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Chemical Constituents from *Sargassum micracanthum* and Antioxidant Activity

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Abstract: By bioactivity-directed fractionation, three antioxidant constituents have been characterized from *Sargassum micracanthum* collected in Jeju Island, Korea. The potent antioxidant elucidated sargaquinoic acid, sargachromenol and fucosterol by NMR and mass spectroscopic data. The free radical scavenging activities of the sargaquinoic acid, sargachromenol and fucosterol were investigated in relation to 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals using an Electron Spin Resonance (ESR) system. The sargaquinoic acid and sargachromenol were found to scavenge DPPH (IC_{50}, 49.3 and 100.2 μM). Therefore, these results resent sargaquinoic acid and sargachromenol with a potent antioxidative activity that could be useful in cosmetics, foods and pharmaceuticals.

Key words: ESR, fucosterol, *Sargassum micracanthum*, sargachromenol, sargaquinoic acid

INTRODUCTION

Natural products obtained from marine resources play an important role in the development of new drugs. Because more than 70% of the earth's surface is covered by oceans, many marine resources such as seaweeds, are used for medical and agricultural areas. In these marine creatures, seaweeds have been investigated for the purpose of searching for biologically useful substances. Some of them had been used and/or examined for an application to food, cosmetic materials and a source of alternative medicines. *Sargassum micracanthum* is a marine brown alga distributed worldwide from temperate to subtropical regions. It has not been considered medicinal; however, its extracts have various biological activities such as antioxidant and antiviral activities, selective vasodilation effect and inhibitory activities on bone resorption (Iwashima *et al*., 2005; Hayashi *et al*., 2006; Komai *et al*., 2006; Park *et al*., 2008). Also, *S. micracanthum* are known to contain structurally unique secondary metabolites containing plastoquinones and chromenes such as sargaquinoic acid and sargahydroquinoic acid (Iwashima *et al*., 2005, 2008; Hayashi *et al*., 2006; Komai *et al*., 2006; Park *et al*., 2008). These compounds show various biological activities due to their unique structure.

Excessive generation of Reactive Oxygen Species (ROS) induced by various stimuli and which exceed the antioxidant capacity of the organism leads to significantly pathological role in human diseases such as arthritis, atherosclerosis, cirrhosis, emphysema and cancer (Gulcin *et al*., 2002). Therefore, antioxidants are screen to prevent oxidative damage by free radical and ROS and may prevent the occurrence of disease, cancer and aging. The most commonly used antioxidants at the present time are BHA, BHT, propyl gallate and tert-butylhydroquinone (Gulcin *et al*., 2003). However, they have been suspected of being responsible for liver damage and carcinogenesis in laboratory animals. Hence, a need for identifying alternative natural and safe sources of food antioxidants has been created and the search for natural antioxidants, especially of natural origin, has notably increased in recent years (Gülçin *et al*., 2004; Ak and Gülçin, 2008).

In the course of investigations on the biologically active metabolites from this alga, three known compounds, sargaquinoic acid, sargachromenol and fucosterol, were isolated as major constituents. In this study, we evaluated their radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals using an electron spin trapping technique.

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MATERIALS AND METHODS

Plant material: *Sargassum micracanthum* were collected in May 2006 from Chuja Island of Jeju, Korea. Voucher specimen number JBR-513 was deposited at the herbarium of Jeju Biodiversity Research Institute (JBRI). 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.

General: The NMR spectra were recorded on a Brucker AVANCE 3 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C), with the residual solvent $CDCl_3$ used as an internal standard for ^{3}H (7.23) and for ^{13}C (77.0). The FAB Mass spectra data were obtained from the Korea Basic Science Institute (Daegu) on a Jeol JMS 700 mass spectrometer. ESI mass spectra analysis was carried out on a Thermo LCQ-Fleet LC-MS/nMS. Column Chromatography (CC) separations were on silica gel 60 (70-230 mesh, Merck) and celite 545 (Celite Korea). The TLC was performed on commercial plates (silica gel and F_{254}, Merck). Compounds were detected by UV (λ = 254 and 366 nm) irradiation and/or with a methanolic solution of anisaldehyde, sulfuric acid and acetic acid (90:5:1 mL), followed by heating.

Isolation of fucosterol, sargaquinoic acid and sargachromenol: The algal powder (370 g) was extracted three times with 80% ethanol and filtered. The filtrate was then evaporated at 40°C to obtain the ethanol extract, which was subsequently sequentially solvent partitioned into Hexane, CH_2Cl_2, EtOAc, butanol and water-soluble extracts. The CH_2Cl_2 extract was fractionated using celite column chromathography (Hexane/CH_2Cl_2 0:1, 10:1, 5:1, 2:1, 0:1, CH_2Cl_2/EtOAc 10:1, 5:1, 2:1, 0:1, methanol). The 0:1 Hexane/CH_2Cl_2 celite column fraction was subjected to repeated silicagel column chromatography (CH_2Cl_2/methanol 40:1) to yield compound 1(43 mg). The 5:1 Hexane/CH_2Cl_2 celite column fraction was also subjected to silica gel column chromatography (Hexane/EtOAc/methanol 3:1:0.1) to afford compound 2 (13.5 mg), 3 (22.8 mg). The structure of these compounds was identified by comparing the NMR spectral data with those in the existing literature.

