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ORIGINAL PAPER

Determination of singlet oxygen quenching and antioxidant activity of Bieckols isolated from the brown alga *Eisenia bicyclis*

Tae-Hyung Kwon · Hwa-Jin Suh · In-Kyoung Lee · Bong-Sik Yun · Tae-Wan Kim · Dai-Il Hwang · You-Jeong Kim · Min-Jeong Kim · Oh-Oun Kwon · Choong-Gon Kim · Nyun-Ho Park

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Abstract In this study, we determined 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activities as well as the reducing power for screening of antioxidant activity of bieckols in *Eisenia bicyclis* (Kjellamn) Setchell (*E. bicyclis*). The compounds 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol are representative members of the phlorotannins family. The isolated bieckols displayed markedly strong DPPH and ABTS radical scavenging effects when compared to those of positive controls (BHA and Trolox). These bieckols were also found to have significant reducing power. The isolated bieckols (6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol) were found to effectively suppress the detrimental effects of $^1O_2$ on type-II photosensitization. The concentrations required to exert a 50 % quenching effect on $^1O_2$ (QC_{50}) were found to be 30.7, 35.7 and 49.4 μM for 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol, respectively. Interestingly, all these bieckols were found to be superior to histidine (5.9 mM), a well-known $^1O_2$ quencher. These results suggested that brown algae phlorotannins, and bieckols in particular, may play an important role in protecting marine organisms against sunlight damage by eliminating $^1O_2$. This knowledge may contribute to the development of natural bioactive products with potential applications in reducing photo-produced oxidative damage involving reactive oxygen species in living organisms.

Keywords Antioxidant · Brown alga · *Eisenia bicyclis* (Kjellamn) Setchell · Phlorotannins · Singlet oxygen quenching

T.-H. Kwon · C.-G. Kim · N.-H. Park (✉)
Gyeongbuk Institute for Marine Bio-Industry, Uljin 767-813, Republic of Korea
e-mail: pnh863660@gimb.or.kr

T.-H. Kwon · T.-W. Kim · D.-I. Hwang · Y.-J. Kim · M.-J. Kim
Food Science and Biotechnology Major, Andong National University, Andong 760-749, Republic of Korea

H.-J. Suh · O.-O. Kwon
Gyeongbuk Natural Color Industry Institute, Yeongcheon 770-906, Republic of Korea

I.-K. Lee · B.-S. Yun
Division of Biotechnology and Advanced Institute of Environment and Bioscience, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan, Republic of Korea

Introduction

Reactive oxygen species (ROS) are implicated in degenerative diseases. There are various forms of ROS; for example, hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), singlet oxygen ($^1O_2$) and superoxide. ROS can be induced by endogenous factors in living organisms during metabolic processes and respiration, along with exogenous factors, like organic solvents, tobacco smoke, ultraviolet rays, processed foods and oxidative stress [1, 2]. Moreover, excess or imbalanced levels of ROS are noxious and lead to cell damage, accumulation of lipid peroxides, oxidative stress and chronic diseases [3]. These include diseases such as cancer, heart diseases, osteoporosis and cerebrovascular diseases [4, 5]. Therefore, antioxidants are regarded as important factors in various fields, including pharmacology, health sciences and medical sciences. Commercial synthetic antioxidants are widely used in the food industry and in food additives. These substances can control ROS production and lipid oxidation. However, synthetic antioxidants, such as butylated hydroxytoluene (BHT),

butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ), have been suspected of inducing carcinogenesis and toxicity. Therefore, they are strictly regulated in the food industry. More studies are currently focusing on the properties of natural antioxidants that are not harmful to the human body [6–8].

Polyphenolic secondary metabolites are part of a large and diverse group of chemical compounds that exist both in terrestrial plants and in aquatic macrophytes [9]. Tannins, a widespread family of phenolic metabolites present in many plants, are commonly divided into three chemically distinct groups based on their structures [10]. Hydrolysable tannins are characterized by a central polyhydroxyl moiety that is esterified with gallic or hexahydroxydiphenic acid. Hydrolysable tannins occur in some green algae and are widely distributed in angiosperms. Flavonoid-based condensed tannins are found mainly in woody plants and in red wine, tea and cocoa beans. The third, less familiar group is the phlorotannins, which consist of polymers of phloroglucinol units and are restricted to brown algae.

