resist the antimicrobial actions of a number of commonly used antimicrobial agents, including calcium hydroxide [Balto et al., 2013]. Of the ITCs, I3C was the most potent (MIC range = 0.125-0.500 mg/mL), followed by BITC (0.047-1.000 mg/mL), PEITC (0.156-1.000 mg/mL), erucin (0.055-1.000 mg/mL), iberin (0.188-1.000 mg/mL), sulforaphene (0.250-1.000 mg/mL), sulforaphane (0.250-1.000 mg/mL), and AITC (0.219-4.000 mg/mL). Radish root hydrolysate also exhibited antimicrobial effects (MIC range: 0.188 to >0.500 mg/mL, as raphasatin).

The MBCs for I3C, BITC, PEITC, erucin, and iberin were either the same or 2×MIC. This difference between MIC and MBC values reflects that the ITCs tested were bactericidal in their activity against the test strains.

However, the MBCs for sulforaphene, sulforaphane, and AITC were less than 4×MIC and those ITCs were bacteriostatic.

We report here for the first time a relationship between specific chemical structures of ITCs and antimicrobial activity against major oral pathogens. Indolyl ITC (containing an indole group) was the most potent inhibitor of growth against the oral pathogens, followed by aromatic ITCs (containing a benzene ring) and aliphatic ITCs. The indolyl structural formula may interfere more effectively in peptidoglycan biosynthesis, reducing assembly and protein synthesis, which are fundamental for bacterial survival [Dias et al., 2014]. Sung and Lee [2007] assessed the *in vitro* antimicrobial activity of I3C and its mode of action. I3C exhibited broad antimicrobial activities against human pathogenic microorganisms and exerted its antimicrobial activity by disrupting the structure of the cell membrane. MICs of I3C against *S. aureus* (wild type), *E. faecium* (wild type), and *C. albicans* have been reported to be 5, 20, and 10 μg/mL, respectively [Sung & Lee, 2007].

BITC and PEITC, both being aromatic with a benzene ring, exhibited potent activities against four of the six oral pathogens. Dias et al. [2014] evaluated the MICs of purified BITC and PEITC from cruciferous plants against 15 isolates of methicillin-resistant *S. aureus* isolated from diabetic foot-ulcer patients. BITC was the most effective, with MICs varying between 2.9 and 110 μg/mL, and the antibacterial activity was mainly bactericidal. PEITC was effective with MICs varying between 7.3 and 183 μg/mL, and the antibacterial activity was mainly bacteriostatic. Due to their lipophilic and electrophilic properties, BITC and PEITC may be more capable of moving throughout bacterial structures, interfering with the bacterial redox system, and consequently stopping the ability of bacteria to maintain their internal potential [Dias et al., 2014]. BITC may also penetrate through the outer

bacterial membrane and possibly interfere with the bacterial redox systems, thus hampering the ability of the bacterium to maintain its membrane potential [Softra et al., 2011]. However, sulforaphane, sulforaphene, iberin, and erucin, being aliphatic compounds, were less effective and showed high antimicrobial activities against only three of the six oral pathogens tested.

The presence of a double bond and a thiol (-S-) group in the chemical structures of ITCs, and ITCs with short carbon chains, increased the antimicrobial activity. The presence of a double bond in the chemical structure of ITCs increased the antimicrobial activity. Sulforaphene, which is similar in structure, but has one double bond, showed higher antimicrobial activity than sulforaphane. Thiol (-S-) or sulfinyl (-SO-) groups in the chemical structure also made a difference to antimicrobial activity. Erucin. which is similar in structure but has a thiol group, showed higher antimicrobial activity than sulforaphane, which has a sulfinyl group. The antimicrobial activity was also dependent on the length of the hydrocarbon chain. BITC, with a short chain, exhibited higher antimicrobial potentials than PEITC, with a longer chain. Iberin also showed higher activities than sulforaphane. Wilson et al. [2013] also reported that compounds with shorter chains had more antibacterial activity.

