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Identification of Chemical Structure and Free Radical Scavenging Activity of Diphlorethohydroxycarmalol Isolated from a Brown Alga, *Ishige okamurae*

Heo, Soo-Jin[1], Jong-Pyung Kim[2], Won-Kyo Jung[3], Nam-Ho Lee[4], Hahk-Soo Kang[2], Eun-Mi Jun[2], Soon-Hye Park[2], Sung-Myung Kang[1], Young-Jae Lee[5], Pyo-Jam Park[6], and You-Jin Jeon[1,7]*

[1]*Faculty of Applied Marine Science, Cheju National University, Jeju 690-756, Korea*
[2]*Functional Metabolites Research Center, KRIBB, Daejeon 305-806, Korea*
[3]*Department of Marine Life Science, Chosun University, Gwangju 501-759, Korea*
[4]*Department of Chemistry, Cheju National University, Jeju 690-756, Korea*
[5]*Department of Veterinary Science, Cheju National University, Jeju 690-756, Korea*
[6]*Department of Biotechnology, Konkuk University, Chungju 380-701, Korea*
[7]*Marine and Environmental Research Institute, Cheju National University, Jeju 695-814, Korea*

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To obtain a natural antioxidant from a marine biomass, this study investigated the antioxidative activity of methanolic extracts from the marine brown alga, *Ishige okamurae* collected off Jeju Island. A potent free radical scavenging activity was detected in the ethyl acetate fraction containing polyphenolic compounds, and the potent antioxidant elucidated as a kind of phlorotannin, diphlorethohydroxycarmalol, by NMR and mass spectroscopic data. The free radical scavenging activities of the diphlorethohydroxycarmalol were investigated in relation to 1,1-diphenyl-2-picrylhydrazyl (DPPH), alkyl, and hydroxyl radicals using an electron spin resonance (ESR) system. The diphlorethohydroxycarmalol was found to scavenge DPPH (IC_{50}=3.41 μM) and alkyl (IC_{50}= 4.92 μM) radicals more effectively than the commercial antioxidant, ascorbic acid. Therefore, these results present diphlorethohydroxycarmalol as a new phlorotannin with a potent antioxidative activity that could be useful in cosmetics, foods, and pharmaceuticals.

Key words: Phlorotannin, diphlorethohydroxycarmalol, ESR, brown algae, *Ishige okamurae*

Free radicals are highly reactive species made of molecules or atoms that are unstable owing to single or unbalanced electrons. Free radicals, such as reactive oxygen species (ROS), are produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions, and abnormal metabolic processes, and have a wide variety of pathological effects, such as DNA damage, atherosclerosis, muscular dystrophy, inflammatory disorders, and neurological disorders [7, 19, 23, 36]. Thus, owing to growing health concerns, interest has significantly increased in finding natural antioxidants for use in food and medicinal materials to replace synthetic antioxidants with harmful effects [32, 42, 43]. Natural antioxidants can protect the human body from serious cellular or molecular damage by free radicals and retard the progress of many chronic diseases, as well as lipid peroxidation in food [21, 22, 41].

Marine algae have recently been identified as an under-exploited plant resource and functional food [11, 29]. In particular, brown seaweed is plentiful around Jeju Island, Korea, although rare in other parts of the world, and is already utilized as an ingredient in food, animal feed, fertilizers, and medicine. In addition, brown algae include xanthopophyll, pigments, fucoxanthin, fucoidans, and phycocolloids, and are an excellent source of alginates, which can be used as viscosifiers (thickeners) in a wide variety of products [8]. Most photosynthesizing plants, including marine algae, are exposed to intense light and high oxygen concentrations that lead to the formation of free radicals and other strong oxidizing agents, yet they are not affected by any photodynamic damage, suggesting that their cells possess antioxidative mechanisms related to bioactive compounds [6, 14, 37]. Potent antioxidative compounds have already been isolated from brown algae and identified as phylopheophytin in *Eisenia bicyclis* (arame), fucoxanthine in *Hijikia fusiformis* (hijiki), and phlorotannin in *Ecklonia stolonifera* [5, 16, 39]. Among these antioxidants, phlorotannins, which are only known as marine algal polyphenols, are found in the form of organic polymers, such as phloroglucinol (1,3,5-trihydroxybenzene). It has also been reported that phlorotannins

*Corresponding author
Phone: 82-64-754-3475; Fax: 82-64-756-3493;
E-mail: youjinj@cheju.ac.kr

are antiplasmin inhibitors and exhibit antiallergic, antitumor, and antioxidant activities [15, 25, 27, 35].