Recently, solvent and enzymatic extracts from seaweeds, such as brown, green and red algae, have been studied to evaluate their biological activity and their potential as natural antioxidants. Seaweeds are widely used in the food industry as carrageenan, and fucoidan, as well as in folk medicine, and in animal feed. Seaweeds are rich in dietary fiber, minerals and vitamins [11, 12]. *Eisenia bicyclis* (Kjellamn) Setchell belongs to the family Laminariaceae (class Phaeophyceae) which are edible seaweeds. It is a perennial brown alga that is widely distributed around Ulleung Island in South Korea. It is well known as a dietary fiber [13], an anti-diabetic agent [14], an antioxidant [15], a BACE1-inhibitory agent [16] and for its role in extracellular secretion [17]. However, the quenching effects on singlet oxygen ($^1O_2$) by the three phlorotannins isolated from *E. bicyclis* and the antioxidant activity of 6,8′-bieckol have not yet been discovered.

Therefore, this study aimed to investigate the antioxidant and singlet oxygen ($^1O_2$) quenching effects against photodynamic damage of phlorotannins isolated from edible brown algae, *E. bicyclis* (Kjellamn) Setchell. Antioxidant activities of phlorotannins were measured assessing their ability for DPPH and ABTS radical scavenging, and by determining their reducing power.

Materials and methods

Materials and Chemicals

The brown alga, *E. bicyclis* (Kjellamn) Setchell, was collected from the coast of Ulleung in Gyeongbuk Province, Korea, between March 2009 and July 2009. The *E. bicyclis* (Kjellamn) Setchell was authenticated and identified by professor Ki-Wan Nam of Department of Marine Biology, Pukyong National University, Busan, Korea. The samples were washed three times with tap water to remove epiphytes, salts and sand. The samples were lyophilized using a freeze drier (Ilshin, Dongducheon, Korea). They were then pulverized and powdered by passing through 80 mesh sieves and stored at −20 °C prior to use. 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, rose bengal, trichloroacetic acid (TCA), dimethyl sulphoxide (DMSO), potassium ferricyanide, butylated hydroxyanisole (BHA), iron (III) chloride and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid were purchased from Sigma chemical Co. (St. Louis, MO, USA). 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Wacko chemical (Tokyo, Japan). All other chemicals used were 99 % or greater purity.

Isolation of three phlorotannins

The dried sample (1.3 kg) was extracted twice with methanol at room temperature for 2 days. Following removal of MeOH under reduced pressure, the resulting solution was partitioned between chloroform and H_2O and then ethyl acetate and H_2O. The ethyl acetate-soluble fraction was subjected to a column of silica gel eluted with chloroform:methanol (50:1–1:1, v/v, stepwise) to give an antioxidant fraction. The active fraction was concentrated and separated by reversed phase (ODS) column chromatography eluted with a gradient of an increasing amount of methanol (10 → 80 %) in water to afford two active fractions. One fraction was concentrated under reduced pressure and subjected to a column of Sephadex LH-20 eluted with methanol to afford three antioxidants, compounds **1**, **2** and **3**.

Structure determination

NMR spectra including ^{1}H NMR, ^{13}C NMR, HMQC and HMBC were obtained using a JEOL JNM-ECA600 600 MHz FT-NMR spectrometer (JEOL, Tokyo, Japan) in CD_3OD with tetramethylsilane as an internal standard. FAB (fast atom bombardment) mass analyses were performed using a JEOL JMS-700 high resolution mass spectrometer (JEOL, Tokyo, Japan) in the negative mode with *m*-nitrobenzyl alcohol as the matrix.

DPPH radical scavenging activity

The antioxidant activity of phlorotannins isolated from *E. bicyclis* (Kjellamn) Setchell was measured using scavenging activities of the stable radical reaction. DPPH radical scavenging assay was measured using Blois method [18].

