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Investigation of Antimicrobial Activity of Brown Algae Extracts and the Thermal and pH Effects on Their Activity

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Abstract The antimicrobial activity of water and ethanol extracts from 30 species of algae was measured using the agar diffusion method and minimum inhibitory concentration (MIC) test. In agar diffusion method, the 95% ethanol extracts from 12 of the algae showed growth inhibition against the tested microorganisms. In particular, *Ishige okamurai*, *Ecklonia stolonifera*, *Sargassum siliquastrum*, *Sargassum thunbergii*, *Colpomenia bullosa*, and *Ecklonia cava* had strong antibacterial activities against Gram-positive bacteria at 4 mg/mL. In the results of the MIC test, *S. siliquastrum* showed the most antimicrobial activity, where its MIC values ranged from 0.005 to 0.0075% against *Listeria monocytogenes*, *Clostridium perfringens,* and *Basillus subtilis*. In the thermal stability test, for the ethanol extracts of *I. okamurai, E. cava, S. siliquastrum, S. thunbergii,* and *C. bullosa,* the extracts proved to maintain high antimicrobial activities when they were treated at 121°C for 15 min. In the pH stability test, the antimicrobial activity of the *S. siliquastrum* ethanol extract was stable from pH 2 to 10, whereas the activity of the other species ethanol extracts were weakened under pH 10 against several microbes.

Keywords: algae, antimicrobial, agar diffusion method, minimum inhibitory concentration (MIC) test

Introduction

The food contamination caused by food spoiling and food poisoning microorganisms is a serious problem in the food industry; thus, many attempts are made to ensure that foods are safe from them. Heat treatment has been used as a sure method to prevent contamination, but it leads to loss in freshness, quality, and nutritional value (1). Based on these losses, food preservatives such as benzoic acid, sorbic acid, and chlorine dioxide have been used to make up for the weaker points of heat treatment, and to keep the food safe from spoilage and pathogenic microorganisms. However, there is a concern that using these preservatives induces carcinogenicity and mutations caused by their toxicity (2, 3). Additionally, along with growing consumer desires to be healthy, and the issues concerning the safety of foods, consumers are increasingly avoiding food prepared with preservatives of chemical origin. Natural alternatives are therefore needed to achieve sufficient and longer shelf-lives of food, as well as a high degree of safety with respect to spoilage and pathogenic microorganisms.

So far, the development of antimicrobial substances from natural sources has focused on terrestrial animals and plants. For instance, conalbumin, avidin, and lysozyme from eggs, and lactoferrin from milk, are known as antimicrobial substances (4); also, from plants, succinic, malic, tartaric, and benzoic acids (5-7) have antimicrobial activity. Additionally, garlic, onion, and *Tymus quinquecostatus* are reported to possess antimicrobial activity against *Aspergillus* spp. (8-10), and the essential oils of cinnamon, oregano, and cloves have been studied for their efficacy in inhibiting the growth of meat spoilage organisms (11). However, the recently developed antimicrobial substances acquired from terrestrial animals and plants have reached their uppermost limits. Incidentally, as a consequence of the advances in marine biology as well as farming and collection techniques, investigators are interested in marine life as a potential and promising source of antimicrobial agents. Algae, along with other marine resources, have been consumed as food for ages; but recently, algae are also being considered as sources of bio-active compounds since they can produce a great variety of secondary metabolites that have cholesterol-lowering and hypolipidaemic effects, as well as antioxidant, anticancer, antiviral, anti-inflammatory, anticoagulant, immunomodulatory, and antimicrobial activities (12-16). The antimicrobial substances that are presently known include secondary metabolites such as bromophenol from Rhodomelaceae (17), sarganin from the ether fraction of *Cymopolia barbata* (18), dolabellane derivatives from *Dictyota dichotomya* (19), phloroglycin from *Fucus versiculosus* (20), acrylic acid from *Phaeocyslis pouchelii* (21), and hydroquinone derivatives from *Dictyopteris zonarioides* (22). However, very few cases have been documented concerning their effects on food spoilage and food poisoning microbes, and even fewer findings have been published pertaining to their unique chemical structures. Furthermore, even in Korea, despite the large amounts of algae consumed as food, studies concerning the development of novel antimicrobial substances from algae have hardly been carried out, and only *Symphyocladia latiuscula* (23), *Hizikia fusiformis* (24), and *Laminaria sinclairii* (25) were investigated. Therefore, the aim of the present study was to investigate