As mentioned in previous reports associated with the bioactivities of brown algae, phlorotannins are surely the key compounds. For example, diphlorethohydroxycarmalol, a kind of phlorotannin, was found to have an inhibitory effect on the human immunodeficiency virus type-1 (HIV-1) reverse transcriptase [3]. Nonetheless, the antioxidant activities of the phlorotannins from the brown alga *Ishige okamurae* have not yet been reported. Accordingly, the present study isolated a strong antioxidant phlorotannin, diphlorethohydroxycarmalol from *Ishige okamurae*, and evaluated its radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, and alkyl radicals using an electron spin trapping technique.

MATERIALS AND METHODS

Materials

The brown alga *Ishige okamurae* was collected along the coast of Jeju Island, Korea, between October 2005 and March 2006. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, and then carefully rinsed with fresh water and maintained in a medical refrigerator at −20°C. Then, the frozen samples were lyophilized and homogenized with a grinder prior to extraction.

For the electron spin trapping technique, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The UV spectra were recorded on a Pharmacia Biotech Ultrospec 3000 UV/visible spectrometer, the NMR spectra recorded on a Varian INOVA 500 MHz NMR spectrometer, and the ESI and HREI mass spectra acquired using a Finnigan Navigator 30086 and JMS-700 Mstation high-resolution mass spectrometer system, respectively. The HPLC was carried out using a Waters HPLC system equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 150× 20 mm, 4 μm; YMC Co.).

Isolation of Diphlorethohydroxycarmalol

The algal powder (500 g) was extracted three times with 80% methanol and filtered. The filtrate was then evaporated at 40°C to obtain the methanol extract, which was dissolved in water, and then partitioned with *n*-hexane, chloroform, ethyl acetate, and butanol (Fig. 1). Since the ethyl acetate fraction exhibited the desirable radical scavenging activities, the active compounds were extracted from the ethyl acetate fraction using a silica gel and Sephadex LH-20 column chromatography. The active compounds were finally purified by high-performance liquid chromatography (HPLC), and the structure of the compound (Fig. 2) was identified by comparing the NMR spectral data with those in the existing literature.

DPPH Radical Scavenging Assay

The DPPH radical scavenging activity was measured using an ESR spectrometer (JES-FA machine; JOEL, Tokyo, Japan) according to the technique described by Nanjo *et al.* [28]. Sixty μl of each

