American Journal of Plant Sciences, **2014, 5, 387-392**
Published Online February 2014 (http://www.scirp.org/journal/ajps)
http://dx.doi.org/10.4236/ajps.2014.53051

The Influence of Phlorotannins and Bromophenols on the Feeding Behavior of Marine Herbivorous Gastropod *Turbo cornutus*

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Received December 6th, 2013; revised January 16th, 2014; accepted January 28th, 2014

ABSTRACT

The influence of phlorotannins and bromophenols on the feeding behavior of marine herbivores was determined using bioassay of *Turbo cornutus*. It was found that phloroglucinol and its oligomers isolated from the brown alga *Eisenia bicyclis* (eckol, fucofuroeckol A, phlorofucofuroeckol A, dieckol, and 8,8'-bieckol) had a deterrent effect against feeding behavior of *T. cornutus* in the concentration of algal body, respectively. In the case of the examination of 0.1 mM concentration, although fucofuroeckol A and phlorofucofuroeckol A significantly reduced feeding by *T. cornutus*, phloroglucinol and 8,8'-bieckol did not show any significant influence on feeding behavior, and eckol and dieckol had stimulating activity. 2,4-Dibromophenol and 2,4,6-tribromophenol, which are major components of extracellular secretions from *Eisenia* and *Ecklonia* species, caused the death, the torpidity, and the decreased appetite of *T. cornutus* at the concentration of 0.1 mM. In addition, 2,4-dibromophenol had strong feeding deterrent activity at the concentration of 1 µM. These results indicate that phlorotannins and bromophenols act as chemical defense agents of brown algae against environmental stresses such as the herbivore attack.

KEYWORDS

Bromophenols; Brown Algae; Chemical Defenses; Feeding Deterrent; *Eisenia bicyclis*; Phlorotannins; Plant-Herbivore Interactions; Turban Shell; *Turbo cornutus*

1. Introduction

Phlorotannins of marine algal polyphenols, which have been found exclusively in brown algae, are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) [1,2]. In marine chemical ecology, phlorotannins (as well as tannins from terrestrial plants) are considered to be an important component of the chemical defense material against natural enemies (e.g., marine herbivores, bacteria, epiphytes and ultraviolet). Many investigators have discussed the biological activities and roles of phlorotannins [1-7]. To our knowledge, however, most of the reports on the defensive function of phlorotannins present no findings regarding a "relationship between chemical structures of the compounds and biological activities", "correlation of the activities and concentration of phlorotannins" and "evaluation of the activities based on actual concentration of the compounds in the algal body".

Previously, we isolated the phlorotannins, these being eckol (a phloroglucinol trimer), fucofuroeckol A (a tetramer), phlorofucofuroeckol A (a pentamer), dieckol and 8,8'-bieckol (hexamers) from Japanese *Eisenia* and *Ecklonia* species and reported their chemical and local distribution in the algae [8]. The brown algae are distrib-

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uted along the temperate coasts of Japan and form a kelp bed called a "marine forest" in the subtidal zone [9]. In the preceding reports [10-12], the following two facts became apparent: 1) the phenolic substances secreted from the living brown algae consisted of the two monomeric bromophenols, 2,4-dibromophenol and 2,4,6-tribromophenol; 2) phloroglucinol and phlorotannins were kept strictly within the algal body, whilst the algae were alive. It is indicated that extracellular phenolic substances of living brown algae have some deterrent activities against natural enemies [1,13-15]. Though it has been thought that the substances are phlorotannins in many past reports [1,13-15], the monomeric bromophenols may play a deterrent role against natural enemies such as marine herbivores.

In this study, we isolated the phlorotannins from *Eisenia bicyclis* and determined the influence of each compound on feeding behavior using a bioassay of marine herbivorous gastropod *T. cornutus*. Then, the influence of bromophenols on their feeding behavior was evaluated. The mechanism of chemical defense *via* phenolic compounds in the brown algae was also discussed.