50 μL of the sample was added to 100 μL of 0.2 mM DPPH solution, vortex-mixed, and allowed to stand at room temperature for 10 min. Absorbance was measured at 517 nm. All the determination was carried out in triplicate. In this study, butylated hydroxyanisole was used as a positive control and the capability of scavenging the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging activity} = \left[1 - \left(A_{\text{sample}}/A_{\text{control}}\right)\right] \times 100.$$

ABTS radical cation decolorization activity

ABTS radical cation decolorization assay was measured using the Re method [19]. 7 mM 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was added to 2.45 mM potassium-persulfate, and the reaction mixture was incubated at room temperature in the dark for 24 h. Next, the absorbance of the reaction mixture was measured for dilution using ethanol to still OD 0.7–0.9 at 734 nm. 50 μL of the sample was added to 100 μL of ABTS solution, and reaction mixture was incubated at room temperature for 5 min. Its absorbance was measured at 734 nm. All the determination was carried out in triplicate. Trolox was used as a positive control, and the capability of scavenging the ABTS radical was calculated using the following equation:

$$\text{ABTS radcal cation activity} = \left[1 - \left(A_{\text{Sample}}/A_{\text{Control}}\right)\right] \times 100$$

Reducing power ability

Reducing power was determined according to the method by Oyaizu [20]. First, 1 mL of the sample was added to 1 mL of 1 % potassium ferricyanide. The reaction mixture was incubated in water bath at 50 °C for 20 min. The mixture was then kept at room temperature, and 1 mL of 10 % trichloroacetic acid was added to the mixture. Finally, 1 mL of the mixture was mixed with 1 mL distilled water and 0.1 mL of 0.1 % ferric chloride. The absorbance of the sample was measured at 700 nm. Ascorbic acid was used as a positive control. All the determination was carried out in triplicate.

Detection of $^1O_2$ quenching activity

In the case of $^1O_2$ photogeneration from the rose bengal (RB, well-known photosensitizer), the imidazole-RNO (*N*,*N*-dimethyl-4-nitrosoaniline) method was employed in which $^1O_2$ was photogenerated from rose bengal (RB, well-known type-II photosensitizer) [21]. The $^1O_2$-mediated bleaching of RNO via imidazole oxidation was further monitored spectrophotometrically at 440 nm in reaction mixtures of 2 μM RB in the 20 mM Tris-succinate buffer (pH 6.5) containing 5 mM imidazole and 4 μM RNO.

The samples were illuminated with white light ($\lambda > 400$ nm, 100 Wm^{-2}) passed through a 6-mm Plexiglass from a 150-W Halogen-lamp (Osram, Augsburg, Germany) using a cutoff filter for 4 min at 25 °C. Histidine was used as a positive control.

Statistical analysis

The results are presented as mean ± standard error of the mean. Statistical comparisons were made using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA), and significance was determined by one-way ANOVA followed by Duncan's multiple range test for multiple comparisons and it was considered significant at $P < 0.05$.

Results

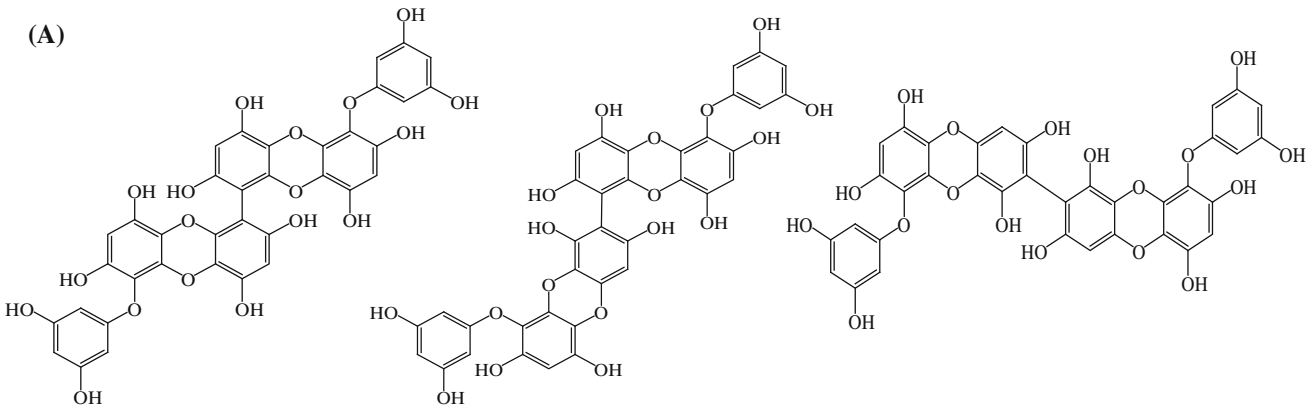
Isolation and structure determination of three phlorotannins