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the antimicrobial activity of algae extracts against microorganisms related to food contamination and food poisoning diseases, and to inspect the thermal and pH stability of the algae extracts that showed activity. Also, the possible use of algae as a natural food preservative is discussed.

Materials and Methods

Algae Thirty species of algae were collected from the coast near Busan in Korea. Salt, epiphytes, and sand were removed using tap water. The algae were dried at room temperature and then grinded. Each algae powder was stored at −70°C for further experiments.

Strains and media For the purpose of antimicrobial evaluation, 10 bacteria, 1 yeast, and 2 molds were employed. These microorganisms were purchased from the Korean Type Culture Collection (KTCC, Daejeon, Korea) and cultured in nutrient broth (NB, Accumedia, Lansing, MI, USA), brain heart infusion (BHI, Difco, Sparks, MD, USA), MRS (Difco), potato dextrose broth (PDB, Difco), Muller-Hinton broth (MHB, Difco), and blood agar plates (Asan Biotech, Hwaseong, Korea). Table 2 presents the test microorganisms and culture media. Each microbial culture was activated by transferring a loopful of the slant culture into the proper broth medium (Table 1) before the experiments. *Clostridium perfringens* and *Lactobacillus plantarum* were incubated at 37°C for 24 hr under anaerobic conditions after they were put in an anaerobic container system with a gas pak (BBL Microbiology System, Cockeysville, MD, USA) and anaerobic indicator. The others bacteria were incubated at 37°C for 24 hr. The yeast was cultured on yeast peptone dextrose (YPD) at 28°C for 48 hr, and the molds cultured on PDB at 25°C for 3-5 days. Minimum inhibitory concentration (MIC) was used for the agar diffusion method assay and MIC testing.

Preparation of extracts The dried and milled algae were extracted with 99% ethanol or water (10 times the sample amount) for 24 hr at room temperature, and then the mixture was stirred. After extraction, the extract centrifuged (UNION 32R; Hanil Co., Anyang, Korea) for 10 min at 2,090×g and the supernatants collected. The residues were re-extracted twice using the same method. The obtained supernatants were filtered over Whatman No. 5 paper, and the filtrate was evaporated to remove the solvent under reduced pressure at a temperature lower than 40°C by using a rotary evaporator (RE 200; Yamato Co., Tokyo, Japan). To completely remove extraction solvent, this concentrate was dried at 37°C. Dried extracts were stored at −20°C.

Agar diffusion method The antimicrobial activities of the algae extracts were evaluated using the agar diffusion method. Inocula of approximately 10^6 CFU were inoculated onto the surface of pre-dried Muller-Hinton agar (MHA). Sterile 6-mm filter paper discs were placed on the plates and impregnated with 20 μL of algae extract dissolved in 99% ethanol or water. After allowing 1 hr at room temperature for the extracts to facilitate diffusion across the surface, the plates were incubated at 37°C for 24 hr for the bacteria, at 28°C for 48 hr for yeast, and at 25°C for 3-5 days for the molds. However, *C. perfringens* and *L. plantarum* were incubated at 37°C for 24 hr after putting them into an anaerobic container system with a gas pak (BBL) and anaerobic indicator. The antimicrobial activity was measured as the size of the clear zone of growth inhibition. The 99% ethanol and water were used as the negative control.