2. Materials and Methods

2.1. Materials

The brown alga *E. bicyclis* (Kjellman) Setchell, without any visible grazing or other tissue damage, was collected from the coasts of the Itoshima Peninsula (33˚37'N, 130˚10'E) in Fukuoka Prefecture, Japan. The alga was washed with filtered seawater, air-dried, and pulverized. The algal powder was stored at −30˚C until use.

T. cornutus (approx., 6.5 cm in shell length) were purchased from the Fisheries Cooperative Association of Yoshimi in Yamaguchi prefecture, Japan. 2,4-Dibromophenol and 2,4,6-tribromophenol were purchased from Wako Pure Chemicals (Osaka).

2.2. Extraction and Purification of Phlorotannins

Extraction of the phlorotannins from the algal powder was carried out according to the method described in previous reports [11,16,17]. Each of the phlorotannins in the crude extracts was partially purified on a silicic acid column according to the same method described in a previous report [16]. Further purification of the phlorotannins was carried out using preparative HPLC system. The HPLC system consisted of LC-6AD pumps (Shimadzu, Japan), a CBM-20A system controller (Shimadzu, Japan), a SPD-20A UV detector (Shimadzu, Japan) and an Inertsil ODS-3 column (10 mm I.D. × 250 mm, GL Science, Japan). Elution was performed at a flow-rate of 4.7 mL/min with a linear gradient from 30% to 100% MeOH for 20 min, and maintained for an additional 20 min. The UV detector was set at 290 nm. The purity was confirmed by three-dimensional HPLC using a photodiode array detector (SPD-M10AV, Shimadzu, Japan) with an Inertsil ODS-3 column (4.6 mm I.D. × 250 mm, GL Science, Japan) [11,16]. The identification of the purified phlorotannins was carried out using an LC/MS system (6120 Quadrupole LC/MS with 1260 Series HPLC System, Agilent, CA, USA) with an Inertsil ODS-3 column (4.6 mm I.D. × 150 mm, GL Science, Japan). Elution was performed at a flow-rate of 0.5 mL/min with a linear gradient from 30% to 100% MeOH for 20 min, and maintained for an additional 20 min. Each phlorotannin was monitored by ESI-MS/APCI-MS multimode analysis in positive and negative mode.

2.3. Bioassays of Feeding Behavior of the Marine Herbivorous Gastropod, *T. cornutus*

The bioassays of feeding behavior were carried out according to the method of Miyasaki and Harada [18]. The experiment herbivore, *T. cornutus* was fed with the dead brown alga *E. bicyclis*, as its daily feed. The experiment food media were prepared as follows: first, each of the phenolic compounds dissolved in MeOH was mixed with dextran. After removal of the MeOH with a lyophilizer, the mixture was homogenized and this was then dissolved in 40 mL of a 2% agar medium in a Petri dish (9.0 cm i.d. × 8.0 mm). Control medium without the phenolic compounds was also prepared. Each of the experimental and control media was placed in pairs in an aquarium (270 w. × 350 d. × 130 h. mm) with running seawater (150 mL/min) and aeration. *T. cornutus* (8 individuals/aquarium) was allowed to feed for 16 h after sunset. Finally, after the bioassay of the feeding, the amount of media consumed was weighed. The data are presented as mean ± standard deviation of analytical values of independent tests (n = 10). The statistical analysis of the data was performed using t-test ($P < 0.05$).

All reagents used in this experiment were of analytical grade.

