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Evaluation for Antioxidative Properties of Phlorotannins Isolated from the Brown Alga *Eisenia bicyclis*, by the H-ORAC Method

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ABSTRACT

The antioxidative properties of phlorotannins isolated from the brown alga *Eisenia bicyclis* were measured using the H-ORAC (Hydrophilic Oxygen Radical Absorbance Capacity) method. The ORAC values of phloroglucinol and it's oligomers: eckol, fucofuroeckol A, phlorofucofuroeckol A, dieckol, 8,8'-bieckol, were 2.57 ± 0.14, 4.97 ± 0.36, 9.82 ± 0.70, 8.97 ± 0.89, 10.22 ± 0.85, 8.62 ± 0.92 μmol Trolox equivalent/μmol, respectively. With the exception of eckol, the ORAC values of tested phlorotannins were higher than those of the well-known antioxidants (epigallocatechin gallate, resveratrol and L-ascorbic acid) used as positive controls. As a result of comparing with known ORAC values, it was found that the dieckol and fucofuroeckol A had stronger antioxidant activity than representative polyphenols (e.g., kaempferol, quercetin, myricetin and chlorogenic acid) derived from terrestrial plants.

Keywords: Antioxidants; Brown Alga; Dieckol; *Eisenia bicyclis*; Fucofuroeckol A; H-ORAC Assay; Phlorotannins

1. Introduction

The brown alga *Eisenia bicyclis*, belongs to the order Laminariales within the class of Phaeophyceae [1]. The brown alga is distributed along the temperate coasts from the central to southern parts of Japan, and form a kelp bed called a "marine forest" in the sub-tidal zone [1]. In Japan, this brown alga has been used since ancient times as an industrial source of alginic acid, and is currently attracting attention as a new raw material in the field of biorefinery.

It is known that the *E. bicyclis* produces polyphenols called phlorotannins [2,3]. Most phlorotannins, with the exception of some compounds, are oligomers of phloroglucinol (1,3,5-trihydroxybenzene), and can be divided into six categories, fucols, phlorethols, fucophlorethols, fuhalols, isofuhalols, and eckols [2,4]. Previously, we isolated the phlorotannins; eckol (phloroglucinol trimer), phlorofucofuroeckol A (a pentamer), dieckol, and 8,8'-bieckol (hexamers) from *E. bicyclis*, and reported their distribution [3], 1,1-diphenyl-2-picrylhydrazyl (DPPH)-radical and superoxide anion radical scavenging activities [5].

Recently, the ORAC assay has received much attention as a new *in vitro* method for measuring antioxidant activity [6], and is used to evaluate the antioxidative potencies of vegetables, fruits, their processed products, and phytochemicals [6-10]. In the case of phlorotannins, Parys *et al*. [11] isolated fucophlorethols from the brown alga *Fucus vesiculosus*, and reported their H-ORAC values. The ORAC values of eckols, however, are still obscure.

In this study, we isolated phloroglucinol and eckols from the brown alga *E. bicyclis*, and evaluated their antioxidative properties using the H-ORAC method. From comparison with well-known antioxidants, the availability of phlorotannins as antioxidants was also discussed.

2. Materials and Methods

2.1. Materials

The brown alga *Eisenia bicyclis* was collected from the coasts of the Itoshima Peninsula (33˚37'N, 130˚10'E) in

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Fukuoka prefecture, Japan. The alga was washed with filtered seawater, air-dried and pulverized. The algal powder was stored at −30˚C until used.

Epigallocatechin gallate (EGCG) was kindly donated by Kurita Water Industries (Tokyo, Japan). Resveratrol and L-ascorbic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan).

All reagents used in this study were of analytical grade.

