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Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*

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The bactericidal activity of phlorotannins from brown algae against food-borne pathogenic bacteria (25 strains), methicillin-resistant *Staphylococcus aureus* (MRSA) (nine strains) and *Streptococcus pyogenes* (one strain) was examined and compared with that of catechins. In addition, the effect of the oral administration of phlorotannins on mice was investigated. Phlorotannins, which are oligomers of phloroglucinol, were extracted from thalli of the brown alga *Ecklonia kurome* and prepared by silicic acid chromatography. The bactericidal activity of polyphenols was determined using a broth microdilution method. Of the bacteria tested, *Campylobacter* spp. were the most susceptible to the phlorotannins. The MBCs of the crude phlorotannins, dieckol and 8,8′-bieckol (hexamers), and that of epigallocatechin gallate (EGCG) against *Campylobacter jejuni* were 50 mg/L, 0.03 μmol/mL and 0.03 μmol/mL, respectively. On the whole, the bactericidal effects of the phlorotannins were more pronounced than those of the catechins. The phlorotannins were as effective against MRSA as against the other bacteria tested. At twice the MBCs, all *Vibrio parahaemolyticus* were killed within 0.5–2 h. However, at the same concentration, catechins showed little bactericidal activity within 4 h. No effect on mice was observed with oral administration of the phlorotannins under the conditions tested.

Introduction

Food-borne illnesses and methicillin-resistant *Staphylococcus aureus* (MRSA) infections are still a major problem in the world today. To help prevent and to treat these illnesses, many researchers have been studying the antimicrobial effects of various plant extracts, such as essential oils and tannins.[1,2] Recently, the antimicrobial activities of tea (*Camellia sinensis*) extracts and/or tea catechins have attracted special interest.[3–5] Among tea catechins, epigallocatechin gallate (EGCG) has been shown to have the strongest antimicrobial activity.[5,6] Similarly, several papers have described antimicrobial activity in algae.[7,8] Horikawa *et al.*[9] reported significant anti-MRSA activity by crude methanol extracts from 11 species of Japanese marine algae, and they isolated four bromindoles from the red alga *Laurencia brongniartii* as antibacterial substances. However, their study did not investigate the antibacterial substances of brown algae. To our knowledge, the antibacterial or bactericidal activity of marine tannins against food-borne pathogenic bacteria and MRSA has yet to be reported. *Ecklonia kurome* has been utilized as a food product in Japan, and phlorotannins, which are oligomers of phloroglucinol, have been reported to be both anti-plasmin inhibitors[10,11] and antioxidants.[12] They have also been found to have algicidal activities against red tide microalgae (our unpublished data).

The present study was undertaken in order to examine the bactericidal effects of crude and purified phlorotannins from the brown alga *E. kurome* on pathogenic bacteria, primarily including food-borne pathogenic bacteria and MRSA. In addition, the effect of the administration of phlorotannins on male and female mice was investigated in order to confirm the safety of phlorotannins for mammals.

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Materials and methods

Bacterial strains and growth conditions for preincubation

Bacillus cereus (two strains), MRSA (nine strains), *S. aureus*, *Streptococcus pyogenes*, *Campylobacter fetus*, *Campylobacter jejuni* (three strains), *Escherichia coli* (three strains), *Salmonella enteritidis* (nine strains), *Salmonellae typhimurium* (four strains) and *Vibrio parahaemolyticus* (two strains) were obtained from our collection or from clinical isolates held within The Chemo-Sero-Therapeutic Research Institute.

The reference strains used in tests were *S. aureus* Smith, *S. aureus* ATCC 25923 (MRSA), *S. pyogenes* ATCC 19615, *B. cereus* ATCC 19637, *C. fetus* CIP 53105, *C. jejuni* CIP 702, *E. coli* ATCC 25922, *E. coli* NIHJ JC-2, *S. enteritidis* S-48, *S. enteritidis* L-248, *S. enteritidis* L-540, *S. typhimurium* L-719, *S. typhimurium* L-767, *S. typhimurium* SIC-8401 and *S. typhimurium* ATCC 14028.

