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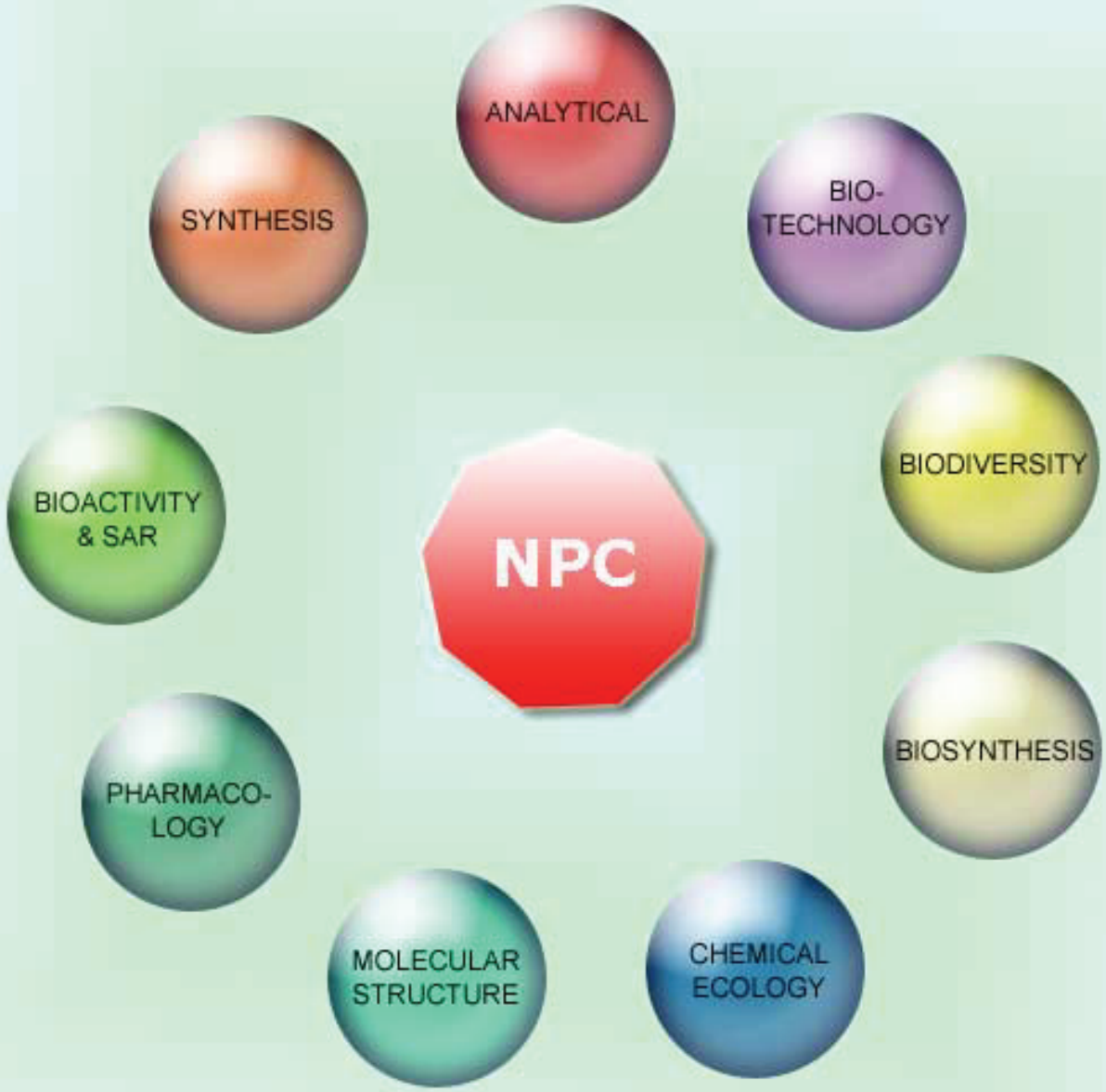

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Evaluation of Anti-glycation Activities of Phlorotannins in Human and Bovine Serum Albumin-methylglyoxal Models