2.2. Extraction and Purification of Phlorotannins

The extraction of the phlorotannins from the algal powder was carried out using the same method described in previous reports [5,12]. 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 [5]. Further purification of the phlorotannins was carried out using a preparative HPLC system. The HPLC system consisted of LC-6AD pumps (Shimadzu, Kyoto, Japan), a CBM-20A system controller (Shimadzu, Japan), a SPD-20A UV detector (Shimadzu, Kyoto, Japan), and an Inertsil ODS-3 column (10 mm I.D. × 250 mm, GL Science, Tokyo, Japan). Elution was performed at a flow-rate of 4.7 ml/min with a linear gradient from 30% MeOH to 100% MeOH for 20 min, and maintained for 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, Kyoto, Japan) with an Inertsil ODS-3 column (4.6 mm I.D. × 250 mm, GL Science, Tokyo, Japan) [5,12]. The identification of the purified phlorotannins was carried out using a LC/MS system (6120 Quadrupole LC/MS with 1260 Series HPLC System, Agilent, CA, USA).

2.3. H-ORAC Assay

The H-ORAC assay of phlorotannins was performed according to the method of Watanabe *et al*. [6] with slight modification. Terrestrial polyphenols (EGCG and resveratrol) and an antioxidant vitamin (L-ascorbic acid) were used as positive controls [9,10]. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) standards (3.125, 6.25, 12.5, 25 μM), fluorescein (110 nM), and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (31.7 mM) solutions were prepared separately by dissolving in a 75 mM phosphate buffer (pH 7.4). Each sample was dissolved in MeOH (100 μg/ml). The phosphate buffer was also used for dilution of the sample solutions.

The measurement was carried out using a 96-well black plate (Costar 3915, Corning, NY, USA) with plate seal (Q-Stick adhesive sheets for qPCR, 4 titude, Surrey, UK) and a 96-well microplate reader (Infinite 200, Tecan Japan, Kanagawa, Japan). The sample solution (10 μl), fluorescein (115 μl) and the phosphate buffer (25 μl) were added to the sample wells in the plate. Fluorescein (115 μl), Trolox, or the phosphate buffer (blank) (35 μl), were added to the Trolox wells or blank wells, respectively. Each plate was incubated at 37˚C. To prevent error of the ORAC value, the plate was preincubated at 37˚C for 5 min before each measurement. The phosphate buffer used for the AAPH solution was also incubated at 37˚C for 30 min before each measurement, and then the AAPH solution was prepared [7,8]. The fluorescence intensity (excitation at 485 nm, emission at 528 nm) was monitored every 2 min for 90 min by a microplate reader. The assays were made in three independent measurements. The area under the curve (AUC) of the fluorescence decay from 8 to 90 min after the addition of AAPH solution was calculated for each well. The net AUC was calculated by subtracting AUC for the sample, or Trolox standard, from that for the blank. A calibration curve was constructed from the net AUCs of Trolox standard solutions, and a power approach was fitted to the results. The H-ORAC value for each sample was calculated on the basis of the standard curve for Trolox and expressed as μmol of Trolox equivalents (TE)/μmol of samples. The final ORAC values were expressed as the mean ± SD (n = 3).

3. Results and Discussion

Figure 1 shows the chemical structures of each of the phloroglucinol and eckols isolated from the brown alga *E. bicyclis*. These phlorotannins were refined to a purity of 98% or more by column chromatography and preparative HPLC (data not shown), and used in an H-ORAC assay.

The ORAC measures the scavenging activity against peroxyl radicals induced by AAPH at 37˚C, using fluorescein as a fluorescence probe [6-10]. The ORAC value is widely used as one of the quantitative standards that shows the antioxidative potency of an antioxidant [6-10]. Generally, antioxidants can be classified by their solubility into two groups: hydrophilic antioxidants (e.g., ascorbic acid and majority of polyphenols) and lipophilic antioxidants (e.g., tocopherols and carotenoids) [13]. The H-ORAC assay is a method that can measure the titer of a hydrophilic antioxidant, and so was used to evaluate the antioxidative properties of phlorotannins in this study. The most suitable dilution ratio of the sample solution for the H-ORAC measurement was selected according to the method of Watanabe *et al*. [6]. As a result, the AUC of 25X and 30X diluted solutions of each sample solution (100 μg/ml) were located between 3.125 μM Trolox AUC, and 25 μM Trolox AUC respectively (data not shown). Therefore, sample solutions diluted 25X and