All the bacteria except for *Campylobacter* spp. were cultured aerobically without shaking in 10 mL of brain heart infusion (BHI) broth (Nissui, Tokyo, Japan) at 37°C for 18 h. NaCl (2.5%) was added to the medium used for *V. parahaemolyticus*. *Campylobacter* spp. were grown on fresh Skirrow agar plates (Merck, Tokyo, Japan) at 37°C for 48 h in a Campy Pouch (Becton Dickinson, San Jose, CA, USA).

Preparation of phlorotannins

The brown alga *E. kurome* Okamura was collected from an undersea forest of algae near Tsuji Island, Kumamoto, Japan, in May 2000 and washed with tap water to remove sediment and epiphytes, before being air-dried and pulverized. The algal powder (800 g, moisture ~10%) was extracted with methanol (2400 mL) with shaking (90 rpm) at 5°C for 48 h. The extracts were concentrated *in vacuo* to a small volume before methanol (240 mL), chloroform (480 mL) and deionized water (180 mL) were added, and then the upper and lower layers were separated.[13] The upper layer was extracted twice with ethylacetate (300 mL). The ethylacetate fraction was evaporated *in vacuo*. The extract is hereafter referred to as crude phlorotannins. The algal powder produced a yield of phlorotannins of ~3%.

The crude phlorotannins were composed of phloroglucinol (2%) and its oligomers: eckol (trimer, 9%), phlorofucofuroeckol A (pentamer, 28%), dieckol (hexamer, 24%), 8,8′-bieckol (hexamer, 7%) and others (unidentified tetramer, etc., 30%). The components were identified by thin-layer chromatography (TLC)[12] and determined quantitatively by high-performance liquid chromatography (Inertsil ODS column, 6 × 150 mm, GL Science, Tokyo, Japan). Elution was performed at a flow rate of 1.0 mL/min using a linear gradient of 30–100% methanol with UV detection at 290 nm. These compounds were fractionated by silicic acid chromatography on a column (15 mm i.d. × 150 cm, Wakogel C-300HG; Wako Pure Chemical Industries, Osaka, Japan) with chloroform: methanol:water (80:20:2, v/v) as the eluent. Each 5 mL fraction was collected and evaporated *in vacuo*. Consecutive fractions that showed a single spot representing eckol, phlorofucofuroeckol A, dieckol or 8,8′-bieckol on TLC were pooled (>90% purity). Phloroglucinol (as dihydrate, >98% purity) was obtained from Wako Pure Chemical Industries. Terrestrial polyphenols, catechin (>90% purity) and EGCG (>95% purity) were obtained from Kurita Water Industries (Tokyo, Japan).

Determination of bactericidal effects

The MBC of the phlorotannins, taken as the lowest concentration that could kill 99.9% of the initial inoculum within 24 h, was determined using a broth microdilution method. All tests were performed in sensitivity test broth (StB; Eiken Chemical, Tokyo, Japan) in 24-well microtitre plates. All cultured cells were suspended in StB to give a concentration of 10^4–10^5 cfu/mL, and 1 mL of broth culture was pipetted into each well. The concentration of each inoculum was determined using viable counts on BHI agar (BHIA) plates for bacteria. Fresh Skirrow agar plates or 2.5% NaCl-added BHIA plates were used for *Campylobacter* or *Vibrio* spp., respectively. The isolated or crude phlorotannins and catechins were dissolved in 70% methanol, and 20 μL of the solution was added to each well, before being incubated with shaking (60 rpm) aerobically at 37°C for 24 h. To determine the MBCs, 20–200 μL of culture was removed from each well at various time points and incubated in BHIA. After aerobic incubation at 37°C for 24 h, the colonies were enumerated. *V. parahaemolyticus* was tested in medium supplemented with 2.5% NaCl. Fresh Skirrow agar plates were used for *Campylobacter* spp. instead of BHIA, and these were incubated for 72 h. The microtitre and fresh agar plates seeded with *Campylobacter* spp. were incubated in a Campy Pouch. The methanol (final concentration, 1.4%) in the medium did not affect the growth of the strains during the experiments. The experiments were repeated two or three times.