3. Results and Discussion

It is shown that the crude extracts from the brown algae including phlorotannins have a feeding deterrent activity against marine herbivores in the past reports [1,3,5,6]. The aim of this study was mainly to clarify the relationship between the chemical structures of phlorotannins and their influence on the feeding of marine herbivores. First, to evaluate the function of each phlorotannins, the compounds were isolated from the *E. bicyclis*. **Figure 1** shows the chemical structures of phlorotannins used in this study. These phlorotannins were refined to a purity

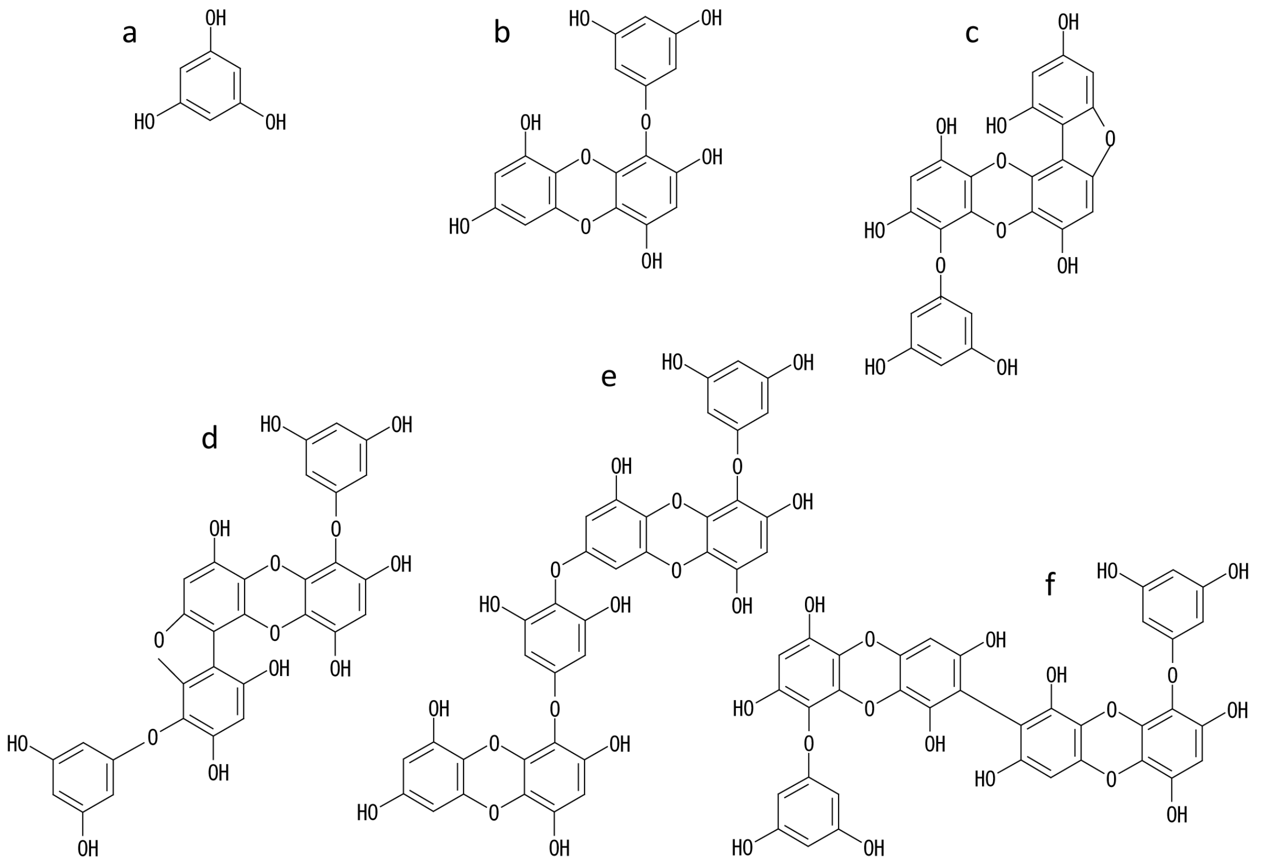

Figure 1. Chemical structures of phloroglucinol and phlorotannins isolated from the brown alga *E. bicyclis*. These compounds were isolated from crude phlorotannins of *E. bicyclis* by column chromatography and preparative HPLC. (a) phloroglucinol; (b) eckol; (c) fucofuroeckol A; (d) phlorofucofuroeckol A; (e) dieckol; (f) 8,8'-bieckol.

