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Inhibition of oral pathogens and collagenase activity by seaweed extracts

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Abstract

Fifty-seven species of common seaweed from the Coast of Korea were screened for antimicrobial (*i.e.* inhibition of *Prevotella intermedia* and *Porphyromonas gingivalis* growth) activity. As a source of bioactive compounds, seaweeds can produce many secondary metabolites with a variety of activities. Using the agar diffusion method, only 17 species (29.8%) showed inhibitory activity. Of these, methanol extracts of *Enteromorpha linza*, *Sargassum sagamianum,* and *Ulva pertusa* showed strong inhibitory effects against both *P. intermedia* and *P. gingivalis*. The MIC values of *E. linza*, *S. sagamianum,* and *U. pertusa* extracts against *P. intermedia* were 625, 78 and 625 μg ml^{-1} and those against *P. gingivalis* were 312, 156 and 625 μg ml^{-1}, respectively. When these three species' extracts were separated into five fractions according to their polarity, the main active agents were determined to be phenolic compounds. We then compared the antimicrobial activities of these phenolic compounds against various periodontal pathogens using a MIC test. Phenolic compound containing extracts at concentrations of 10 to 100 μg ml^{-1} showed a moderate to significant inhibitory effect on collagenase 1, 2 and 3 activity.

Key words

Antimicrobial activity, Seaweed extracts, Periodonitis, *Prevotella intermedia, Porphyromonas gingivalis,* Collagenase

Introduction

Periodontitis is a bacterial inflammatory disease, characterized by destruction of connective tissue, including alveolar bone, that can lead to tooth loss. Periodontitis is typically initiated by a group of Gram-negative periodontal pathogens, such as *Prevotella intermedia* and *Porphyromonas gingivalis* (Socransky *et al*., 1999).

Systemic or topical antibiotics have been used as an adjunct in the treatment of periodontal disease (Slots and Ting, 2002). *Prevotella* and *Porphyromonas* species, including the main oropharyngeal pathogenic species, have traditionally been considered susceptible to penicillin (Niederau *et al*., 1980). However, a gradual increase in the rate of resistance to penicillin has been noted by several investigators over recent years (Appelbaum, 1992). At present, the antibiotics used most frequently against anaerobic bacteria include metronidazole, imipenem and meropenem. These are active against almost all clinically relevant anaerobic bacteria, although strains resistant to these agents have been reported sporadically (Falagas and Siakavellas, 2000; Aldridge *et al.,* 2001). For this reason, extensive research is now being carried out to find novel antimicrobial compounds.

Interest in marine organisms as promising sources of pharmaceutical agents has increased in recent years (Mayer and

Hamann, 2004; Newman *et al*., 2003). As a source of bioactive compounds, seaweeds can produce many secondary metabolites with a variety of activities. Compounds with antiviral, antihelminthic, antifungal and antibacterial activities have been detected in green, brown and red algae (Newman *et al*., 2003; Del Val *et al*., 2001). There are many reports on the biological activities of macroalgae against human pathogens, fungi, and yeasts, but only a few contain data regarding effects against *P. intermedia* and *P. gingivalis*.

The destruction of collagen fibers by collagenase is one of the unique characteristics of periodontal disease. Thus, collagenase is an important pathogenic factor for the development of periodontal disease (Robertson and Simpson, 1976; Larivee *et al*., 1986). Vertebrate collagenases are members of the matrix metalloproteinase (MMP) family of proteolytic enzymes that are involved in extracellular matrix degradation and remodelling during the course of periodontal diseases.

We thus assessed the *P. intermedia*- and *P. gingivalis*-inhibitory activities of seaweed extracts and of the active fractions of three seaweeds against several oral pathogens. Additionally, we investigated their inhibitory effect on collagenase 1, 2 and 3 activity. In this way we hope to discover therapeutic agents for periodontitis that have few or no side effects and that are highly antimicrobial.