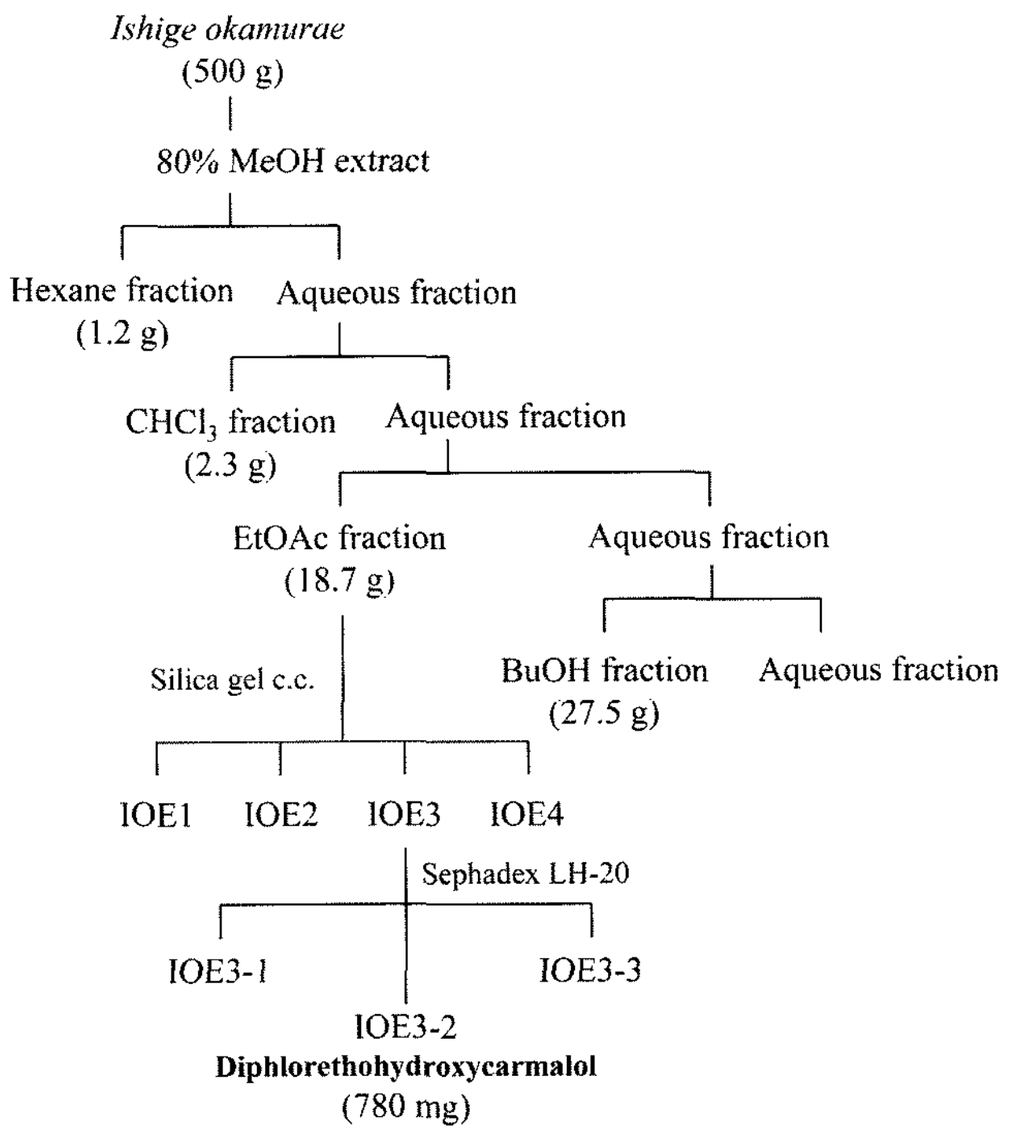

Fig. 1. Extraction and isolation scheme for diphlorethohydroxycarmalol from *I. okamurae*.

sample was added to 60 μl of DPPH (60 μmol/l) in ethanol. After 10 sec of vigorous mixing, the solutions were transferred to Teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adduct was determined by the ESR spectrometer exactly 2 min later under the following measurement conditions: central field 3,475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, and temperature 298 K. All the radical scavenging activities (%) in the present study were calculated using the following equation, in which H and H_0 were the relative peak heights of the radical signals with and without a sample, respectively.

$$\text{Radical scavenging activity}=[1-(H/H_0)]\times100$$

Alkyl Radical Scavenging Assay

Alkyl radicals were generated *via* AAPH. Thus, reaction mixtures containing 10 mmol/l AAPH and 10 mmol/l 4-POBN were mixed with the tested samples. The solutions were incubated for 30 min at

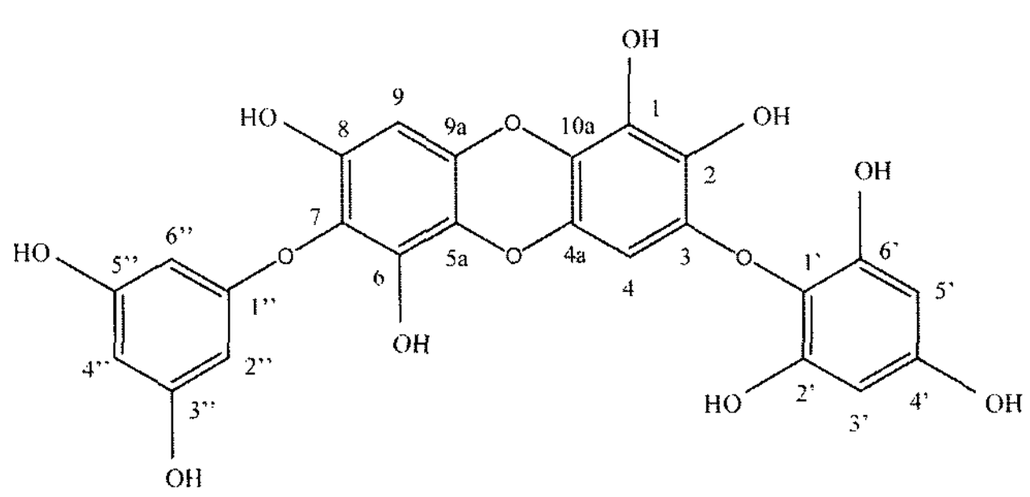

Fig. 2. Chemical structure of diphlorethohydroxycarmalol.