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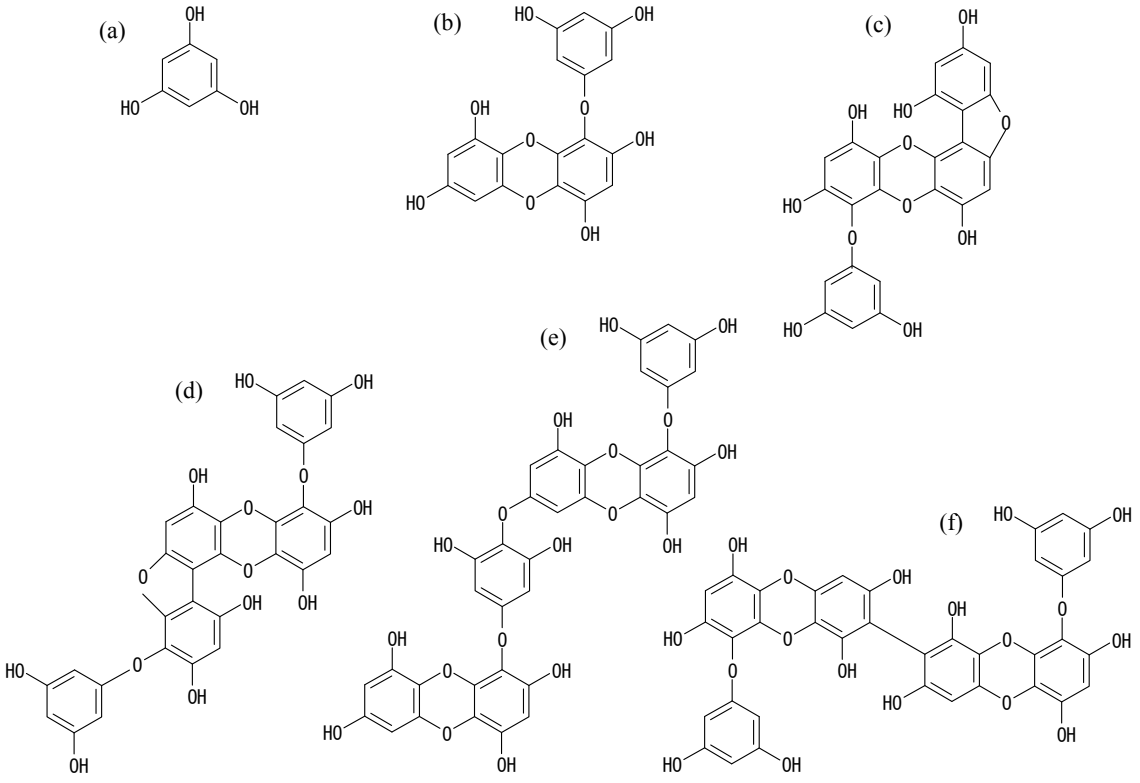

Figure 1. Chemical structures of phloroglucinol and phlorotannins isolated from the brown alga *E. bicyclis*. These compounds were isolated from crude phlorotannins of *E. bycyclis* by column chromatography and preparative HPLC. (a) phloroglucinol, (b) eckol, (c) fucofuroeckol A, (d) phlorofucofuroeckol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