Minimum inhibitory concentration (MIC) test Algae extracts were diluted with sterilized MHA medium that had not yet hardened to desired final concentration in sterile test tubes. Then the test tubes were inoculated with 10^6 CFU of the test microorganisms. When test microorganisms were inoculated, the temperature of the MHA medium was about 40-45°C. At this temperature, growth inhibition against test microorganisms was not observed (data not shown.). This mixture was poured into a plate and then dried. The plates were incubated as indicated above. The lowest concentration of algae extract with no microbial growth observed under a microscope was determined as the MIC.

Table 1. List of strains and media used for experiments

Category	Strain	Media
Bacteria	*Bacillus subtilis* KFCC 35421	Nutrient broth
	Escherichia coli ATCC 25922	Nutrient broth
	Clostridium perfringens KCTC 5014	Nutrient broth
	Lactobacillus plantarum KCTC 1048	MRS broth
	Listeria monocytogenes KCTC 3569	Brain heart infusion
	Listeria inoccua ATCC 33090	Brain heart infusion
	Pseudomonas aeruginosa KCTC 1636	Nutrient broth
	Salmonella enteritidis ATCC 13076	Nutrient broth
	Salmonella typhimurium ATCC 14028	Nutrient broth
	Serratia liquefaciens KCTC 2925	Nutrient broth
	Staphylococcus aureus ATCC 6538	Nutrient broth
Yeasts	*Saccharomyces cerevisiae* KCTC 7905	Yeast peptone dextrose
Molds	*Aspergillus niger* KCTC 6906	Potato dextrose broth
	Penicillum expansum KCTC 6436	Potato dextrose broth

Thermal stability test To evaluate the effects of heat treatment on the antimicrobial activities of the algae extracts, diluted samples were processed at 60°C for 10, 30, and 60 min; at 80 and 100°C for 10 and 20 min; and at 121°C for 15 min. After the treated samples had quickly cooled, the heat effects were assayed using the agar diffusion method.

pH stability test To evaluated the pH effects on the antimicrobial activities of the algae extracts, diluted samples were adjusted to pH 2, 4, 6, 8, and 10 with 0.1 N NaOH and 0.1 N HCl, and remained at room temperature for 24 hr. After the pH values were readjusted to the original value, the pH effects were assayed using the agar diffusion method.

Results and Discussion

Antimicrobial activity by extracting solvent To evaluate the antimicrobial ability of algae and consider the possible use as natural food preservative, the antimicrobial activity of algae was examined by using their water and ethanol extract. The results for antimicrobial activity of the water and 99% ethanol algae extracts against *Bacillus subtilis* and *Escherichia coli* are summarized in Table 2. Only the ethanol extracts had significant growth inhibitory effects. Twelve species of the ethanol extracts exhibited antibacterial activity against *B. subtilis,* and only the ethanol extract of *Codium fragile* showed antibacterial activity against *E. coli* at 4 mg/mL. These results agree with previous research where antimicrobial activity of algae organic solvent extracts was higher than that of water extracts (26). It can be related to fact that most antimicrobial substance from algae, including terpenoids (19), hydroquinone (22), and bromophenol (17), are extracted by organic solvent.

The active species were *I. okamurai, C. fragile, Calliarthron tuberculosum, Halymenia acuminate, Corallina pilulifera, Corallina vancouverensis, Corallina officinalis, Ecklonia stolonifera, Sargassum thunbergii, Colpomenia bullosa, S. siliquastrum, and E. cava* belonging in the Rhodophyceae or Phaeophyceae except for *C. fragile*. The high efficiency of seaweeds belonging to the Rhodophyceae and Phaeophyceae are in accordance with the results of previous findings (27,28).