37°C in a water bath [12], and then transferred to Teflon capillary tubes. The spin adduct was recorded using a JES-FA ESR spectrometer under the following measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW, gain 6.3×10^5, and temperature 298 K. The alkyl radical scavenging activity (%) was presented as described above.

Hydroxyl Radical Scavenging Assay

Hydroxyl radicals were generated *via* the Fenton reaction, and reacted rapidly with nitrone spin trap DMPO. The resultant DMPO-OH adducts were detected using an ESR spectrometer [33]. Reaction mixtures containing 100 µl of 0.3 M DMPO, 100 µl of 10 mM $FeSO_4$, and 100 µl of 10 mM H_2O_2 were mixed with the test samples, and then transferred to a Teflon capillary tube. The spin adduct was measured on an ESR spectrometer exactly 2.5 min later under the following measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 1 mW, gain 6.3×10^5, and temperature 298 K. The hydroxyl radical scavenging activity (%) was presented as described above.

Statistical Analysis

The data are expressed as the mean±standard error (SE). A statistical comparison was performed *via* a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *P*-Values of less than 0.05 were considered to be significant.

Results and Discussion

Ishige okamurae is abundant along the coast of Jeju Island and regarded as an edible alga. However, since biological studies of this alga are relatively rare, this study screened its antioxidant activity *via* its scavenging effect on DPPH free radicals using various solvent fractions. As the ethyl acetate fraction showed the highest DPPH radical scavenging activity of 91.58% at a concentration of 100 µg/ml, the active compound of this fraction was isolated and identified as diphlorethohydroxycarmalol based on a comparison of NMR spectroscopic data and previous literature [24]. The diphlorethohydroxycarmalol was isolated as a brownish yellow amorphous powder, and its molecular formula deduced as $C_{24}H_{16}O_{13}$ based on NMR (Table 1) and HRESI-MS analyses (M^+, *m/z*: 512.0589 Calcd. for $C_{24}H_{16}O_{13}$ *m/z*: 512.0591). The diphlorethohydroxycarmalol

Table 1. NMR spectroscopic data for diphlorethohydroxycarmalol in DMSO-d_6.

Position	^{13}C	^{1}H (mult. J=Hz)
1	135.1	
2	125.5	
3	133.8	
4	92.4	5.69 (1H, s)
4a	143.0	
5a	146.0	
6	139.6	
7	138.8	
8	124.1	
9	94.3	6.06 (1H, s)
9a	126.4	
10a	130.7	
1'	122.9	
2', 6'	151.3	
3', 5'	95.0	5.87 (2H, s)
4'	154.9	
1"	160.1	
2", 6"	93.7	5.67 (2H, d, J=2)
3", 5"	158.9	
4"	96.1	5.78 (1H, t, J=2)

*500 MHz for ^{1}H and 125 MHz for ^{13}C.