Administration of crude phlorotannins to mice

Two hundred inbred 4-week-old ICR (Institute of Cancer Research) mice were obtained from Japan SLC (Shizuoka, Japan). After they had been acclimatized for 1 week, they were maintained throughout the study in a specific pathogen-free environment with a temperature of 24 ± 2°C, a humidity of 60 ± 15% and a 12 h light–dark cycle. The mice were housed in aluminum cages, each of which held 10 mice, and they were provided with the pellet diet CE-2 (CLEA Japan, Tokyo, Japan) *ad libitum*. In Experiment 1, crude phlorotannins were freely available. The mice were provided with the phlorotannins in solution (5000, 2500, 1250, 625 or 0 mg/L

in tap water) *ad libitum* in 200 mL plastic water bottles. In Experiment 2, the crude phlorotannins were administered only once. After intragastric administration of 1 mL of the phlorotannin solution (5000, 2500, 1250, 625 or 0 mg/L in phosphate buffered saline), the mice were allowed free access to food and tap water. Each group comprised 10 male and 10 female mice. Body weights were recorded every 2 or 3 days: the initial weights of the male and female mice were 30.1 ± 1.6 and 25.8 ± 1.4 g (mean ± S.D.), respectively. The consumption of the phlorotannin solution was measured every day in Experiment 1. The experiments continued for 14 days. This study was approved by the Ethics Committee for Animal Experiments, the Chemo-Sero-Therapeutic Research Institute.

Results

Bactericidal activity of crude phlorotannins

The crude phlorotannins showed bactericidal activity against all 35 strains tested: *S. aureus* (MRSA) (MBCs, 100–200 mg/L), *S. aureus* (MBC, 100 mg/L), *S. pyogenes* (MBC, 400 mg/L), *B. cereus* (MBCs, 200–400 mg/L), *C. fetus* (MBC, 50 mg/L), *C. jejuni* (MBC, 50 mg/L), *E. coli* (MBCs, 200–400 mg/L), *S. enteritidis* (MBCs, 200–800 mg/L), *S. typhimurium* (MBC, 200 mg/L) and *V. parahaemolyticus* (MBC, 200 mg/L). Overall, *Campylobacter* spp. were the most susceptible, followed by *S. aureus*. There was considerable variation in the MBCs among the different strains of *S. enteritidis*. Of all the bacteria tested, only two strains of *Vibrio* were killed within 2 h at twice the MBC. The other bacteria were largely unaffected by this concentration, even at 4 h (data not shown).

Bactericidal activity of purified phlorotannins

The MBCs of five purified phlorotannins (phloroglucinol, eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol), catechin and EGCG against six reference strains and the clinical isolate of *V. parahaemolyticus* were determined (Table 1). For all the polyphenols, the MBCs were lowest for *C. jejuni* among the bacteria tested. The lowest MBCs against *C. jejuni* and the other bacteria were 0.03 and 0.13–0.54 μmol/mL of both dieckol and 8,8′-bieckol; 0.08 and 0.33–1.08 μmol/mL of both eckol and phlorofucofuroeckol A; and 0.03 and 0.44–1.75 μmol/mL for EGCG, respectively. On the whole, the bactericidal effects of eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol were more pronounced than those of the catechins, although EGCG had a similar MBC to both dieckol and 8,8′-bieckol against *C. jejuni*.

Like the crude phlorotannins, the purified phlorotannins killed only *V. parahaemolyticus* within a short time (Figure 1). At twice the MBCs, eckol, phlorofucofuroeckol A and dieckol all killed the bacteria within 0.5 h, and 8,8′-bieckol killed them within 2 h; on the other hand, EGCG did not show any bactericidal activity, and yet it inhibited the growth of the bacteria within 4 h.