Radish root hydrolysate showed high antimicrobial effects against *S. mutans* (MIC 0.188 mg/mL), and *C. albicans* and *L. casei* (MIC 0.500 mg/mL), in terms of the concentration of raphasatin. Beevi et al. [2009] also reported that ethyl acetate extract of radish root had potent antibacterial activity, with significant inhibition of pathogenic bacteria such as *B. subtilis*, *S. aureus*, *S. epidermidis*, *E. faecalis*, *S. typhimurium*, *E. coli*, *E. aerogenes*, *E. cloacae*, and *P. aeruginosa*. Turgis et al. [2009] also reported that an extract from radish root showed the strongest antibacterial activity against *L. casei* with

an MIC of 0.84 mg/mL and an MBC of 1.67 mg/mL. The ITCs extracted from radish root seem to have multiple mechanisms of action in metabolic pathways, membrane integrity, cellular structure, and higher release of the cell compounds from microorganisms [Turgis et al. 2009].

The results above showed that the chemical structure was related to antimicrobial effectiveness. Side chains of each ITC differ because they are derived from different amino acids, and structural differences in ITCs reflect not only lipophilic and hydrophilic properties, but also their antimicrobial, antioxidant, and anticancer potential [O'Callaghan et al., 2000; Fahey et al., 2001]. The variations in the specific side chain structures of ITCs also influence the speed at which ITCs penetrate the cell, and the persistence and accumulation of each ITC in cells [Zhang & Talalay, 1998; Ye & Zhang, 2001; Dufour et al., 2015].

5. Conclusions

The current study provides valuable information on the antimicrobial activity of a wide range of ITCs in preventing oral microbial diseases and maintaining oral health. To the best of our knowledge, this is the first report of the antimicrobial activities of 10 ITCs and radish root hydrolysate against oral pathogens. Comparing activities of ITCs showed that, within a structural group, the activity of ITCs can vary dramatically, and that structural features of ITCs may be of importance, such as the presence of particular functions (e.g., thiol group and double bond), molecular size, or the length of a hydrocarbon chain. These features certainly affect the ITC's capacity to interact with microbial cells, to penetrate the cell envelope, and probably to cross the plasma membrane thereby hindering enzymatic activities [Wilson et al., 2013].

The results of this study suggest that ITCs from cruciferous vegetables have strong antimicrobial activities and may be useful intra oral medicaments (for example, as a mouthwash or root canal irrigant). ITCs are released by hydrolysis of glucosinolates through the action of the enzyme myrosinase. This enzyme-mediated release of ITCs also occurs when cruciferous vegetables, such as broccoli, cabbage, and radish, are chewed on prior to swallowing. Indeed, chewing may give rise to a brush-like effect (with natural antimicrobial agents, i.e., ITCs). A mechanical action, like chewing, facilitates deep penetration of the freshly released ITCs into the teeth, to remove plaque mechanically.

We have demonstrated the antimicrobial potential of 10 ITCs with six oral pathogens in these in vitro studies. However, the antimicrobial susceptibilities

and relationships between ITC structures and antimicrobial activities need to be evaluated further using more clinical strains. Also, further cellular and in vivo studies are necessary to confirm their beneficial activities and potential use for the prevention and treatment of dental decay.