Antimicrobial activity using the agar diffusion method Of the 30 algae samples, the 12 algae that indicated antimicrobial activity against *B. subtilis* or *E. coli* were re-evaluated for their antimicrobial activity against other test microorganisms. *I. okamurai, E. stolonifera, H. acuminate, S. thunbergii, C. pilulifera, S. siliquastrum, and E. cava* indicated growth inhibition against all of the tested Gram-positive bacteria at 4 mg/mL, and the others indicated growth inhibition against 5 Gram-positive bacteria. However, these algae showed antibacterial activity against only some tested Gram-negative bacteria. Our data revealed that the tested Gram-negative bacteria were not susceptible to the 12 algae of ethanol extracts, as compared to the Gram-positive bacteria. These results follow the common pattern in antibacterial screening of extracts from natural product including terrestrial plants and algae, according to several reports in the literature (29-31). The observed behavior can be related to fact that Gram-negative bacteria have cell wall lipopolysaccharides that can shield out certain substances (32). As the results of antifungal activity, none of the ethanol extracts of the 12 algae performed against *Aspergillus niger* and *Penicillium expansum;* but, *S. siliquastrum, S. thunbergii, C. bullosa,* and *E. cava* showed growth inhibition effects against *Saccharomyces cerevisiae* at 4 mg/mL (Table 3). In previous studies regarding antimicrobial substances from algae, crinitol from *S. siliquastrum* (33) and the 80% methanol extract of *S. thunbergii* (34) possessed bactericidal activities against Gram-positive bacteria and Gram-negative, respectively. Also, Awad *et al.* (12) reported that the hydrocarbon from the *n*-hexane extracts of *C. officinalis* had antimicrobial activity against Gram-positive bacteria as well as antifungal activity. From this study by Awad *et al.* (12), it is inferred that the antimicrobial substances in the ethanol

Table 2. Antimicrobial activity of algae ethanol extract by the paper disc method[1)] (concentration: 4 mg/mL)

Species	*B. subtilis*[2)]		*E. coli*	
	WE	EE	WE	EE
Chlorophyceae				
Codium fragile	-	+	-	+
Zostera marina	-	-	-	-
Ulva pertusa	-	-	-	-
Rhodophyceae				
Ishige okamurai	-	+	-	-
Ecklonia stolonifera	-	+	-	-
Diophus okamurai	-	-	-	-
Colpomenia sinuosa	-	-	-	-
Sargassum silliquastrum	-	++	-	-
Chorda filum	-	-	-	-
Sargassum thunbergii	-	+	-	-
Sargassum confusum	-	-	-	-
Sargassum micracanthum	-	-	-	-
Colpomenia bullosa	-	+	-	-
Laminaria japonia	-	-	-	-
Ecklonia cava	-	+	-	-
Kjellmaniella crassifolia	-	-	-	-
Phaeophyceae				
Porphyra pseudolinearis	-	-	-	-
Lomentaria catenata	-	-	-	-
Calliarthron tuberculosum	-	+	-	-
Meristotheca papulasa	-	-	-	-
Grateloupia turuturu	-	-	-	-
Chondrus ocellatus	-	-	-	-
Halymenia acuminate	-	+	-	-
Corallina pilulifera	-	+	-	-
Corallina vancouvrensis	-	+	-	-
Lomentaria hakodatensis	-	-	-	-
Laurencia pinnata	-	-	-	-
Grateloupia okamurai	-	-	-	-
Pachymeniopsis elliptica	-	-	-	-
Corallina officinalis	-	+	-	-

[1)]Growth inhibition size of clear zone: -, not detected; +, smaller than 1.5 mm; ++, 1.5-3 mm; +++, 3-5 mm; ++++, lager than 5 mm.
[2)]WE, water extract; EE, ethanol extract.