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In this study, the anti-glycation activities of phlorotannins contained in the Japanese Lessoniaceae (*Ecklonia cava*, *Eck. kurome*, *Eck. stolonifera*, *Eisenia arborea*, and *Eis. bicyclis*) were tested using serum albumin-methlglyoxal (MGO) models. In the human serum albumin (HSA)-MGO model and the bovine serum albumin (BSA)-MGO model, the concentrations of crude phlorotannins at 50% inhibition (IC_{50}) of fluorescent advanced glycation end products (AGEs) formation was in the range of 0.45 to 0.53 mg/mL and 0.43 to 0.53 mg/mL, respectively. Among the purified phlorotannins, phlorofucofuroeckol A with the benzobisbenzofuran skeleton had the highest inhibitory activities (IC_{50}: 4.8 x 10^2 μM in HSA-MGO model and 4.1 x 10^2 μM in BSA-MGO model) against fluorescent AGEs formation, and showed about 13 times the inhibition in the HSA-MGO model, and about 20 times the inhibition in the BSA-MGO model, with respect to aminoguanidine hydrochloride, which was used as a positive control. In the test on MGO-scavenging activity using HPLC analysis, it showed that eckol and its simple dimers (dieckol and 8,8′-bieckol) had higher scavenging rate than aminoguanidine hydrochloride. In addition, it was revealed by liquid chromatography-electrospray ionization-mass spectrometry that eckol reacted with MGO and formed two adducts with it. These results strongly suggest that phlorotannins are novel and effective anti-glycation substances derived from natural plants.

Keywords: Advanced glycation end products, Anti-glycation, Eckol, Methylglyoxal, Phlorotannins, Phlorofucofuroeckol A, Lessoniaceae, Liquid chromatography-electrospray ionization-mass spectrometry.

In vivo, reducing sugars react nonenzymatically with lysine and arginine residues of proteins, and cause irreversible denaturation of proteins. This reaction is called a glycation reaction of proteins (glycation), which leads to the production of advanced glycation end products (AGEs) after the formation of sugar metabolic intermediates such as glyoxal, methylglyoxal (MGO), and 3-deoxyglucosone [1]. It has been pointed out that AGEs play key roles in the development of diabetic complications [2,3,4], atherosclerosis [2,4], osteoporosis [5], and aging [6]. From this scientific background, studies regarding the development of compounds, and the search for natural products, having an inhibitory effect on AGEs formation are conducted competitively. Among sugar metabolic intermediates, MGO of α-dicarbonyl compound is well known for the following facts: (1) MGO is more reactive with proteins than glucose [7], (2) the precursor of AGEs in vascular endothelial cells is MGO [8,9], and (3) the blood level of MGO in type I diabetics is about 6 times higher than in healthy subjects [10]. Therefore, AGEs formation pathway *via* MGO can be regarded as one of the most significant pathways in the AGEs formation pathways.

In a study of patients with chronic renal failure, the relationship between the increase in oxidative stress and the promotion of the glycation reaction of proteins is clarified. It has been reported that plant foodstuffs rich in antioxidants exhibit excellent anti-glycation activity [11-13]. Therefore, it is highly possible that antioxidants (e.g. polyphenols) derived from plants act as anti-glycation substances. Phlorotannins are polyphenols which occur only in brown algae and have a structure in which phloroglucinol is simply polymerized [14].

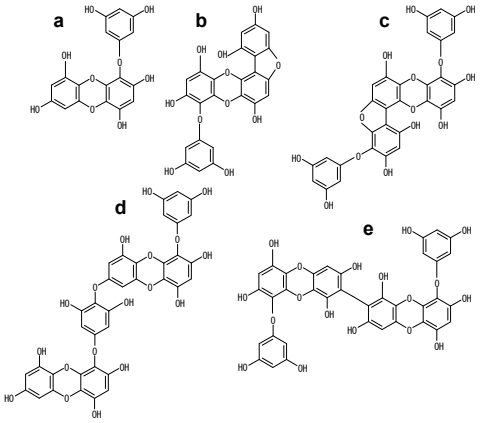

Figure 1: Chemical structures of eckols isolated from the Japanese Lessoniaceae. **a**: eckol, **b**: fucofuroeckol A, **c**: phlorofucofuroeckol A, **d**: dieckol, **e**: 8,8′-bieckol.