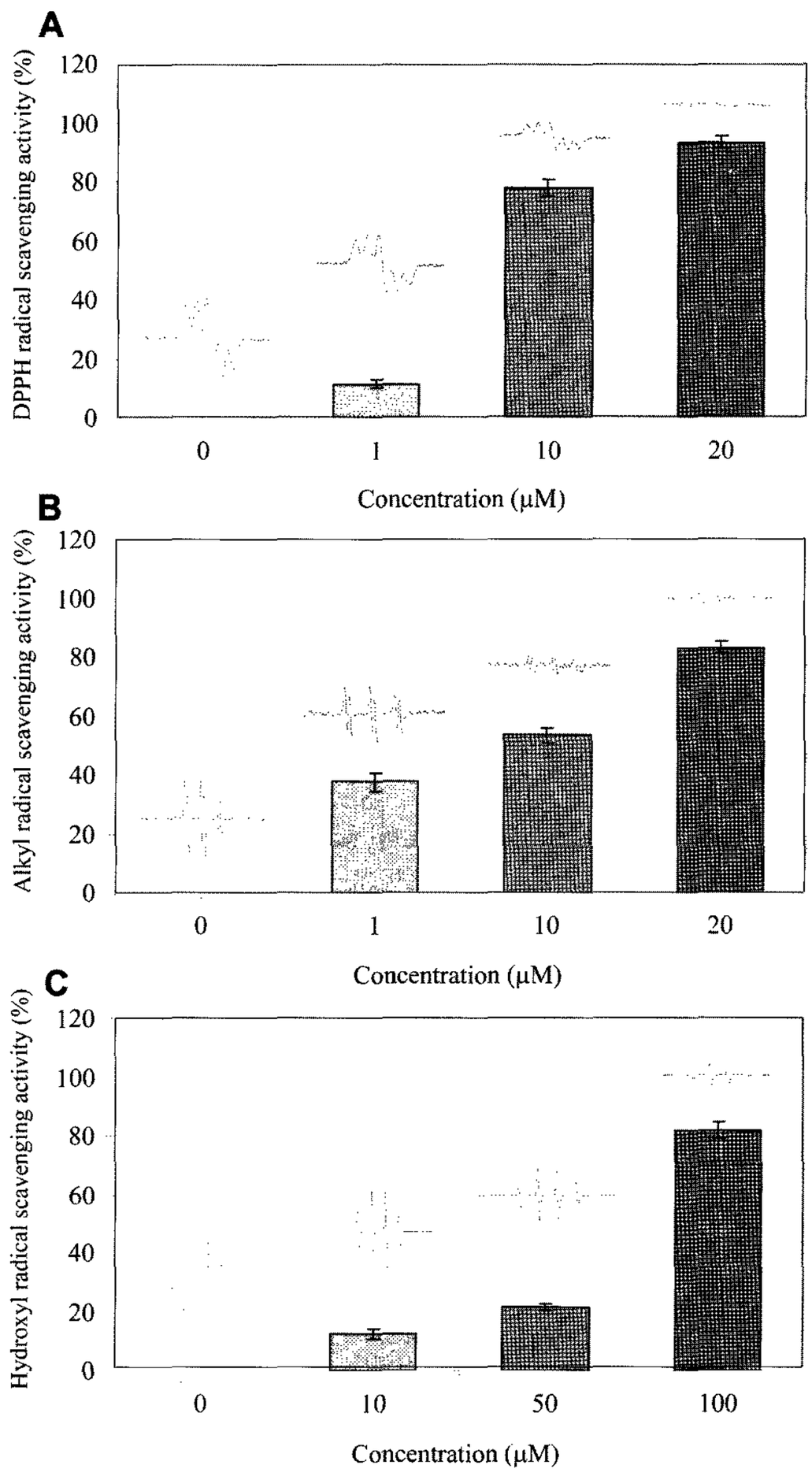

Fig. 3. Radical scavenging activities of diphlorethohydroxycarmalol in dose-dependent manner.
(**A**) DPPH radicals, (**B**) alkyl radicals, and (**C**) hydroxyl radicals.

Table 2. Radical scavenging capacities of diphlorethohydroxycarmalol *via* the ESR system.

	IC_{50} (μM)		
	DPPH	Alkyl	OH
Diphlorethohydroxycarmalol	3.41	4.92	114.80
Ascorbic acid	19.94	19.26	69.75

was then used in further experiments with an ESR spectrometer.

ESR spin trapping provides a sensitive, direct, and accurate means of monitoring reactive species [9]. Therefore, this study used ESR to compare the DPPH, alkyl, and hydroxyl radical scavenging abilities of the diphlorethohydroxycarmalol isolated from *I. okamurae* with those of a commercial antioxidant, such as ascorbic acid. DPPH is a stable free radical donor that is widely used to test the free radical scavenging effect of natural antioxidants. The scavenging activity of the diphlorethohydroxycarmalol towards DPPH free radicals is shown in Fig. 3A, where 74.68% of the DPPH free radicals were scavenged even at 10 μM, plus the radical scavenging occurred in a dose-dependent manner. In addition, the diphlorethohydroxycarmalol (IC_{50}=3.41 μM) exhibited stronger DPPH free radical scavenging than ascorbic acid (Table 2). Several previous reports have emphasized an association between DPPH radical scavenging and phenolic compounds. For example, Tepe and Sokmen [38] reported that a high total phenolic content was correlated with high antioxidant activity in a DPPH assay, whereas Yuan *et al.* [40] noted a positive relationship between phenolic functional groups and DPPH free radical scavenging activity. Phenols are particularly effective antioxidants for polyunsaturated fatty acids, and can easily transfer a hydrogen atom into lipid peroxyl radicals to form aryloxyl, which being incapable of acting as a chain carrier, then couples with other radicals, thereby quenching the radical propagation process [34]. Phlorotannins are also known to exist in marine alga polyphenols, as evidenced by the positive effect on DPPH free radicals in this study. Therefore, the diphlorethohydroxycarmalol isolated from *I. okamurae* would appear to be a good potential DPPH free radical scavenger.