Materials and Methods

Seaweed extracts: In total, 57 species (10 green, 29 brown, 18 red) of seaweed were collected from various locations in South Korea from November 2005 to April 2006. Seaweed tissues were washed with tap water to remove salt, epiphytes and sand, and dried for 1 day at room temperature. They were then ground to a powder using a coffee grinder for 5 min. For methanol and water extractions, 1 l of methanol was added per 20 g powder and incubated for 1 day. This was repeated three times and the combined extracts were evaporated to dryness. Distilled water (1 l) was then added to the remaining powder to extract water-soluble components. Stock solutions were prepared by addition of 1 ml methanol or distilled water per 100 mg of dried extract. Stock solutions were filtered through a 0.22 μM filter and stored at 20°C until required (Jin *et al*., 1997).

For constituent separation, seaweed powders (20 g) were extracted with 1 l of methanol-water (4:1) three times. Crude extracts were evaporated under vacuum and then fractionated according to their polarity: saccharides, lipids, phenolics, alkaloids, and nitrogen compounds (Harborne, 1998).

Bacterial strains and culture conditions: Standard bacterial strains and culture conditions were used to screen for antimicrobial activities against oral pathogens, *Prevotella intermedia* and *Porphyromonas gingivalis* were used. To determine the MIC values of the active fractions against several oral pathogens, *Aggregatibacter actinomycetemcomitans*, *Candida albicans*, *Fusobacterium nucleatum subsp. vincenti,* and *Streptococcus mutans* were used. All strains obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). Anaerobic conditions were maintained at 5% H_2, 5% CO_2 and 90% N_2 using the Bactron™ Anaerobic Chamber system (SHELLAB, USA).

Disk diffusion assay: *In vitro* antimicrobial activity against *P. intermedia* and *P. gingivalis* was determined by a disk diffusion assay. Bacteria were incubated in GAM broth agar, supplemented with 10% (v/v) sheep blood, 5 μg ml^{-1} hemin and 1 μg ml^{-1} menadione at 37°C for 48 hr under anaerobic conditions and then adjusted to a density of approximately 2.0 x 10^8 CFU ml^{-1}. This microbial suspension (1 ml) was aseptically spread on the surface of an agar plate. Filter-paper disks (8 mm diameter) were impregnated with seaweed extracts and then placed on the agar surface. Plates were incubated at 37°C for 48 hr under anaerobic conditions. Antimicrobial activity was assessed by measuring the diameter of the growth inhibition zone (mm) (NCCLS, 2004). Controls were run simultaneously. The antimicrobial agent chloramphenicol (Sigma

Table - 1: Bacterial strains, culture conditions and NCCLS guidelines used

Bacterium/Strains	Culture media/Culture conditions	NCCLS guideline
Aggregatibacter actinomycetemcomitans KCTC 3698	Brucella broth + 3% horse serum 37°C, 72 hr, anaerobic conditions	M11-A6
Candida albicans KCTC 17485	RPMI 1640 medium 37°C, 48 hr, aerobic conditions	M27-A2
Fusobacterium nucleatum subsp. *vincenti* KCTC 5105	Schaedler broth 37°C, 72 hr, microaerobic conditions	M11-A6
Porphyromonas gingivalis KCTC 381	GAM broth+ 10% sheep blood + 5 μg ml^{-1} hemin + 1 μg ml^{-1} menadione 37°C, 48 hr, anaerobic conditions	M11-A6
Prevotella intermedia KCTC 25611	GAM broth+ 10% sheep blood + 5 μg ml^{-1} hemin + 1 μg ml^{-1} menadione 37°C, 48 hr, anaerobic conditions	M11-A6
Streptococcus mutans KCTC 3065	BHI broth + 3% horse serum 37°C, 24 hr, aerobic conditions	M7-A6

Table - 2: Antimicrobial activity against *P. intermedia* and *P. gingivalis* in seaweed methanol extracts, as determined by the disk diffusion method.