In the preceding reports [15,16], we isolated phloroglucinol and eckols (eckol, fucofuroeckol A, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol) from the brown alga *Eisenia bicyclis* (Figure 1) and revealed that they have superior antioxidant properties compared to catechins, ascorbic acid, and resveratrol. In this study, we extracted and isolated phlorotannins from several species of the

Japanese Lessoniaceae (Laminariales, Phaeophyceae) including *Ecklonia cava*, *Eck. kurome*, *Eck. stolonifera*, *Eisenia arborea*, and *Eis. bicyclis*, and evaluated their inhibitory activities against the production of fluorescent AGEs in the serum albumin-MGO models. Furthermore, the measurement of the MGO scavenging activity of phlorotannins was also carried out using HPLC and liquid chromatography-electrospray ionization-mass spectrometry (LC/ESI/MS).

Table 1: IC_{50} values of crude phlorotannins from Lessoniaceae against fluorescent AGEs formation.

Algae	Specific area of origin	HSA-MGO (mg/mL)	BSA-MGO (mg/mL)
Eck. cava	Mie	0.53	0.51
Eck. kurome	Fukuoka	0.45	0.46
Eck. kurome	Kumamoto	0.53	0.50
Cultured *Eck. kurome*	Kumamoto	0.46	0.46
Eck. stolonifera	Yamaguchi	0.52	0.47
Eis. arborea	Mie	0.46	0.53
Eis. bicyclis	Fukuoka	0.45	0.43

All the data are expressed as the mean of three independent measurements. The IC_{50} values of aminoguanidine hydrochloride were 0.70 mg/mL in the HSA-MGO model and 0.90 mg/mL in the BSA-MGO model.

Table 2: IC_{50} values of phloroglucinol and isolated phlorotannins against fluorescent AGEs formation.

Compounds	HSA-MGO (μM)	BSA-MGO (μM)
Phloroglucinol	3.9×10^3	2.4×10^3
Eckol	1.3×10^3	1.6×10^3
Fucofuroeckol A	5.5×10^2	7.4×10^2
Phlorofucofuroeckol A	4.8×10^2	4.1×10^2
Dieckol	6.9×10^2	7.4×10^2
8,8′-Bieckol	6.3×10^2	6.9×10^2

All the data are expressed as the mean of three independent measurements. The IC_{50} values of aminoguanidine hydrochloride were 6.4×10^3 μM in the HSA-MGO model and 8.1×10^3 μM in the BSA-MGO model.

In both human serum albumin (HSA)-MGO and bovine serum albumin (BSA)-MGO models, all samples tested in this study inhibited the formation of fluorescent AGEs in a dose-dependent manner. The concentrations (IC_{50}) of crude phlorotannins at 50% inhibition of fluorescent AGEs formation were calculated and shown in Table 1. In the HSA-MGO model, the IC_{50} values of the crude phlorotannins were in the range of 0.45 to 0.53 mg/mL. Among the tested samples, crude phlorotannins prepared from *Eck. kurome*, *Eis. bicyclis*, *Eis. arborea*, and cultured *Eck. kurome* showed effective inhibitory activities of fluorescent AGEs formation, and their activities were about 1.6 times higher than that of aminoguanidine hydrochloride used as a positive control. In the BSA-MGO model, the crude phlorotannins of *Eis. bicyclis* exhibited the highest inhibitory activity against fluorescent AGEs formation, and its IC_{50} value was 0.43 mg/mL. Since the IC_{50} value of aminoguanidine hydrochloride was 0.90 mg/mL in the model, it was revealed that crude phlorotannins prepared from *Eis. bicyclis* had about 2.1 times the inhibitory activity. In another study using brown algae rich in phlorotannins, Liu *et al*. [17] prepared a 70% acetone extract (phlorotannins rich fraction) from brown alga *Fucus vesiculosus* and reported its inhibitory activity on the formation of fluorescent AGEs in the BSA-MGO model. As a result of comparing data with the IC_{50} values described in the report, it was found that the anti-glycation activity of crude phlorotannins from Lessoniaceae are equivalent to that of a 70% acetone extract from *F. vesiculosus*.