Eisenia bicyclis (Kjellamn) Setchell was extracted with methanol, and the methanolic extract was separated by partitioning between ethyl acetate and H_2O. The ethyl acetate-soluble fraction was further purified by sequential column chromatography using silica gel, ODS and Sephadex LH-20 to afford three antioxidative compounds **1–3**. Compounds **1–3** were identified by spectroscopic analyses including FAB-mass in the negative mode, ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, HMQC and HMBC spectra. Their spectra were in good agreement with those reported in the literature for 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol, respectively (Fig. 1), [22, 23].

DPPH radical scavenging activity

The free radical scavenging activity of phlorotannins isolated from *E. bicyclis* (Kjellamn) Setchell was investigated using DPPH and ABTS radical scavenging assays. DPPH is a stable free radical, and assaying DPPH scavenging ability is a widely used method for evaluating antioxidant activity in a relatively short time. This approach was therefore used to screen the capacity of the isolated phlorotannins for singlet oxygen ($^1O_2$) quenching [24]. DPPH radical scavenging effects of the phlorotannins isolated from *E. bicyclis* (Kjellamn) Setchell are shown in Fig. 2. The scavenging activity of the three pure compounds significantly increased at concentrations from 10 to 100 μg/mL. The scavenging effect of the three pure compounds on the DPPH radical followed this order: 6,6′-bieckol > 6,8′-bieckol > 8,8′-bieckol. The concentrations of 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol required for 50 % inhibition of the DPPH radical (IC_{50}) were found to be 49.38, 71.21

(A)



1 (6,6'- bieckol) **2 (6,8'- bieckol)** **3 (8,8'- bieckol)**

(B)

Chemical name	Molecular formula	Molecular weight
6,6'-bieckol	$C_{36}H_{22}O_{18}$	742
6,8'-bieckol	$C_{36}H_{22}O_{18}$	742
8,8'-bieckol	$C_{36}H_{22}O_{18}$	742

(C)