Table 3. Antimicrobial activity of algae ethanol extract[1)]

(concentration: 4 mg/mL)

	Ishige okamurai	*Codium fragile*	*Ecklonia stolonifera*	*Callicarthron tuberculosum*	*Halymenia acuminata*	*Corallina pilulifera*	*Corallina vancouverensis*	*Sargassum siliquastrum*	*Sargassum thunbergii*	*Colpomenia bullosa*	*Ecklonia cava*	*Corallina officinalis*
B. subtilis	+	+	+	+	+	+	+	++	+	+	+	+
S. aureus	+	+	+	+	+	+	-	+	+	+	+	+
L. inoccua	+	-	+	-	+	+	+	+	+	+	+	-
C. perfringens	++++	+++	+++	++	++	++	++	++++	++++	+++	++	++
L. monocytogenes	+	+	+	+	+	+	+	++	+	+	+	+
L. plantarum	+	+	+	+	+	+	+	+	+	-	+	+
E. coli	-	+	-	-	-	-	-	-	-	-	-	-
S. liquefaciens	+	-	-	+	+	+	-	+	+	+	+	-
S. enteritidis	+	-	-	-	-	-	+	-	-	-	+	-
S. typhimurium	+	-	-	+	+	-	-	+	+	-	+	-
P. aerugenosa	+	+	-	-	-	-	-	-	+	-	-	-
A. niger	-	-	-	-	-	-	-	-	-	-	-	-
P. expansum	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae	-	-	-	-	-	-	-	+	+	+	+	-

[1)]Growth inhibition size of clear zone: -, not detected; +, smaller than 1.5 mm; ++, 1.5-3 mm; +++, 3-5 mm; ++++, lager than 5 mm.

Table 4. Minimum inhibitory concentrations of algae ethanol extracts on various microbial strains

(unit: %)

	B. subtilis	*S. aureus*	*C. perfringens*	*L. monocytogenes*	*L. plantarum*	*S. liquefaciens*	*S. typhimurium*	*S. cerevisiae*
Ishige okamurai	0.1	0.4	0.01	0.075	0.3	0.6	0.8	ND[1)]
Ecklonia stolonifera	0.3	0.1	0.05	0.06	0.3	ND	ND	ND
Sargassum siliquastrum	0.0075	0.1	0.005	0.005	0.1	0.8	0.4	0.4
Sargassum thunbergii	0.3	0.3	0.01	0.1	0.8<	0.8<	0.8<	0.4
Colpomenia bullosa	0.06	0.5	0.03	0.1	0.3	0.8<	ND	0.4
Ecklonia cava	0.075	0.06	0.05	0.075	0.075	0.2	0.3	0.4

[1)]Not done.

extracts of *C. officinalis* tested in our study contained hydrocarbon, given the extractive efficiency by the polarity between ethanol and hexane.

MIC test The results of the MIC test for the ethanol extracts of *I. okamurai, E. stolonifera, S. siliquastrum, S. thunbergii, C. bullosa,* and *E. cava* indicated they had efficient antimicrobial activities, and are summarized in Table 4. The 6 algae showed week antimicrobial activity against Gram-nagative bacteria and *S. cerevisiae*, with MIC values of 0.2-0.8% or above. On the other hand, they had superior antimicrobial activity against the Gram-positive bacteria. By these 6 algae, the 2 most susceptible organisms were *C. perfringens* and *L. monocytogenes*, and the algae possessing the strongest effects were *S. siliquastrum* and *E. cava*. The MIC values of *S. siliquastrum* against *B. subtilis, L. monocytogenes,* and *C. perfringens* were 0.005-0.0075%, and those of *E. cava* were 0.05-0.075%. *I. okamurai* and *E. stolonifera* exhibited moderate antimicrobial activity. The MIC values against Gram-positive bacteria were 0.01-0.1% for *I. okamurai,* and 0.05-0.3% for *E. stolonifera*. *S. thunbergii* inhibited the growth of *B. subtilis, Staphylococcus aureus,* and *L. plantarum* at concentrations of 0.1-0.3%, and inhibited *C. perfringens* at 0.01%. *C. bullosa* indicated growth inhibition against *S. aureus, L. plantarum,* and *L. monocytogenes* at concentrations of 0.1-0.5%, and inhibited *B. subtilis* and *C. perfringens* at 0.06 and 0.03%, respectively. The antimicrobial activities of the 6 algae against *L. monocytogenes* were more effective as compared to those of methanol extracts from *Symphyocladia latiuscula* (MIC 0.125%) (23). *S. siliquastrum* and *E. cava,* possessing the strongest effects, showed greater antimicrobial activity against *B. subtilis* and *L. plantarum* than green tea, which is well known as having antimicrobial activity (35). Additionally, the sodium propionate and benzoic acid, which are used as food preservatives, inhibited the growth of *L. monocytogenes* at concentrations of 0.15-1.1 and 0.6% and over, respectively (36), and potassium sorbate and sodium benzonate inhibited the growth of *S. aureus* and *Salmonella typhimurium* at concentrations of 0.53-0.98% (37). When compared with these results, the 6 algae may have possible uses as natural food preservatives. Moreover, considering the fact that the 6 algae extracts were crude extracts which are complex mixture of many compounds and the portion of active compounds is very low, their possible use for natural food preservative is great.