To further analyze the inhibitory activity of phlorotannins against fluorescent AGEs formation in serum albumin-MGO models, a test was carried out using phloroglucinol and five kinds of purified eckols (eckol, fucofuroeckol A, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol). These phlorotannins are common compounds contained within the Japanese Lessoniaceae [14,18-20]. Phloroglucinol and the purified eckols inhibited the glycation of serum albumin in a concentration-dependent manner in both serum albumin-MGO models. Calculation of the IC_{50} value showed that fucofuroeckol A, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol had the most effective activities out of all of the tested compounds, and the range of values were 4.8×10^2 to 6.9×10^2 μM in the HSA-MGO model and 4.1×10^2 to 7.4×10^2 μM in the BSA-MGO model (Table 2). In particular, phlorofucofuroeckol A showed the highest inhibitory activity of fluorescent AGEs formation in both models and the IC_{50} values were 4.8×10^2 μM and 4.1×10^2 μM, respectively (Table 2). In comparison of the IC_{50} values, phlorofucofuroeckol A showed anti-glycation activity about 13 times greater in the HSA-MGO model, and about 20 times greater in the BSA-MGO model, with respect to aminoguanidine hydrochloride. MGO is known to be formed by the degradation and oxidation of Amadori products in the middle stage of protein glycation [21]. Therefore, the test using the albumin-MGO model evaluates the anti-glycation activity of the target sample in this middle stage. From the data obtained in the present study, it was suggested that phlorotannins from *Eisenia* and *Ecklonia* species, especially phlorofucofuroeckol A, may exert excellent inhibition of AGEs formation in the middle stage of protein glycation.

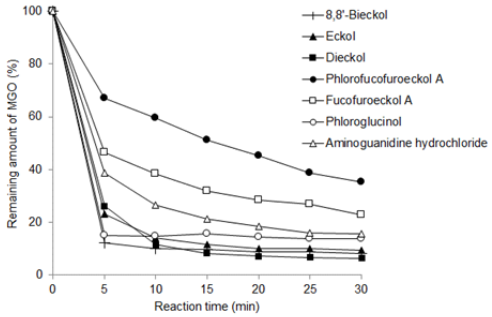

Figure 2: MGO scavenging activities of isolated phlorotannins, phloroglucinol, and aminoguanidine hydrochloride. All the data are expressed as the mean of three independent measurements.

In addition to involvement in the formation of AGEs *in vivo*, MGO causes inactivation of superoxide dismutase and increases production of reactive oxygen species [22,23]. Consequently, scavenging of MGO is considered to possibly result in a reduction of both glycation stress and oxidative stress. As a result of experimentation at a concentration of 1 mg/mL, each crude phlorotannins from the Lessoniaceae scavenged MGO over time, and the scavenging rate was in the range from 75.2% to 86.7% after 120 min of incubation (data not shown). The MGO-scavenging activities of phloroglucinol and five kinds of purified eckols were measured, and the obtained data are shown in Figure 2. Differences in MGO-scavenging activities were observed in the tested eckols depending on the presence or absence of the benzobisbenzofuran skeleton in their molecules. After 30 min of incubation, eckol and simple dimers of eckol (dieckol and 8,8′-bieckol) showed higher MGO-scavenging activities (90.5%, 93.6%, and 91.8%, respectively) as compared to aminoguanidine hydrochloride (84.4%) (Figure 2). In contrast, the MGO-scavenging activity of phlorofucofuroeckol A, which was the highest in inhibiting fluorescent AGEs formation, showed the lowest value of 64.6% (Figure 2). The scavenging activity of fucofuroeckol A, having a benzobisbenzofuran skeleton in its molecule similar to phlorofucofuroeckol A, was also lower than that of aminoguanidine hydrochloride. Phloroglucinol exhibited a scavenging activity just below that of eckol, and its proportion was 86.0%. Using LC/ESI/MS^n analysis, Liu *et al*. [17] reported that phloroglucinol reacts with reactive carbonyls (glyoxal and MGO) and forms phloroglucinol-carbonyl adducts. In analogy with phloroglucinol,