Seaweeds	*P. intermedia*			*P. gingivalis*		
	1 mg disk^{-1}	3 mg disk^{-1}	5 mg disk^{-1}	1 mg disk^{-1}	3 mg disk^{-1}	5 mg disk^{-1}
Ahnfeltiopsis flabelliformis	-	-	-	-	-	+
Costaria costata	-	-	-	+	++	++
Desmarestia viridis	-	-	-	+	++	++
Enteromorpha linza	-	++	+++	+	++	+++
Ishige okamurae	-	-	-	+	++	++
Ishige sinicola	-	-	-	-	+	++
Laminaria japonica	-	+	++	+	+	++
Lomentaria catenata	-	-	-	-	-	+
Myagropsis myagroides	-	-	-	-	++	++
Myelophycus simplex	-	-	-	-	-	+
Pachydictyon coriaceum	-	-	-	++	+++	++++
Petalonia fascia	-	-	-	-		+
Sargassum horneri	-	-	-	+	++	++
Sargassum sagamianum	-	++	+++	++	++	+++
Scytsiphon lomentaria	-	+	+	+	+	++
Ulva pertusa	-	++	+++	++	+++	++++
Undaria pinnatifida	-	+	++	+	+	++

Inhibition zone diameter was measured (+ = < 4 mm, ++ = 4 - 8 mm; +++ = 8 - 12 mm; ++++ = > 12 mm). Data are mean of triplicate determinations.

857440) was included in the assays as a positive control. All disk diffusion tests were performed independently in triplicate.

Determination of MIC values: Seaweed extracts and fractions that showed strong activity were investigated further by a broth microdilution assay, following the guidelines of the National Committee for Clinical and Laboratory Standards (NCCLS) for anaerobic bacteria M11-A6 (NCCLS, 2004), aerobic bacteria M7-A6 (NCCLS, 2003) and yeasts M27-A2 (NCCLS, 2002) in a 96-well U-shaped microplate. Inocula were prepared from 24 hr broth cultures and suspensions were adjusted to 0.5 McFarland standard solution turbidity. Seaweed extracts or fractions were first diluted to the highest concentration (10 μg ml^{-1}) to be tested and then serial two-fold dilutions were made, resulting in concentrations ranging from 19.5 μg to 10 μg ml^{-1}. Microtitre plates were prepared by dispensing the inoculum and sample (100 μl of each) into each well. The first well of each strip contained 100 μl broth with no compound and 100 μl inoculum, and represented the negative control. The second well on each strip contained 90 μl broth, 10 μl methanol and 100 μl inoculum, and represented the positive control. The final volume in each well was 200 μl. Plates were incubated appropriately for each microorganism (Table 1). The MIC value was defined as the lowest concentration that yielded no bacterial cell growth. All MIC tests were performed independently in triplicate. The antimicrobial agent chloramphenicol (Sigma 857440) was included in the assays as a positive control.

Measurement of collagenase activity: To investigate collagenase inhibition, 10 and 100 μg ml^{-1} concentrations of the phenolic fraction of extracts were used. Collagenase 1 (MMP-1), 2 (MMP-8) and 3 (MMP-13) activity was measured using a MMP Colorimetric Drug Discovery Kit: AK-404, AK-414 and AK-412 (Enzo Life Science, Plymouth, PA, USA). Briefly, aliquots (50 μl) of buffer solution were distributed into wells of a 96 well plate. Diluted MMP enzymes and phenolic compounds (20 μl of each) were added, reaction mixtures were incubated for 30 min at 37°C and diluted substrate (thiopeptide; 10 μl) was added. The final volume was brought up to 100 μl immediately. Inhibition was measured by continuously reading plates at A_{412} for 20 min in a microplate reader (Spectra MAX 190, Molecular Devices, CA, USA). Inhibition percent activity remaining were analyzed by dividing reaction velocity of inhibitor with control and quotient multiplied with 100. All assays were performed independently in triplicate.