of 98% or more *via* column chromatography and preparative HPLC (data not shown). In the previous paper [8], it was revealed that the yield of total phlorotannins among Japanese *Eisenia* and *Ecklonia* species was about 3.0% of the algal powder (dry weight) and the content and composition of the phlorotannins did not vary seasonally. Therefore, based on these findings, each of the phloroglucinol and isolated phlorotannins, which was equivalent to the natural concentration in an algal body, was added to the experiment food medium and used to the bioassay using marine herbivorous gastropod *T. cornutus* (**Table 1**). As a result, without any relation to extent polymerization of phloroglucinol, it is found that all examined compounds have deterrent activity against feeding behavior of *T. cornutus* in the concentration of algal body (**Table 1**). Under the condition of certain concentration, the influence that each compound exerted on the feeding behavior of *T. cornutus* was also examined (**Table 2**). As a result of comparative test, fucofuroeckol A and phlorofucofuroeckol A significantly deterred feeding by *T. cornutus* at the concentration of 0.1 mM. On the other hand, eckol and dieckol have a stimulating effect on feeding activity at the concentration of 0.1 mM and the opposite results were shown (**Table 2**). It was found that phloroglucinol and 8,8'-bieckol did not have significant influence on feeding behavior (**Table 2**).

2,4-Dibromophenol and 2,4,6-tribromophenol, which are halo metabolites, are major components of extracellular secretions from *Eisenia* and *Ecklonia* species [11,12]. Then, an examination of the influence of these bromophenols on the feeding behavior of *T. cornutus* was conducted. In order to compare with the data of phlorotannins shown in **Table 2**, the effect of each bromophenol was measured at the concentration of 0.1 mM. As a result, both monomeric bromophenols caused the death, the torpidity, and the decreased appetite of *T. cornutus* (data not shown). Therefore, it was found that 2,4-dibromophenol and 2,4,6-tribromophenol demonstrated deterrent activity much stronger than that of phlorotannins at the concentration of 0.1 mM. **Table 3** shows the effect of each bromophenol when the concentration in a medium is 1 μM or less. At the concentration of 1 μM, although 2,4-dibromophenol had a strong deterrent effect on feeding activity, 2,4,6-tribromophenol

Table 1. Influence of phloroglucinol and phlorotannins on feeding behavior of marine herbivorous gastropod *T. cornutus*.

Compounds**	Concentration (mM)	Amount of media consumed (g)		Relative to control (A/C)
		Addition (A)	Control (C)	
Phloroglucinol	0.444	15.4 ± 9.2*	26.1 ± 8.9	0.59
Eckol	1.410	13.4 ± 5.2*	19.8 ± 6.9	0.68
Fucofuroeckol A	0.615	4.5 ± 1.2*	9.1 ± 0.9	0.49
Phlorofucofuroeckol A	2.440	14.4 ± 5.7*	23.8 ± 7.5	0.61
Dieckol	2.170	11.7 ± 6.7*	22.6 ± 6.7	0.52
8,8'-Bieckol	1.600	9.4 ± 5.8*	23.5 ± 6.8	0.40

*$P < 0.05$. **The amount of each compounds added to the agar, based on the concentration in the brown alga *E. bicyclis* (Shibata *et al*., 2004). The data are presented as mean ± standard deviation of analytical values of independent tests ($n = 10$).

Table 2. Influence of phloroglucinol and phlorotannins on feeding behavior of *T. cornutus* at the concentration of 0.1 mM.