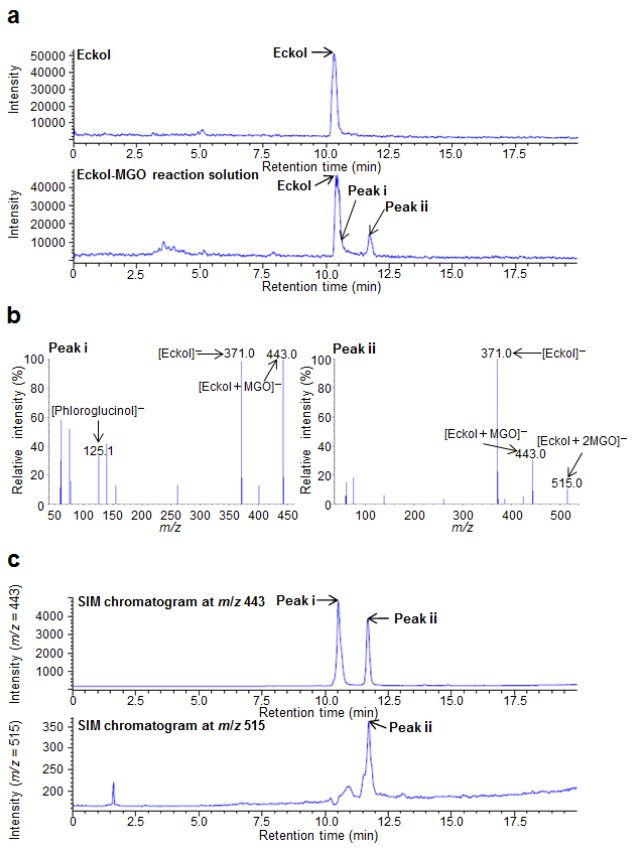

Figure 3: Detection and analysis of eckol-MGO adducts using LC/ESI/MS. **a**: total ion chromatograms of eckol and eckol-MGO reaction solution. The peak of eckol was detected in total ion chromatogram with a retention time of 10.3 min. **b**: MS spectra of eckol-MGO adducts, **c**: SIM chromatograms of eckol-MGO adducts.

eckol and the dimers, having MGO-scavenging activity, may possibly form adducts with MGO. In fact, an analysis of eckol and MGO reaction solution suggested that there were two kinds of eckol-MGO adducts (Figure 3). In the negative mode of LC/ESI/MS measurement, the reaction solution of eckol-MGO was analyzed. The first eckol-MGO adduct eluted at 10.5 min (peak i) (Figure 3a), which gave rise to *m/z* 443 $[M-H]^-$ (Figure 3b). This yielded a product ion at *m/z* 371 that was consistent with eckol moiety. Therefore, this adduct was identified as an adduct of one eckol molecule and one MGO molecule. The second adduct eluted at 11.7 min (peak ii) (Figure 3a) and had *m/z* 515 $[M-H]^-$ (Figure 3b). Fragment ions at *m/z* 443 and 371 result from the elimination of one molecule of MGO and two molecules of MGO molecules, respectively. This was consistent with an adduct composed of one eckol molecule and two MGO molecules. As a result of measurement using the selected ion monitoring (SIM) mode of the mass spectrometer, chromatograms showing each of the two adducts were obtained (Figure 3c). These results suggest that the scavenging activity of MGO may partially contribute to the anti-glycation activity of eckol and its dimers (dieckol and 8,8′-bieckol).

To our knowledge, this is the first study to evaluate the anti-glycation activities of phlorotannins in human and bovine serum albumin-MGO models. Recently, Corona *et al*. [24] evaluated the bioavailability of phlorotannins extracted from *Ascophyllum nodosum* in healthy subjects. In that literature, it was reported that three types of low molecular phlorotannins (hydroxytrifuhalol A, 7-hydroxyeckol, and dimer of phloroglucinol) were detected as metabolites in both samples of plasma and urine. Okada *et al*. [25] clarified that eckol and dieckol isolated from the *Eis*. *bicyclis* inhibit the formation of N_ε-carboxymethyl-lysine, a kind of AGEs, *in vitro* test. The evidence obtained in the previous and present studies suggest that phlorotannins may prevent or ameliorate chronic diseases (e.g. diabetes and atherosclerosis) in which AGEs are involved, as novel anti-glycation substances from natural products.

Experimental

Materials: The brown algae *Eisenia bicyclis* (Kjellman) Setchell, *Eis*. *arborea* Areschong, *Ecklonia cava* Kjellman, *Eck*. *kurome* Okamura, and *Eck*. *stolonifera* Okamura, approximately 90-120 cm in length, were collected from the coasts of the Itoshima peninsula (33°37′N, 130°10′E) in Fukuoka prefecture, Itsuwa (32°54′N, 130°13′E) in Kumamoto prefecture, Sakishima peninsula (34°14′N, 136°50′E) in Mie prefecture, and Kawatana (34°9′N, 130°53′E) in Yamaguchi prefecture, Japan, in 2015. The cultured *Eck*. *kurome* was purchased from the Fisheries Cooperative Association of Amakusa in Kumamoto prefecture, Japan, in 2015. The algae used for the extraction of phlorotannins were washed with filtered seawater, air-dried, and pulverized. The algal powders were stored at -30°C until use.