Acute toxicity test: Acute toxicity of the moderately polar fractions was assessed in BALB/c mice (8-10 weeks old; 20-25 g body weight) (Cho *et al*., 2007). Animals were kept at room temperature (24±1°C) on a 12/12 hr light/dark cycle with free access to food and water. For the acute toxicity test, mice were fasted for 6 hr with water provided *ad libitum*. Phenolic fractions were evaporated under vacuum at 35°C using a rotary evaporator and then administered orally (5 g 10 ml^{-1} corn oil of 5% DMSO kg b.wt.$^{-1}$) to mice (n = 5). Animals were observed for any abnormal behavior for 3 hr and any mortality was recorded for up to 2 weeks. A group of animals treated with DMSO only served as a control. Animal experiments were performed in accordance with the U.S. NIH Guidelines for the Care and Use of Laboratory Animals.

Statistics: Each independent assay was repeated at least three times with separate cultures. Treatment means were compared to controls using student's *t*-test.

Results and Discussion

Screening of antimicrobial activity: Of the 57 seaweed species screened, only six (10.5%) showed antimicrobial activity against *P. intermedia* and 17 (29.8%) against *P. gingivalis* (Table 2). Among

the 10 species of Chlorophyta (green algae) screened, only two (*Enteromorpha linza* and *Ulva pertusa*; 20.0%) inhibited both microbial pathogens. Phaeophyta (brown algae) showed the highest activity (37.9%) among the three classes of seaweeds screened. Two species showed anti *P. intermedia* and 11 showed anti-*P. gingivalis* activity. Of the Rhodophyta (red algae), antimicrobial activity was present only at low levels in two species. The strongest activities against both microbial pathogens among chlorophyta seaweed species were exhibited by *E. linza* and *U. pertusa*; while among Phaeophyta, the activity of *S. sagamianum* was highest. The inhibition zones of chloramphenicol (positive control; 0.5 mg disk^{-1}) were 14 and 15 mm, respectively.

MIC values determination: The MIC values were determined using a two-fold serial dilution method. The MIC values of *E. linza*, *S. sagamianum* and *U. pertusa* extracts against *P. intermedia* were 625, 78 and 625 μg ml^{-1}, respectively. The MIC values of *E. linza*, *S. sagamianum* and *U. pertusa* extracts against *P. gingivalis* were 312, 156 and 625 μg ml^{-1}, respectively. The MICs of chloramphenicol (positive control) against *P. intermedia* and *P. gingivalis* were 2 and 1 μg ml^{-1}, respectively (Table 3).

Table - 3: MIC values (μg ml^{-1}) against *P. intermedia* and *P. gingivalis* of methanol extracts, as determined by broth microdilution assay.

Seaweeds	*P. intermedia*	*P. gingivalis*
Enteromorpha linza	625	312
Sargassum sagamianum	78	156
Ulva pertusa	625	625

The MIC values were measured for 48 hr after incubation

Fractionation of crude extracts: Each seaweed powder (20 g) was extracted in 1 l of a methanol-water (4:1) mixed three times, and the crude extract was evaporated, yielding a dark greenish-brown gummy residue. The fraction that was acidified to pH 2 and extracted with chloroform yielded a moderately polar mixture of phenolic compounds (0.68, 1.30 and 0.42 g), which contained the majority of the antimicrobial activity. The MIC values of the phenolic fractions of *E. linza*, *S. sagamianum,* and *U. pertusa* extracts against *P. intermedia* were 156, 78 and 78 μg ml^{-1}, respectively. The MIC values of the phenolic fractions of *E. linza*, *S. sagamianum* and *U. pertusa* extracts against *P. gingivalis* were 156, 78 and 312 μg ml^{-1}, respectively (Table 4).