Base on the previous researches about antimicrobial activity of brown algae, hydroquinone (22), terpenoid (38), chromanol (39), and phlorotannin (40) were reported as antimicrobial substance from brown algae. Especially, the genus *Sargassum* are known to contain sargaquinoic acid derivatives (41) and acryl diterpene alcohol (38) having antibacterial effect against Gram-positive, and the genus of *Ecklonia* are known to contain phlorotannins such as eckol and eckol-related compound having bactericical activity

Table 5. Effects of heat treatment on the antimicrobial activity of the algae ethanol extract[1)] (concentration: 4 mg/mL)

		B. subtilis[2)]			*S. aureus*			*C. perfringens*			*L. monocytogenes*			*L. plantarum*		
		IO	ES	SS	IO	ES	SS	IO	ES	SS	IO	ES	SS	IO	ES	SS
Untreated		+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
60°C	10 min	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
	30 min	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
	60 min	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
80°C	10 min	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
	20 min	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
100°C	10 min	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
	20 min	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
121°C	15 min	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+

[1)]Growth inhibition size of clear zone: -, not detected; +, smaller than 1.5 mm; ++, 1.5-3 mm; +++, 3-5 mm; ++++, lager than 5 mm.
[2)]IO, *Ishige okamurai*; ES, *Ecklonia stolonifera*; SS, *Sargassum siliquastrum*.

Table 6. Effects of heat treatment on the antimicrobial activity of the algae ethanol extract[1)] (concentration: 4 mg/mL)

		B. subtilis[2)]			*S. aureus*			*C. perfringens*			*L. monocytogenes*			*L. plantarum*		
		ST	CB	EC	ST	CB	EC	ST	CB	EC	ST	CB	EC	ST	CB	EC
Untreated		+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
60°C	10 min	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
	30 min	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
	60 min	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
80°C	10 min	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
	20 min	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
100°C	10 min	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
	20 min	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
121°C	15 min	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+

[1)]Growth inhibition size of clear zone: -, not detected; +, smaller than 1.5 mm; ++, 1.5-3 mm; +++, 3-5 mm; ++++, lager than 5 mm.
[2)]ST, *Sargassum thunbergii*; CB, *Colpomenia bullosa*; EC, *Ecklonia cava*; ND, not done.

(40). Although we do not yet purified active compound in this study, it is considered that *S. siliquastrum, S. thunbergii, E. cava,* and *E. stolonifera* contain terpenoid or phlorotannin, given the similarity of the chemical taxonomy of secondary metabolites produced from the same genus. So, study to purify and identify the active compound from these brown algae ethanol extracts is in progress now.