Extraction and purification of phlorotannins: Extraction of phlorotannins from algal powder was prepared according to the method described in the previous report [20]. Phlorotannins were partially purified by silica column chromatography (1.5 cm i.d. x 150 cm) using Wakogel C-300 HG (Wako Pure Chemical) with a mobile phase of $CHCl_3$:MeOH:water (80:20:2, *v*/*v*) according to the method described in the previous reports [15,16]. Further purification of phlorotannins was carried out using preparative HPLC with an Inertsil ODS-3 column (10 mm i.d. x 250 mm, GL Science) [16]. Each of the obtained phlorotannins (eckol, fucofuroeckol A, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol) was confirmed to have a purity of 98% or more by three-dimensional HPLC (SPD-M10AV, Shimadzu) with an Inertsil ODS-3 column (4.6 mm i.d. x 250 mm, GL Science) [16]. The purified phlorotannins were stored at -30°C until use.

Serum albumin-MGO assay: The antiglycation assay of phlorotannins in the serum albumin-MGO model was performed according to the method of Liu *et al*. [17] with slight modification. Sample, MGO (60 mM), and albumin (HSA or BSA, 20 mg/mL) solutions were prepared separately by dissolving in 100 mM phosphate buffer (pH 7.4). The measurement was carried out using a 96-well black plate (FLUOTRAC600, Greiner) and a 96-well microplate reader (Infinite 200, Tecan). The sample solution (50 µL), MGO solution (50 µL), and albumin solution (50 µL) were added to the wells in the plate. Sodium azide was added to each well to a final concentration of 0.2 mg/mL. Measurement was carried out with fluorescence intensity at an excitation of 340 nm and an emission of 420 nm, and the obtained value was taken as a control value. After incubation at 37°C for 7 days, the fluorescence intensities of each well were measured under the same measurement conditions. For blank wells, phosphate buffer was used instead of sample solution. Aminoguanidine hydrochloride was used as a positive control. The inhibition rate (%) of fluorescent AGEs formation was calculated using the following formula:

Inhibition rate (%) = [1 - (fluorescence intensity of sample after incubation for 7 days - fluorescence intensity of control of sample) / (fluorescence intensity of blank after incubation for 7 days - fluorescence intensity of control of blank)].

The IC_{50} value was calculated from the logarithmic function obtained by plotting the inhibitory rate of fluorescent AGEs formation against the sample concentration.

MGO-scavenging assay: The MGO-scavenging activity of phlorotannins was carried out using a modification of the method of Liu *et al* [17]. Five mM MGO and 20 mM *o*-phenylenediamine were prepared using 100 mM phosphate buffer (pH 7.4). The crude phlorotannins extracted from each brown algae was dissolved in the phosphate buffer to a concentration of 1 mg/mL. Purified phlorotannins, phloroglucinol, and aminoguanidine hydrochloride were dissolved in the phosphate buffer to a concentration of 5 mM each. Each sample solution (25 μL) and a MGO solution (25 μL) were mixed and incubated at 37°C. In the test using crude phlorotannins, incubation was carried out for 5, 10, 20, 40, 60, and 120 min, respectively. In the test using purified phlorotannins, incubation was carried out for 5, 10, 15, 20, 25 and 30 min, respectively. After incubation, *o*-phenylenediamine solution (5 μL) was added to the mixture and maintained at 25°C for 30 min to be derivatized. For the blank test, the phosphate buffer was used instead of a sample solution. The amount of 1-methylquinoxaline produced by the reaction of *o*-phenylenediamine and MGO remaining in the mixture, was quantified using HPLC (LC-2000Plus, JASCO) with LUNA Phenyl-hexyl column (4.6 mm i.d. x 150 mm, Phenomenex). Elution was performed at a flow rate of 1 mL/min with 50% MeOH as a mobile phase. The UV detector was set at 315 nm.

Detection and identification of Eckol-MGO adduct using LC/ESI/MS: Eckol and MGO were dissolved in ultrapure water to a concentration of 5 mM each. The eckol solution (300 μL) and the MGO solution (300 μL) were mixed and incubated at 37°C for 2 hours. Detection and identification of eckol-MGO adducts were performed using LC/ESI/MS (Agilent 6120, Agilent). The condition of LC/ESI/MS was utilized for the analysis condition of phlorotannins reported in the preceding study [20]. The *m*/*z* was set from 50 to 1000.

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