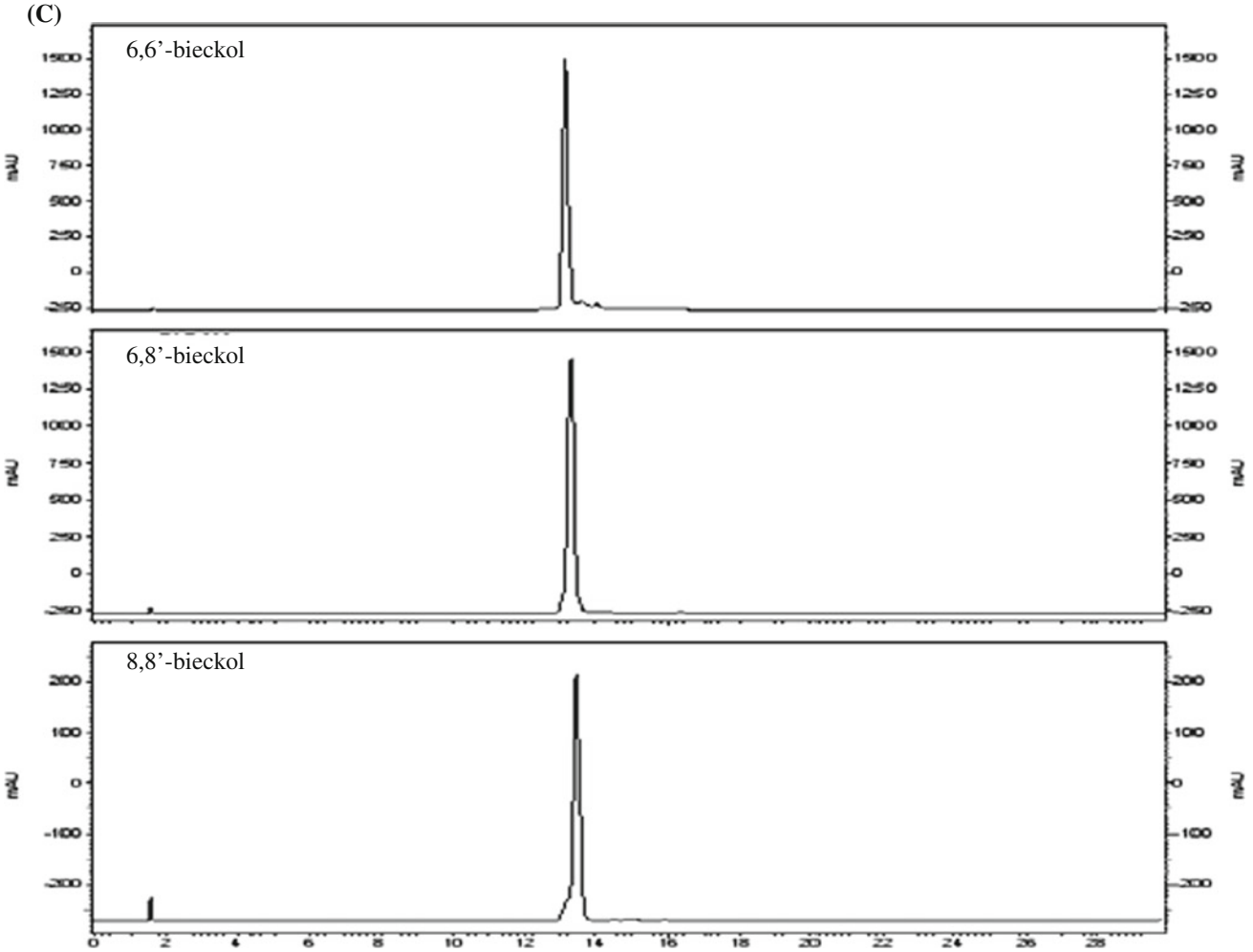


Fig. 1 Chemical structures **a** of phlorotannins isolated from *E. bicyclis.* Chemical information **b** of 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol, and their HPLC profile (**c**)

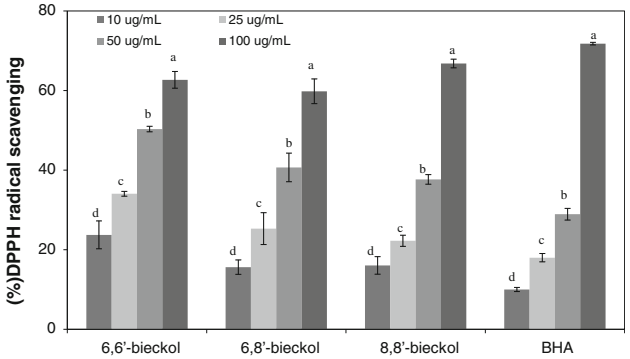


Fig. 2 DPPH radical scavenging activity of phlorotannins isolated from *E. bicyclis*. Sample allowed standing at room temperature for 10 min and absorbance measured at 517 nm. Data represent the mean ± SD of three determinations. BHA; butylated hydroxy anisole

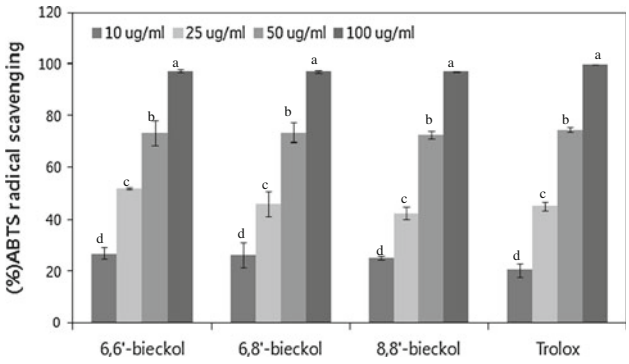


Fig. 3 ABTS radical scavenging activity of phlorotannins isolated from *E. bicyclis*. Sample allowed standing at room temperature for 5 min and absorbance measured at 734 nm. Data represent the mean ± SD of three determinations