Administration of crude phlorotannins to mice

In Experiment 1, the maximum daily intake of phlorotannins was 1500 mg/kg of initial body weight in males and 1286 mg/kg in females. In Experiment 2, the maximum dose of phlorotannins was 168.2 mg/kg in males and 193.7 mg/kg in females. Survival in all groups at the end of 14 days was 100% (Table 2). Moreover, no harmful effects were observed even with the largest administration of crude phlorotannins, and the rate of weight gain in all groups was no less than that of the controls. No signs or symptoms of disorder were observed in any of the groups, in either experiment.

Discussion

This is the first study to demonstrate bactericidal activity of brown algal phlorotannins against a range of pathogenic bacteria. All the crude and purified phlorotannins, with the

Table 1. MBCs of five purified phlorotannins, catechin and EGCG against seven strains of pathogenic bacteria

	MBC (μmol/mL)						
Strain	phloroglucinol	eckol	phlorofucofuroeckol A	dieckol	8,8′-bieckol	catechin	EGCG
Gram-positive bacteria							
S. aureus ATCC 25923 (MRSA)	>6.35	0.54	0.33	0.13	0.13	>2.76	0.44
B. cereus ATCC 19637	>6.35	1.08	0.66	0.54	0.54	>2.76	>1.75
Gram-negative bacteria							
C. jejuni CIP 702	0.79	0.08	0.08	0.03	0.03	0.34	0.03
E. coli ATCC 25922	>6.35	0.54	0.66	0.27	0.27	>2.76	1.75
S. enteritidis S-48	>6.35	0.54	0.33	0.27	0.27	>2.76	1.75
S. typhimurium ATCC 14028	>6.35	0.54	0.33	0.27	0.27	>2.76	1.75
V. parahaemolyticus KR1151	>6.35	1.08	0.33	0.27	0.27	>2.76	0.44

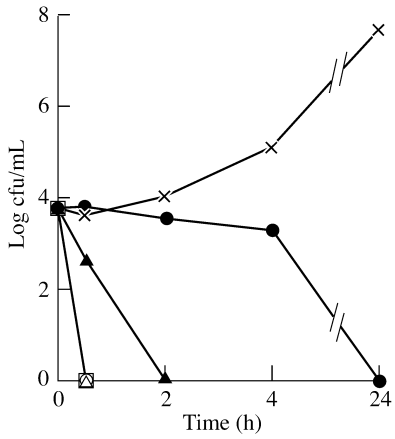

Figure 1. Time–kill curve for *V. parahaemolyticus* KR1151 obtained using isolated phlorotannins and EGCG at twice the MBC. Crosses, without phlorotannins; open squares, eckol; open triangles, phlorofucofuroeckol A; open circles, dieckol; closed triangles, 8,8′-bieckol; closed circles, EGCG.

exception of phloroglucinol, showed bactericidal activity against all the strains tested.

Several studies have found that catechins exhibit stronger antibacterial effects on Gram-positive bacteria than on Gram-negative bacteria.[14,15] However, in the present study, we could not find any remarkable difference in susceptibility to phlorotannins between Gram-positive and Gram-negative bacteria. For example, *Campylobacter* spp., which are Gram-negative, were the most susceptible among the bacteria tested. Moreover, the species specificity of the bactericidal effect of the phlorotannins may be smaller than that of EGCG (Table 1). However, since the sample size (the number and kinds of strains) was small, further experiments may be necessary to confirm these tendencies. Moreover, tests to determine whether or not mutants resistant to the phlorotannins can be generated need to be performed in the future.

Interestingly, the phlorotannins killed *V. parahaemolyticus* within 0.5–2 h when administered at twice the MBC (Figure 1). The reason for this is unclear; however, a similar effect of phlorotannins was found against other *Vibrio* spp., *Vibrio vulnificus*, *Vibrio colerae* and *Vibrio anguillarum* (data not shown).