Compounds	Amount of media consumed (g)		Relative to control (A/C)
	Addition (A)	Control (C)	
Phloroglucinol	11.2 ± 9.0	13.3 ± 8.9	0.84
Eckol	11.8 ± 5.5*	7.5 ± 4.5	1.57
Fucofuroeckol A	10.9 ± 5.6*	16.9 ± 5.4	0.64
Phlorofucofuroeckol A	12.2 ± 5.8*	17.7 ± 6.9	0.69
Dieckol	13.0 ± 2.9*	7.2 ± 2.9	1.81
8,8'-Bieckol	13.3 ± 4.3	17.3 ± 7.8	0.77

*$P < 0.05$. The data are presented as mean ± standard deviation of analytical values of independent tests ($n = 10$).

Table 3. Influence of bromophenols on feeding behavior of *T. cornutus*.

Compounds	Concentration	Amount of media consumed (g)		Relative to control (A/C)
		Addition (A)	Control (C)	
2,4-Dibromophenol	1 μM	3.9 ± 3.2*	6.6 ± 7.2	0.59
	1 nM	14.0 ± 6.9	11.3 ± 3.3	1.24
	1 pM	19.3 ± 6.1*	11.8 ± 4.7	1.64
2,4,6-Tribromophenol	1 μM	6.8 ± 1.7	8.7 ± 2.9	0.78
	10 nM	7.3 ± 1.8	7.9 ± 3.3	0.92
	1 nM	6.4 ± 2.2	7.6 ± 2.6	0.84
	0.1 nM	14.6 ± 4.8	11.7 ± 6.0	1.25
	1 pM	15.6 ± 7.7	16.9 ± 6.6	0.92

*$P < 0.05$. The data are presented as mean ± standard deviation of analytical values of independent tests ($n = 10$).

was not shown. In addition, it was found that 1 pM 2,4-dibromophenol stimulates predation action of *T. cornutus* (**Table 3**). At other concentrations, a difference was not seen between the consumption of a sample medium and control medium in either of the bromophenols (**Table 3**).

In this study, it demonstrated that phloroglucinol and all the examined phlorotannins at concentrations which exist in algal bodies, had feeding deterrent activity against turban shell *T. cornutus*, and furthermore, bromophenols, in particular 2,4-dibromophenol, were the strong feeding deterrent reagents. On the other hand, it was suggested that phenolic compounds of low concentration (eckol, dieckol and 2,4-dibromophenol) have the possibility of stimulating feeding behavior to *T. cornutus*. Previously, we reported that phlorotannins showed inhibitory activity of glycosidases present in the viscera of the *T. cornutus* [10]. Thus, it was suggested that phlorotannins are effective chemical defense agents with the functions of digestive enzyme inhibitor and feeding activity deterrent. Bromophenols are phenols that exist in many algal species [19] and their contents are especially high in red algae and brown algae [19,20]. In the previous report [12], we demonstrated that the secretion of bromophenols was active in the young plants of species *Eisenia* and *Ecklonia*, and their cumulative amount was equivalent to 2.2 mg 2.4-dibromophenol/g wet weight of the plant after 48 h cultivation. Agatsuma [21] and Li [22] reported that 2,4-dibromophenol and 2,4,6-tribromophenol had inhibition of larval survival and metamorphosis of the sea urchin. The sea urchin is a predator of marine algae as well as *T. cornutus*. The *T. cornutus* is a representative marine herbivorous gastropod along the Japanese coast. It is well known that the *T. cornutus* prefers eating dead and/or aged *Eisenia* and *Ecklonia* species rather than live ones. From these findings and the present study, it can be considered that the secretion of bromophenols is greatly advantageous for the survival of brown algae. In conclusion, brown algae produce two types of phenolic compounds, storage phenolic compounds comprising phlorotannins, and secretory phenolic compounds comprising bromophenols, and the compounds act as chemical defense agents of brown algae against environmental stress such as herbivore attack.

Acknowledgements

We acknowledge that the English was revised by Mr. Andrew Rother. This research was supported by Japan Science and Technology Agency, CREST.

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