Table 1 Antioxidant activity and singlet oxygen quenching of phlorotannins isolated from *E. bicyclis*

Pure compound	DPPH radical scavenging activity IC_{50}^{a} (μg/mL)	ABTS radical scavenging activity IC_{50} (μg/mL)	Singlet oxygen quenching QC_{50}^{b} (μM)
6,6′-bieckol	49.38 ± 1.5	27.57 ± 2.1	30.74 ± 2.4
6,8′-bieckol	76.47 ± 2.6	32.13 ± 1.7	35.71 ± 2.4
8,8′-bieckol	71.21 ± 2.4	34.24 ± 1.5	49.35 ± 1.7
Positive control[c]	72.08 ± 0.2	34.19 ± 1.4	5.9 ± 1.8 (mM)

[a] IC_{50}, the concentration of sample required for 50 % inhibition

[b] QC_{50}, the concentration of sample required for 50 % quenching

[c] Positive controls were used butylated hydroxy anisole, trolox and histidine sequentially

and 76.47 μg/mL, respectively (Table 1), as compared to that of butylated hydroxyanisole (BHA; 72.08 μg/mL).

ABTS radical scavenging activity

The ABTS radical scavenging activity assay is often used with the DPPH radical scavenging activity assay to evaluate potential antioxidant capacities. The ABTS radical scavenging assay involves a stable radical reaction and is one of the readily available and popular methods for determining antioxidant activity [25]. The antioxidant activity of phlorotannins isolated from *E. bicyclis* (Kjellamn) Setchell to the ABTS radical was compared to that of Trolox, and the results are shown in Fig. 3. The scavenging activity of the three purified compounds significantly increased at concentrations from 10 to 100 μg/mL. At all concentrations, the ABTS radical scavenging activity of the three pure compounds significantly increased in a dose-dependent manner. The IC_{50} values of 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol on ABTS radical scavenging activity were found to be 27.57, 32.13 and 34.24 μg/mL, respectively (Table 1). Trolox, used as a positive control, had an IC_{50} value of 34.19 μg/mL. These results indicated that the ABTS radical scavenging activity of 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol is higher than that of the positive control.

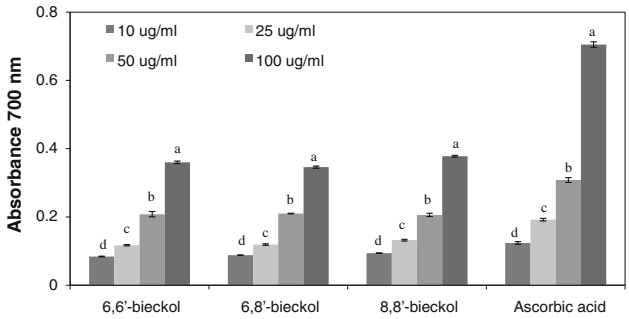


Fig. 4 Reducing power ability of phlorotannins isolated from *E. bicyclis*. Data represent the mean ± SD of three determinations

Reducing power

The ability to scavenge radicals such as the DPPH radical, OH- radical and ABTS radical has been used to evaluate the antioxidant activity of numerous food, plant, marine and biological samples. Assessing the reducing power of a compound, another methods used to evaluate antioxidant activity. In this method, the presence of reduction is assessed utilizing the change in color of the test solution from yellow to green and blue. The reducing power of the three purified compounds and that of a positive control (ascorbic acid) is shown in Fig. 4. The reducing power of the three purified compounds isolated from brown algae *E. bicyclis* (Kjellamn) Setchell increased significantly with increasing concentrations. The absorbance of 6,6′-bieckol,