In this paper, the MBCs of polyphenols are expressed as μmol/mL to allow a comparison of activities per molecule

Table 2. Effect of phlorotannins on weight gain in mice

Experiment[a]	Sex	Concentration of crude phlorotannins (mg/L)	Intake of phlorotannins[b] (mg/kg)	Weight gain (%)[c]	
				7 days	14 days
Expt 1	male ($n = 10$)	5000	1500	116.9 ± 1.1	127.1 ± 2.9
		2500	680	116.5 ± 0.9	127.4 ± 1.6
		1250	347	115.0 ± 0.7	125.5 ± 1.1
		625	170	115.0 ± 1.4	124.7 ± 1.5
		control	0	117.3 ± 0.6	128.7 ± 1.1
	female ($n = 10$)	5000	1286	110.6 ± 2.8	120.1 ± 1.3
		2500	619	107.3 ± 0.9	117.0 ± 1.6
		1250	345	107.9 ± 1.4	115.4 ± 2.4
		625	199	108.8 ± 0.9	119.6 ± 2.3
		control	0	109.2 ± 1.3	118.1 ± 1.3
Expt 2	male ($n = 10$)	5000	168.2	115.6 ± 1.0	126.6 ± 1.4
		2500	80.7	115.2 ± 0.9	126.7 ± 1.3
		1250	42.3	118.9 ± 1.8	130.1 ± 1.8[d]
		625	20.8	114.3 ± 0.5	125.5 ± 1.0
		control	0	114.7 ± 1.0	125.1 ± 1.2
	female ($n = 10$)	5000	193.7	107.7 ± 0.8	117.9 ± 1.1
		2500	92.0	106.1 ± 0.9	115.8 ± 2.0
		1250	49.3	109.2 ± 0.9	121.3 ± 1.8
		625	24.5	108.1 ± 1.2	116.4 ± 1.8
		control	0	108.0 ± 1.4	116.2 ± 1.4

[a]In Experiment 1, the mice were provided with phlorotannins in solution *ad libitum*. In Experiment 2, the mice were administered 1 mL of phlorotannin solution.
[b]Daily intake of phlorotannins per initial weight in Experiment 1.
[c]Weight gain is shown as the mean ± S.E. of 10 mice.
[d]Significantly different from control ($P < 0.05$, Student's *t*-test).

(Table 1). The bactericidal effects of phlorotannins tend to increase with polymerization of phloroglucinol. This tendency is different from the case of tea catechins, whose effects are augmented with the addition of the gallate group.

Schulz *et al*.[16] suggested that the antimicrobial activity of polyphenols may result from their interaction with bacterial enzymes and proteins. Ikigai *et al*.[14] reported that catechins damage bacterial cell membranes. Furthermore, strong interactions between algal phlorotannins and proteins have been reported by Stern *et al*.[17] Although the mode of activity of phlorotannins is still obscure, the interactions of phlorotannins with bacterial proteins may play an important role in the bactericidal action of phlorotannins.

E. kurome as well as *Eisenia bicyclis* is used as a food product in some areas of Japan. Accordingly, it is of interest to note that no harmful effects were observed on oral administration of a large quantity of phlorotannins in mice, corresponding to a human dose of 90.0 g/60 kg per day in males and 64.3 g/50 kg per day in females, or a single dose of 10.1 g/60 kg in males and 9.7 g/50 kg in females.

The results obtained in the present study suggest that phlorotannins have pronounced bactericidal activity against pathogenic bacteria. Therefore, phlorotannins as well as *E. kurome* and *E. bicyclis* may be useful as a food product or as a drug with antibacterial activity.

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References

1. May, J., Chan, H. C., King, A., Williams, L. & French, L. G. (2000). Time–kill studies of tea tree oils on clinical isolates. *Journal of Antimicrobial Chemotherapy* **45**, 639–43.