MIC values of phenolics against several oral pathogens: To determine the antimicrobial activities of phenolic fractions against recognized oral pathogens, MIC values were determined by the broth microdilution assay (Table 5). The MIC values of phenolic fractions of *E. linza*, *S. sagamianum*, and *U. pertusa* extracts against *Candida albicans* were 625, 78 and 312 μg ml^{-1}, respectively. The MIC values against *Fusobacterium nucleatum* subsp. *vincenti* were 625, 78 and 156 μg ml^{-1} and against *Haemophilus actinomycetemcomitans* were 625, 312 and 625 μg ml^{-1}, respectively. The MIC values of *S. sagamianum* and *U. pertusa* against *Streptococcus* mutans were 625 and 1,250 μg ml^{-1}, respectively. The phenolic fraction of E. linza extract showed no inhibitory effect against *Streptococcus mutans*. The three phenolics exhibited moderate to significant inhibition of all oral pathogens tested, except the phenolics of *E. linza* against *S. mutans*. The MICs of chloramphenicol (positive control) against *P. intermedia* and *P. gingivalis* were 2 and 1 μg ml^{-1}, respectively.

Table - 4: Comparison of antimicrobial activity against *P. intermedia* and *P. gingivalis* of seaweed extract fractions by MIC test

Seaweeds	MIC values against *P. intermedia* (μg ml^{-1})				
	Saccharides	Lipids	Phenolics	Alkaloids	Nitrogen compounds
Enteromorpha linza	-	312	156	312	-
Sargassum sagamianum	-	-	78	-	-
Ulva pertusa	-	-	78	625	-
	MIC values against *P. gingivalis* (μg ml^{-1})				
Enteromorpha linza	-	625	156	625	-
Sargassum sagamianum	-	-	78	625	-
Ulva pertusa	-	-	312	-	-

The MIC values were measured for 48 hr after incubation and "-" indicates no inhibition at 10,000 mg ml^{-1}

Table - 5: MIC values of seaweed extract phenolic fractions against several oral pathogens.

Oral pathogens	MIC values of phenolics (μg ml^{-1})		
	E. linza	*S. sagamianum*	*U. pertusa*
Candida albicans	625	78	312
Fusobacterium nucleatum subsp. *vincenti*	625	78	156
Aggregatibacter actinomycetemcomitans	625	312	625
Streptococcus mutans	-	625	1,250

The MIC values were measured for 24~72 hr after incubation and "-" indicates no inhibition at 10,000 mg ml^{-1}