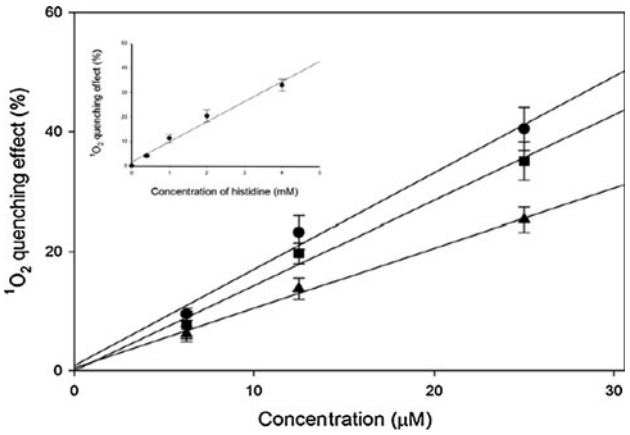


Fig. 5 Kinetics of singlet oxygen quenching capacity was measured by increase in concentration of phlorotannins isolated from *E. bicyclis*. Averaged results from triplicate experiments are given, with *error bars* representing SD. *Filled circle*: 6,6′-bieckol, *filled square*: 6,8′-bieckol, *filled triangle*: 8,8′-bieckol

6,8′-bieckol and 8,8′-bieckol at 700 nm indicating reducing power was 0.361, 0.346 and 0.378, respectively, when compared to that of ascorbic acid (0.705), at a concentration of 100 μg/mL.

Singlet oxygen quenching effect

The $^1O_2$ quenching effects of the isolated samples on RNO-imidazole bleaching as a result of the reaction of imidazole with $^1O_2$ produced by RB in irradiation are shown in Fig. 5. The RNO-mediated bleaching decreased in inverse proportion to the sample concentrations in the photolysis system. The ratio of decrease in RNO bleaching was expressed as the quenching efficacy. The concentrations of 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol required for 50 % quenching of $^1O_2$ (QC_{50} values) were found to be 30.7, 35.7 and 49.4 μM, respectively. Histidine was used as a positive control and had QC_{50} of 5.9 mM. Interestingly, 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol thus appeared be superior to histidine, a well-known synthetic $^1O_2$ quencher (Table 1).

Discussion

Seaweeds are popularly used in the food and cosmetic industries. They are low in calories, and rich in vitamins, dietary fibers and minerals. Seaweeds are consumed as food mostly in Asian countries, such as Korea, Japan, China and South-East Asia [26]. Seaweeds also have numerous biological activities as antioxidants and anti-inflammatory agents, as well as providing protection against DNA damage due to oxidative stress [27]. Therefore, seaweeds are attractive sources to be developed as drugs and functional foods. Moreover, recently, several studies have reported antioxidant activities of enzymatic extracts from brown seaweeds. Cho et al. have reported on the antioxidant properties of brown seaweed (*Sargassum siliquastrum*) extract, and Ganesan et al. have reported on the antioxidant properties of the methanol extract and its solvent fractions obtained from selected Indian red seaweed, using free radical scavenging assays, such as DPPH and ABTS radical scavenging assays and reducing power assays [4, 26, 28]. Furthermore, recently, various assays, such as DPPH, ABTS and oxygen radical absorbance capacity (ORAC), as well as ferric reducing ability of plasma (FRAP), have been applied to evaluate antioxidant activities of food and biological materials [29].

In the present study, we extracted brown algae twice with methanol, and this extract was then progressively partitioned. Subsequently, from the ethyl acetate fraction, we isolated the major compounds by assessing antioxidant activity. Consequently, we identified three compounds, viz., 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol derived form phlorotannins, and the molecular weights of these compounds were 742. The scavenging activities of the phlorotannins isolated from *E. bicyclis* (Kjellamn) Setchell were related to the concentration of the compounds used in the assays. The scavenging effects of the phlorotannins on the DPPH radical followed the order: 6,6′-bieckol > 6,8′-bieckol > 8,8′-bieckol and were found to be 50.32, 40.68 and 37.67 %, respectively, at a concentration of 50 μg/mL. The isolated phlorotannins showed markedly strong antioxidant effects as compared to positive control, a well-known synthetic antioxidant. We also performed an ABTS decolorization assay. The scavenging effect of 6,6′-bieckol, 6,8′-bieckol and 8,8′-bieckol on the $ABTS^{\dot{+}}$ radical was 73.49, 73.64 and 72.70 %, respectively, at a concentration of 50 μg/mL. Nakamura et al., when evaluating the antioxidant activity of phlorotannins isolated from the brown alga *E. bicylis*, also found that antioxidant activities increased with increasing sample concentrations. Moreover, Shibata et al. reported on the antioxidant activities of phlorotannins (phloroglucinol, eckol, phlorofucofuroeckol A, dieckol, 8,8-bieckol) isolated from Japanese Laminariaceae by assessing radical scavenging activity [15, 30].