2. Burapadaja, S. & Bunchoo, A. (1995). Antimicrobial activity of tannins from *Terminalia citrina*. *Planta Medica* **61**, 365–6.

3. Yam, T. S., Shah, S. & Hamilton-Miller, J. M. T. (1997). Microbiological activity of whole and fractionated crude extracts of tea (*Camellia sinensis*), and of tea compounds. *FEMS Microbiology Letters* **152**, 169–74.

4. Kolodzeij, H., Kayser, O., Latte, K. P. & Ferreira, D. (1999). Evaluation of the antimicrobial potency of tannins and related compounds using the microdilution broth method. *Planta Medica* **65**, 444–6.

5. Amarowicz, R., Pegg, B. R. & Bautista, A. D. (2000). Antibacterial activity of green tea polyphenols against *Escherichia coli* K12. *Nahrung* **44**, 60–2.

6. Mabe, K., Yamada, M., Oguni, I. & Takahashi, T. (1999). *In vitro* and *in vivo* activities of tea catechins against *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy* **43**, 1788–91.

7. Caccamese, S., Azzolina, R., Furnari, G., Cormaci, M. & Grasso, S. (1981). Antimicrobial and antiviral activities of some marine algae from eastern Sicily. *Botanica Marina* **14**, 365–7.

8. Bennamara, A., Abourriche, A., Berrada, M., Charrouf, M., Chaib, N., Boudouma, M. *et al*. (1999). Methoxybifurcarenone: an antifungal and antibacterial meroditerpenoid from the brown alga *Cystoseira tamariscifolia*. *Phytochemistry* **52**, 37–40.

9. Horikawa, M., Noro, T. & Kamei, Y. (1999). *In vitro* anti-methicillin-resistant *Staphylococcus aureus* activity found in extracts of marine algae indigenous to the coastline of Japan. *Journal of Antibiotics* **52**, 186–9.

10. Nakayama, Y., Takahashi, M., Fukuyama, Y. & KinZyo, Z. (1989). An anti-plasmin inhibitor, eckol, isolated from the brown alga *Ecklonia kurome* OKAMURA. *Agricultural and Biological Chemistry* **63**, 3025–30.

11. Fukuyama, Y., Kodama, M., Miura, I., KinZyo, Z., Mori, H., Nakayama, Y. *et al*. (1990). Anti-plasmin inhibitor. VI. Structure of phlorofucofuroeckol A, a novel phlorotannin with both dibenzo-1,4-dioxin and dibenzofuran elements, from *Ecklonia kurome* OKAMURA. *Chemical and Pharmaceutical Bulletin* **38**, 133–5.

12. Nakamura, T., Nagayama, K., Uchida, K. & Tanaka, R. (1996). Antioxidant activity of phlorotannins isolated from the brown alga *Eisenia bicyclis*. *Fisheries Science* **62**, 923–6.

13. Folch, J., Less, M. & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipid from animal tissues. *Journal of Biological Chemistry* **36**, 93–8.

14. Ikigai, H., Nakae, T., Hara, Y. & Shimamura, T. (1993). Bactericidal catechins damage the lipid bilayer. *Biochimica et Biophysica Acta* **1147**, 132–6.

15. Toda, M., Okubo, S., Ikigai, H. & Shimamura, T. (1990). Antibacterial and anti-hemolysin activities of tea catechins and their structural relatives. *Japanese Journal of Bacteriology* **45**, 561–6.

16. Schulz, J., Hunter, M. & Appel, H. (1992). Antimicrobial activity of polyphenols mediates plant–herbivore interactions. In *Plant Polyphenols* (Hemingway, R. W. & Lakes, P. E., Eds), pp. 621–37. Plenum Press, New York, NY, USA.

17. Stern, J., Hagerman, A., Steinberg, P. & Magon, P. (1996). Phlorotannin–protein interactions. *Journal of Chemical Ecology* **22**, 1877–99.