30X were used for the experiment. **Figure 2** shows the fluorescence decay curves in the presence of the sample solutions diluted 25X and AAPH, as typical data. The calculated H-ORAC values of samples are shown in **Table 1**. Among phloroglucinol and it's oligomers, it was found that the compounds more than phloroglucinol tetramer had potent antioxidant activity with H-ORAC values in the range of 8.62 - 10.22 μM TE/μM. In contrast, the H-ORAC values of positive controls were in the range of 0.76 - 7.18 μM TE/μM. The data of EGCG and L-ascorbic acid were also almost in agreement with the values showed by Ishimoto *et al*. [10]. Although phloroglucinol and eckol had little antioxidant activities (H-ORAC values: 2.57 μM and 4.97 μM) in the tested samples, they were more effective than L-ascorbic acid. Ishimoto *et al*. [10] reported the H-ORAC values of 43 terrestrial polyphenols and their metabolites. Compared with their H-ORAC values in the report, phlorotannins, in particular dieckol and fucofuroeckol A, had H-ORAC values that were higher than terrestrial polyphenols (ellagitannins, condensed tannins, chlorogenic acids, catechins and flavonoids). Therefore, it was suggested that phlorotannins had more potent antioxidant activity than a lot of the representative polyphenols (e.g., EGCG, resveratrol, kaempferol, quercetin, myricetin and chlorogenic acid).

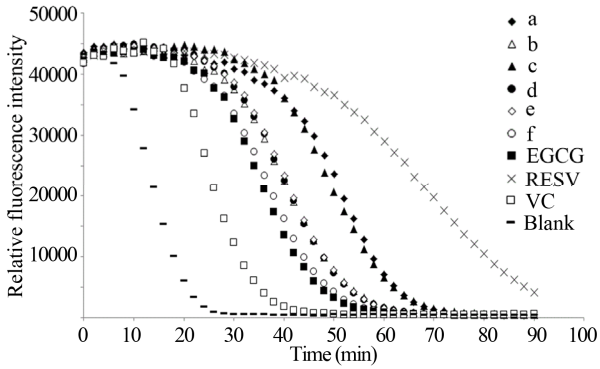

Figure 2. Fluorescence decay curves induced by AAPH in the presence of phlorotannins and positive controls. The figure shows the data of a 25× diluted solution of each sample solution (100 μg/ml) as typical data. (a) phloroglucinol, (b) eckol, (c) fucofuroeckol A, (d) phlorofucofuroeckol A, (e) dieckol, (f) 8,8'-bieckol, EGCG; epigallocatechin gallate, RESV; resveratrol, VC; L-ascorbic acid.

Phlorotannins, which are shown in **Figure 1**, are also commonly found in the Laminariaceous brown algae, *Eisenia arborea*, *Ecklonia cava* and *Ecklonia kurome* [3,14,15]. Previously, we showed that eckols had significant radical scavenging activities against the superoxide anion (50% effective concentration values: 6.5 - 8.4 μM)

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Table 1. H-ORAC values of phloroglucinol and phlorotannins isolated from the brown alga *E. bicyclis*.

Samples	ORAC (μmol TE/μmol)
Phloroglucinol	2.57 ± 0.14
Phlorotannins	
Eckol	4.97 ± 0.36
Fucofuroeckol A	9.82 ± 0.70
Phlorofucofuroeckol A	8.97 ± 0.89
Dieckol	10.22 ± 0.85
8,8'-Bieckol	8.62 ± 0.92
Positive controls	
L-Ascorbic acid	0.76 ± 0.24
Epigallocatechin gallate	4.65 ± 0.80
Resveratrol	7.18 ± 0.24

The data is expressed as the mean ± standard deviation from three independent measurements.

and DPPH (50% effective concentration values: 12 - 26 μM), and were more effective than L-ascorbic acid and *α*-tocopherol [5]. Nagayama *et al*. [16] reported that phlorotannins had no toxicity following oral administration to mice. The evidence obtained in the previous and present studies suggest that phlorotannins are potent antioxidants and that Laminariaceae, which are rich in phlorotannins, are useful as a novel functional food or supplement with antioxidative properties.

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