Alkyl radicals are a primary intermediate in many hydrocarbon reactions, and can be easily detected with ESR, a technique that has been found to be very useful in the characterization of solid surfaces and in the elucidation of active surface sites, as well as surface reactions [1]. The ability of the diphlorethohydroxycarmalol to scavenge alkyl radicals is presented in Fig. 3B, where 83.24% of the alkyl radicals were scavenged at 20 μM, plus the scavenging activities increased when increasing the compound concentration. Moreover, the diphlorethohydroxycarmalol exhibited a higher alkyl radical scavenging activity (IC_{50}= 4.92 μM) than the commercial antioxidant ascorbic acid (IC_{50}=19.26 μM). In previous studies, Park *et al.* [31] observed that *Sargassum thunbergii* had a strong radical scavenging effect on alkyl radicals, whereas Ahn *et al.* [2] reported that the dieckol isolated from *Ecklonia cava* scavenged 90% of alkyl radicals at 50 μg/ml. Although phenolic antioxidants are well known for trapping peroxy radicals to prevent organic materials from oxidative degradation, trapping alkyl radicals is also important to prevent such degradation, as autoxidation includes both peroxy and alkyl radicals as chain carriers [29]. Therefore, the scavenging abilities of the phlorotannin towards alkyl radicals confirm their potential as a source of alkyl radical scavengers.

In cells, hydroxyl radicals are generated from hydrogen peroxide *via* the so-called Fenton reaction [13]. Therefore, to investigate the ability of the phlorotannins to scavenge hydroxyl radicals, the following *in vitro* reaction was generated: $Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+{}^{-}OH+{}^{\bullet}OH$. The hydroxyl radical scavenging activities of the diphlorethohydroxycarmalol are shown in Fig. 3C, where a decrease in the amount of DMPO-OH adducts was exhibited by the ESR signals after the addition of the diphlorethohydroxycarmalol from *I. okamurae*, plus the values were dose-dependent. However, the diphlorethohydroxycarmalol exhibited relatively lower levels of activity (IC_{50}=114.80 μM) than the commercial antioxidant ascorbic acid (IC_{50}=69.75 μM). Hydroxyl radicals are an extremely reactive oxygen species, capable of modifying almost every molecule in living cells and causing strand damage in DNA, leading to carcinogenesis, mutagenesis, and cytotoxicity. Moreover, hydroxyl radicals can rapidly initiate the lipid peroxidation process by abstracting hydrogen atoms from unsaturated fatty acids [4, 18]. Thus, because of this high reactivity of hydroxyl radicals, measurements based on scavenging hydroxyl radicals, such as nonsite-specific methods, are not accurate reflections of the oxidative protection of an antioxidant molecule *in vivo* [10], as the radicals are more likely to be scavenged by direct reactions with other surrounding molecules before attacking the target molecule. Therefore, the present study used the ESR system, where the hydroxyl radicals were trapped by DMPO, forming a spin adduct that could be detected. The findings indicated that the diphlorethohydroxycarmalol exerted a protective effect against reactive radical species, such as hydroxyl radicals.

Brown algae are rich in polyphenolic compounds, called phlorotannins, and many researchers have already reported that the phlorotannins from brown algae exhibit antioxidative capacities. For example, Kang *et al.* [16] isolated the phlorotannins dieckol, eckol, eckstolonol, and phlorofucofuroeckol from *Ecklonia stolonifera*, all of which showed potent radical scavenging activity with IC_{50} values of 8.8, 11.5, 4.6, and 6.2 μM, respectively. Nakai *et al.* [26] also isolated a bifuhalol oligomer, a kind of phlorotannin from *Sargassum ringgoldianum*, that exhibited

strong superoxide anion radical scavenging activity (IC_{50}= 1 μg/ml), whereas Kim *et al.* [20] and Kang *et al.* [17] reported that several phlorotannins demonstrated a lipid peroxidation inhibitory effect and cytoprotective effect on oxidative stress-induced cell damage. However, the diphlorethohydroxycarmalol isolated from *Ishige okamurae* showed a powerful radical scavenging activity compared with other phlorotannins in the existing literature.

The present study isolated diphlorethohydroxycarmalol from *I. okamurae* as a potential reactive radical scavenger. The diphlorethohydroxycarmalol effectively scavenged various reactive radicals, including DPPH, alkyl, and hydroxyl radicals. In addition, the diphlorethohydroxycarmalol was more effective than the commercial antioxidant ascorbic acid. Therefore, based on the results, the diphlorethohydroxycarmalol from *I. okamurae* may be a useful natural radical scavenger and a potential supplement for the food, pharmaceutical, and cosmetic industries because of its antioxidant capacities.

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