국문요약

십자화과 채소의 주요 성분인 10종의 isothiocyanates(ITCs)와 무 가수분해물을 대상으로 구강 병원성 미생물 6종에 대하여 항균활성을 측정하여 ITCs의 화학적 구조와 항균성과의 관계를 비교하였다. 항균활성은 Indole-3-caribinol > benzyl ITC > phenylethy ITC > erucin > iberin > sulforaphene > sulforaphane > allyl ITC > phenyl ITC > hexyl ITC 순으로 높았다. 구강내 미생물의 ITCs에 대한 민감성은 *C. albicans* > *S. mutans* > *L. casei* > *S. aureus* > *S. iniae* > *E. faecalis* 순으로, 효모가 세균에 비하여 민감성이 높았다. 무 가수분해물의 최소저해농도(MIC)는 *S. mutans*에 대해서 0.188 mg/mL, *L. casei*와 *C. albicans*에 대해서 0.500 mg/mL로 높은 항균활성을 나타내었다. ITCs의 화학구조에 따른 구강균에 대한 항균활성은 indolely ITCs인 indole-3-carbinol이 가장 높았으며, 다음으로는 benzene ring을 함유하고 있는 aromatic ITCs인 BITC와 PEITC가 높았다. 이중결합이 있는 sulforaphene이 이중결합이 없는 sulforaphane보다 *L. casei*와 *C. albicans*에 대해서 항균활성이 높았으며, thiol group을 가지고 있는 erucin은 sulfinyl group을 가지고 있는 sulforaphane에 비하여 높은 항균활성을 나타내었다. 탄소사슬이 짧은 BITC는 탄소사슬이 긴 PEITC보다 대부분의 균주에 대해서 높은 항균활성을 나타내었다. 이상의 결과로부터 십자화과 채소 유래 ITCs는 구강 병원성 미생물에 대하여 항균제로 활용할 수 있을 것으로 추정되었다.

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감사의 글

식품이라는 분야의 전공자로써 한없이 부족하기만 하였던 제가, 어느덧 시간이 흘러 벌써 감사의 글을 올리게 되었습니다. 저에게 있어서 대학원 시절은, 힘든 일도 많았지만 “백문이 불여일견, 백견이 불여일행”이라는 말처럼 많은 경험과 배움을 얻을 수 있었던 값진 시기였습니다. 이 모든 것은 저 혼자서 한 것이 아니라 제 주변에서 아낌없는 응원과 격려로 큰 힘이 되어주신 분들이 있었기에, 감사의 마음을 전하고자 합니다.

전공에 있어서 자신감이 부족하였고, 그로 인해 방황하던 저를 학문의 길로 이끌어주시고, 남보다 한없이 부족하고 한없이 느리게 걷던 저를 끝까지 믿고 기다려 주시며, 많은 경험과 가르침을 주시는 임상빈 지도교수님께 가장 큰 무한한 감사를 드립니다. 또한, 바쁘신 와중에도 논문 지도와 심사까지 많은 관심과 가르침을 주신 김현정교수님과 천지연교수님께도 진심으로 감사드립니다. 더불어 학부 때부터 지금까지 부족한 저에게 격려와 가르침을 주신 고영환교수님, 박은진교수님, 강영주교수님, 하진환교수님, 홍근표교수님께도 정말 감사드립니다.

실험과 더불어 대학원생활까지 가까이에서 많은 도움을 주신 김미보 박사님, 재원오빠, 김경민 선생님, 조만재 선생님, 이수경 선생님, 오화정 선생님, 김소연 선생님, 그리고 항상 먼저 안부 물어봐주시고 챙겨주시는 산업대학원의 한지령 선생님, 고재필 선생님, 실험실 막내 동신오빠에게 정말 감사드립니다. 아울러 대학원 생활에 있어서 가장 가까이에서 함께하였기에 큰 힘이 되어준 효진이와 정연이에게 고마운 마음을 전합니다.

자주 만나지 못해도, 항상 힘들 때마다 누구보다 걱정해주고 응원해주던 절친한 친구들 나영, 윤아, 은지, 그리고 든든한 대학동기들과 오빠들, 그리고 친동생 같은 동생들에게 고마움을 전합니다.

오늘이 있기까지 한결같이 같은 자리에서 기다려주고, 응원해주었던, 함께한 날보다 함께할 날이 더 많은, 세상에서 가장 사랑하는 동석오빠에게 말로는 표현할 수 없을 만큼의 무한한 사랑과 감사의 마음을 전합니다.

그리고 마지막으로, 말보다는 마음으로 항상 믿고 응원해주는 아버지와 항상 걱정하시고 사랑해주시는 어머니, 티격태격해도 항상 듬직한 친오빠, 무뚝뚝한 저에게 친동생처럼 사소한 것까지 챙겨주는 새언니, 그리고 큰아빠, 큰엄마를 포함한 가족들에게 감사의 마음을 전하며, 이 논문을 바칩니다. 감사합니다.