Effects of temperature and pH on antimicrobial activity

Preservatives must be stable at heat and pH because most processed foods usually undergo heat and pH treatments to improve their acceptability and shelf-life, or to maintain the properties of products. Therefore, we evaluated the effects of heat and pH on the ethanol extracts of the 6 brown algae that showed great growth inhibition. In the thermal stability test, all the algae, except *E. stolonifera,* maintained high antimicrobial activities, even when treated at 121°C for 15 min; while the antimicrobial activity of *E. stolonifera* against *C. perfringens* was just slightly reduced in all conditions, except by the treatment at 60°C for 10 min (Table 5, 6). When referring to our results above, it is suggested that the antimicrobial substances of the 6 brown algae are low molecular weight and have stable chemical structures, such as phenolic or halogen compounds (34). Consequently, the ethanol extracts of the 6 brown algae may have used as food preservatives with heat processing. Particularly, these ethanol extracts could be used with sterile canned and retort products because they maintained their antimicrobial activity against *C. perfringens,* which can cause spoilage in retort and canned products even when they are treated at 121°C for 15 min.

In the pH stability test, among the 6 algae, *S. siliquastrum* maintained its antimicrobial activity under all of the pH conditions (pH 2-10), but the other algae indicated weak activity for several of the test microorganisms at pH 10. The antimicrobial activity of *I. okamurai* against *C. perfringens* was reduced, and those of *E. stolonifera, S. thunbergii,* and *E. cava* against the test microbes (except *B. subtilis*) were reduced, and the activity of *C. bullosa* against *C. perfringens* and *L. monocytogenes* was reduced (Table 7, 8). Yet, having reduced antimicrobial activity at pH 10 is not a problem in terms of use as natural food preservatives because the pH of foods is generally sub-acid or neutral.

In conclusion, the results of the present study suggest that the 6 brown algae are an interesting source that can be used as natural food preservative. However, before starting the use of algae for natural food preservatives, the investigations regarding toxicity of algae extracts, fraction and purified compounds have to be done.

Table 7. Effects of pH treatment on the antimicrobial activity of the algae ethanol extract[1)] (concentration: 4 mg/mL)

pH	*B. subtilis*[2)]			*S. aureus*			*C. perfringens*			*L. monocytogenes*			*L. plantarum*		
	IO	ES	SS	IO	ES	SS	IO	ES	SS	IO	ES	SS	IO	ES	SS
Untreated	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
2	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
4	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
6	+	+	++	+	+	+	++++	+++	++++	+	+	++	+	+	+
8	+	+	++	+	+	+	++++	++	++++	+	+	++	+	+	+
10	+	+	++	+	-	+	+++	++	++++	+	-	++	+	-	+

[1)]Growth inhibition size of clear zone: -, not detected; +, smaller than 1.5 mm; ++, 1.5-3 mm; +++, 3-5 mm; ++++, lager than 5 mm.
[2)]IO, *Ishige okamurai*; ES, *Ecklonia stolonifera*; SS, *Sargassum siliquastrum*.

Table 8. Effects of pH treatment on the antimicrobial activity of the algae ethanol extract[1)] (concentration: 4 mg/mL)

pH	*B. subtilis*[1)]			*S. aureus*			*C. perfringens*			*L. monocytogenes*			*L. plantarum*		
	ST	CB	EC	ST	CB	EC	ST	CB	EC	ST	CB	EC	ST	CB	EC
Untreated	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
2	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
4	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
6	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
8	+	+	+	+	+	+	++++	+++	++	+	+	+	+	ND	+
10	+	+	+	-	+	-	+++	++	+	-	-	-	-	ND	-

[1)]Growth inhibition size of clear zone: -, not detected; +, smaller than 1.5 mm; ++, 1.5-3 mm; +++, 3-5 mm; ++++, lager than 5 mm.
[2)]ST, *Sargassum thunbergii*; B, *Colpomenia bullosa*; EC, *Ecklonia cava*; ND, Not done.

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