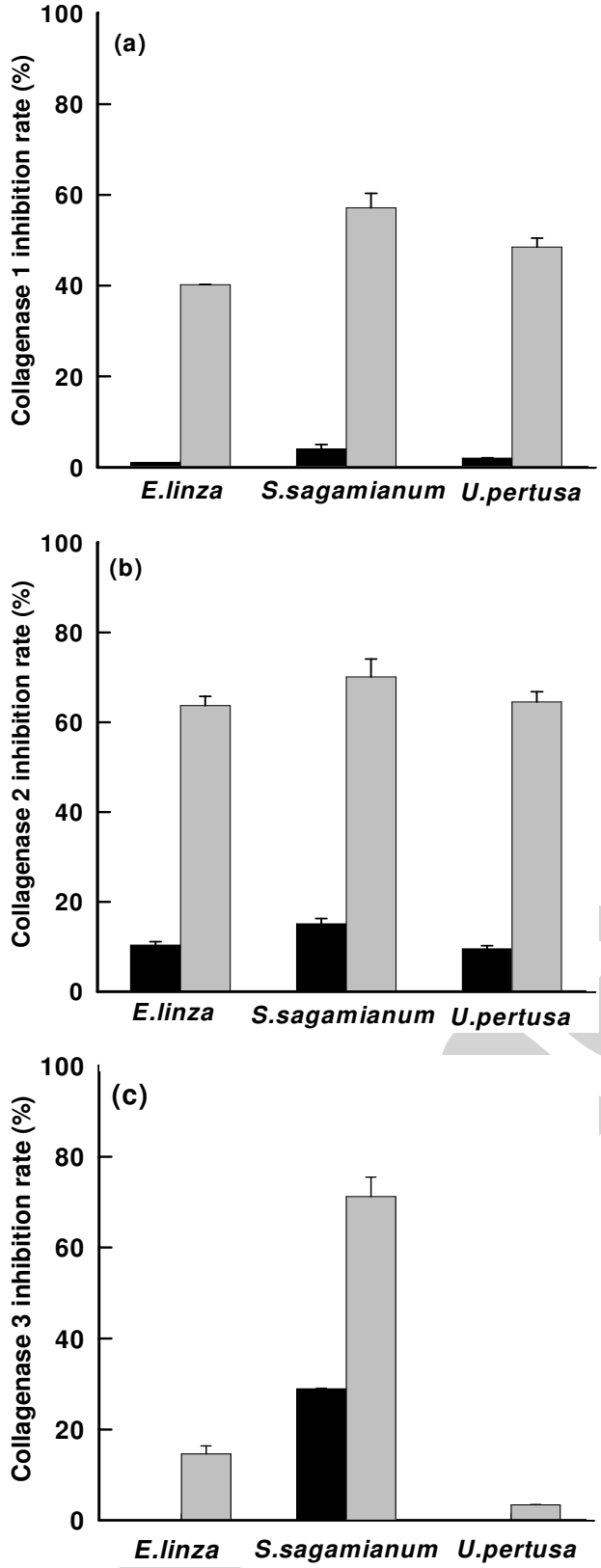

Fig. 1: Inhibition of collagenase 1(a), 2(b) and 3(c) activities by phenolic compounds from *E. linza, S. sagamianum* and *U. pertusa* extracts at 10 (■) and 100 (□) μg ml^{-1}. Statistically significant at $p<0.01$

Inhibitory effect on collagenase activity: Because collagenase is important in the pathogenesis of periodontal disease, we tested the effect of phenolics on collagenase activity using the collagenase (MMP) assay system. In the collagenase 1 (MMP-1) assay system, the phenolic fraction from *E. linza*, *S. sagamianum* and *U. pertusa* extracts inhibited greater than 40.2, 57.1 and 48.5% of collagenase activity, respectively, at 100 μg ml^{-1}. Collagenase 2 (MMP-8) activity levels were inhibited by 63.7, 70.1 and 64.5%, respectively and collagenase 3 (MMP-13) activity levels were inhibited by 3.4, 71.2 and 14.6% at 100 μg ml^{-1}, respectively. In the collagenase 2 assay system, even at 10 μg ml^{-1}, collagenase activity was inhibited by about 10 ~ 15%, but in the collagenase 1 and 3 assay systems, inhibitory effects were either absent or present at only low levels when phenolics were applied at 10 μg ml^{-1} concentration (Fig. 1).

Acute toxicity: We evaluated the acute toxicity of the extract phenolic fractions in mice. Over the 2 week observation period, no death occurred in any mice administered a dose of 5 g kg^{-1} b.wt. Mice administered seaweed extract reacted by wandering briefly and returned to normal behavior after ~10 min. According to the WHO (1992), a herbal medicine is considered toxic if the LD_{50} is lower than 5 g kg^{-1} b.wt. On this basis, these extracts would be classified as non-toxic. Our data suggests, therefore, that these fractions can be safely used by humans at moderate doses.

Periodontitis is a widespread disease that affects as many as 10, 17% of the population (Brown and Löe, 1993; Choi and Kim, 2009). Bacteria commonly detected in the non-healthy gingival pocket are the anaerobic *Aggregatibacter* spp., *Fusobacterium* spp., *P. gingivalis,* and *P. intermedia*. Previous studies have shown that *P. gingivalis* (Hallen *et al*., 2008) and *P. intermedia* (Kim *et al*., 2007) possess a large array of virulence factors, including the ability to adhere to and invade oral epithelial cells and the production of proteolytic enzymes.