Based on the findings that these phlorotannins showed DPPH and ABTS radical scavenging activities, it could be conjectured that phlorotannins hold promise as antioxidant agents. Natural antioxidants can be use to alleviate oxidation and increase the shelf-life in the food industry. Moreover, according to Ganesan et al. [28], consumption of antioxidants via addition of antioxidants to foods can be helpful in protecting the body as well as increasing shelf-life of foods. Taken together, phlorotannins have potential application in foods and as a drug against ROS-associated diseases.

Among free radicals, $^1O_2$ is one of the important factors in reactive oxygen species-mediated disease. Thus, $^1O_2$ is important in all biological systems. These oxidants can react with the contents of living cells, including proteins, lipids and DNA. Proteins are one of the important targets for photo-oxidation in living cells. Photo-oxidation occurs via two major routes [31]. First, it involves direct absorption of UV radiation by protein structures. Second, it involves direct oxidation of proteins via subsequent reactions of singlet oxygen. Reaction of singlet oxygen with peptides, proteins and amino acids can occur via two pathways; viz., chemical and physical routes. However, most reactions occur via chemical rather than physical route except for reactions involving tryptophan [32]. Therefore, singlet oxygen can be generated by radical reactions involving proteins, lipoxygenases and leukocytes.

The incidence of diseases such as cancer, obesity, diabetes, hypertension, dementia and cardiovascular diseases in adults is increasing. Reactive oxygen species are associated with these human diseases and can be generated through living organisms, via pathways including proteins, lipids and DNA. For this reason, the roles and functions of phlorotannins have been the subject of many studies, particularly for those investigating plant-herbivore interactions and anti-fouling agents [33, 34]. It has been suggested that tannins and related phenolic substances play key roles in marine plants, as they may serve as osmoregulatory substances in sea grasses and as cell wall components in both marine vascular plants and brown algae [35]. Tannins may also affect palatability due to their astringent taste and may further act as antioxidant agents. It is also possible that they are strongly involved in redox reactions in plants.

We demonstrated in this study that phlorotannins can suppress free radicals (Figs. 2, 3 and 4). UV screening by phlorotannins apparently had no relation to the phlorotannin effects that were observed in our study, because the samples were subjected to photosensitization treatments in light devoid of UVA and UVB (note that phlorotannins absorb mostly UVB, with a peak at 280–340 nm). There can be little doubt that the isolated phlorotannins quench $^1O_2$ effectively (Fig. 5), and will thus efficiently remove this ROS from biological systems and will provide protection under photosensitization conditions. It can be conjectured that phlorotannins per se act as photosensitizers to UV light, because they exhibit strong UV light absorption. De rosso et al. [36] have reported as observed for other phenolic-like derivatives, the quenching of $^1O_2$ by anthocyanins was mediated by a charge-tansfer mechanism, which was modulated by the total number of –OR substituent. Therefore, in this study, from the results, we estimated the concentration of bieckols required for quenching of 50 % of singlet oxygen. In methanol, the lifetime of singlet oxygen is about 10 us. Therefore, using the classical Stern–Volmer eq., we estimated a total quenching rate constant (kt) about 3 × 109 M-1s-1 for the bieckols. This value is higher for most polyphenols (107–108 M-1s-1). From these finding, taken together, the bieckols isolated from *E. bicyclis* have been higher antioxidant activities. Its effect can be related with the higher number of -OH groups in the bieckols.

Our previous and current $^1O_2$-quenching experiments with various marine organisms showed that, in addition to phlorotannins, several members of the mycosporine-like amino acid family [37] quench $^1O_2$ to some extent. In particular, 6,6′-bieckol, which is biosynthetically related to phlorotannins, shows high $^1O_2$ reactivity. Such preliminary observations together with the results presented here may indicate that phlorotannins, as a group of secondary metabolites, play a role in protecting marine organisms against sunlight damage, not only by screening out energetic UV radiation, but also more importantly by scavenging $^1O_2$ produced by certain endogenous photosensitizers.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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