Systemic or topical antibiotic therapies have been reported to be useful in treating periodontal disease (Slots and Ting, 2002). However, these antibiotics are known to induce side-effects (Falagas and Siakavellas, 2000), thus, many researchers have sought to develop new therapeutic agents for periodontitis (Park *et al*., 2005).

Seaweeds are able to produce many secondary metabolites with a variety of activities. Compounds with antiviral, antifungal and antibacterial activities have been detected in green, brown and red algae (Del Val *et al*., 2001; Newman *et al*., 2003). Additionally, enzyme inhibitory activities have been detected in seaweeds (Mayer *et al*., 1993; Chang *et al*., 2008; Cho *et al*., 2008).

Many reports have demonstrated a positive correlation between the occurrence of high collagenase activity in the exudate from the gingival crevice and the severity of periodontal disease (Lee *et al*., 1995; Larivee *et al*., 1986). It has been reported that collagenase is produced mainly by inflammatory infiltrative cells and tissue cells, such as neutrophils,

macrophages, fibroblasts, and epithelial cells (Lazarus *et al*., 1968; Wahl *et al*., 1975). Furthermore, periodontal disease-related bacteria, including *Prevotella* and *Porphyromonas* species, are capable of producing collagenase (Gibbons and MacDonald, 1961).

Vertebrate collagenases are members of the matrix metalloproteinase (MMP) family of enzymes. Resident fibroblasts, epithelial cells, and macrophages synthesize MMP-1 (collagenase 1) while infiltrating neutrophils release MMP-8 (collagenase 2) (Birkedal-Hansen *et al*., 1993). A third collagenase (MMP-13) has recently been shown to be produced by both epithelial and mesenchymal cells in inflamed (Freije *et al*., 1994; Uitto *et al*., 1998). All three enzymes could contribute to the collagenolytic activity in diseased periodontal tissues. Thus, we tested the inhibitory effect of the most active phenolic fractions on collagenase 1, 2 and 3 activities using the commercial MMP (collagenase) assay systems. The strongest inhibitory effect on all three collagenase activities was exhibited by *S. sagamianum*. According to Chang *et al*. (2008), monoglycerides from *S. sagamianum* exhibited significant inhibition of phospholipase A_2 and cyclooxygenase-2 activity. The phenolics of *E. linza* and *U. pertusa* exhibited moderate to significant inhibition of collagenase. Kim *et al*. (2006) found that phlorotannins in brown seaweed *Ecklonia cava* inhibited MMP-2 and MMP-9 activities. These enzymes degrade type IV collagen of the basement membrane, which is the first barrier against cancer invasion. The main active compounds described here are now being purified for further study.

The green seaweeds *E. linza* and *U. pertusa* are commonly used as a human foodstuff and as animal fodder in Korea and Japan (Oh *et al*., 1990). These seaweeds are widespread on rocks and other substrates in intertidal zones in whole coasts. The brown alga *S. sagamianum* grows on rocks in subtidal zones of the eastern coast of Korea, and is known to have antimicrobial activity against several Gram-positive and negative strains (Baik and Kang, 1986). To our knowledge, this is the first report that this seaweed contains inhibitory compounds. Although these seaweeds have no known toxicities, we evaluated the acute toxicity of the most active phenolic fractions in mice.

In conclusion, this study demonstrated that extracts from three seaweeds, *E. linza*, *S. sagamianum* and *U. pertusa*, possessed strong anti oral pathogen and anti collagenase activity, with no serious toxic effect at the moderate doses assessed. These extracts are thus promising sources of novel antibacterial agents that may be useful for oral care products and in the development of new therapies for oral diseases.

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