DPPH radical scavenging assay: DPPH radical scavenging activity was measured using the method described by Nanjo *et al*. (1996). An ethanolic solution of 60 µL of each sample (or ethanol itself as the control) was added to 60 µL of DPPH (60 µmol L^{-1}) in ethanol. After mixing vigorously for 10 sec, the solution was transferred into a 100 µL Teflon capillary tube and fitted into the cavity of the ESR spectrometer (JES-FA machine, JOEL, Tokyo, Japan). The spin adduct was measured on an ESR spectrometer exactly 2 min later. The measurement conditions used were: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5 and a temperature of 298 K.

Hydroxyl radical scavenging assay: Hydroxyl radicals were generated by the Fenton reaction and reacted rapidly with nitrone spin trap DMPO; the resultant DMPO-OH adducts was detectable with an ESR spectrometer (Rosen and Rauckman, 1984). The ESR spectrum was recorded 2.5 min after addition of a phosphate buffer solution (pH 7.4) with 0.3 M DMPO 0.2 mL, 10 mM $FeSO_4$ 0.2 mL and 10 mM H_2O_2 0.2 mL using an ESR spectrometer set at the following conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 1 mW, gain 6.3×10^5 and a temperature of 298 K.

Statistical analysis: The data are expressed as the Mean±SE. A statistical comparison was performed via a one-way Analysis of Variance (ANOVA) followed by Duncan's multiple range test (Duncan, 1955). The p-values of less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Brown algae of the genus *Sargassum*, (Sargassaceae, Fucales) are known to contain structurally unique secondary metabolites, such as plastoquinones, chromanols, a cyclopentenone and polysaccharides. These compounds show various biological activities due to their unique structure. We also previously reported that *S. micracanthum* showed strong anti-inflammatory activity. Since, the bioactive compounds, not only the structure-activity-relationship toward the development of new drugs or supplements, but also the biological functions in the algae themselves was still unclear, this study screened its antioxidant activity via its scavenging effect on DPPH free and hydroxyl radicals. As the CH_2Cl_2 fraction showed the strong DPPH radical scavenging activity of 83.1% at a concentration of 125 µg mL^{-1}. (IC_{50} values, 45.3 µg mL^{-1}), the active compounds of this fraction was isolated and identified as sargaquinoic acid, sargachromenol and fucosterol, based on a comparison of NMR spectroscopic data and previous literature as follows Lee *et al*. (2003), Seo *et al*. (2004) and Cañabate-Díaz *et al*. (2007).

Table 1: NMR spectroscopic data for sargaquinoic acid and sargachromenol

	Sargaquinoic acid		Sargachromenol	
Position	^{13}C	^{1}H (mult, J = Hz)	^{13}C	^{1}H (mult, J = Hz)
1	27.6 t	3.11 (2H, d, 7.0)	122.8 d	6.23 (1H, d, 10.0)
2	118.2 d	5.14 (1H, t, 7.5)	130.6 d	5.55 (1H, d, 10.0)
3	140.0 s		77.7 s	
4	39.7 t	2.08 (2H, m)	40.7 t	1.64 (2H, m)
5	26.4 t	2.09 (2H, m)	22.5 t	2.09 (2H, m)
6	124.7 d	5.11 (1H, m)	124.9 d	5.13 (1H, t,6.5)
7	135.0 s		134.3 s	
8	39.2 t	2.09 (2H, m)	39.0 t	2.05 (2H, t, 7.5)
9	28.4 t	2.59 (2H, q,7.0)	28.1 t	2.59 (2H, dt,, 7.5, 7.5)
10	145.3 d	5.98 (1H, t,7.5)	145.5 d	5.97 (1H, t, 7.0)
11	130.7 s		130.5 s	
12	34.8 t	2.25 (2H, t, 7.5)	34.5 t	2.25 (2H, t, 7.5)
13	28.1 t	2.12 (2H, m)	27.8 t	2.12 (2H, m)
14	123.6 d	5.07 (1H, m)	123.4 d	5.08 (1H, t, 7.0)
15	132.4 s		132.2 s	
16	25.7 q	1.65 (3H, s)	25.6 q	1.65 (3H, s)
17	17.9 q	1.56 (3H, s)	17.6 q	1.56 (3H, s)
18	172.1 s		173.1 s	
19	16.1 q	1.58 (3H, s)	15.4 q	1.55 (3H, s)
20	16.3 q	1.60 (3H, s)	25.8 q	1.33 (3H, s)
1'	188.3 s		144.8 s	
2'	148.7 s		121.3 s	
3'	132.4 d	6.45 (1H, m)	110.3 d	6.30 (1H, d, 3.0)
4'	188.2 s		148.5 s	
5'	133.4 d	6.53 (1H, quin, 1.5)	117.0 d	6.45 (1H, d, 3)
6'	146.1 s		126.3 s	
Ar-Me	16.1 q	2.03 (3H, d, 1.5)	15.7 q	2.10 (3H, s)

Compound 1 fucosterol: $C_{29}H_{48}O$, white amorphous powder, FABMS : positive mode m/z 395.5 $(M+H-H_2O)^+$: 1H NMR (500 mHz, $CDCl_3$) δ_H (ppm): 5.33 (1H, br d, J = 5.0 Hz, H-6), 5.17 (1H, q, J = 6.5 Hz, H-28), 3.53 (1H, m, H-3), 1.55 (3H, d, J = 7.0 Hz, H-29), 0.98 (3H, s, H-19), 0.97 (3H, br s, H-21), 0.96 (3H, d, J = 1.2 Hz, H-27), 0.95 (3H, d, J = 1.5 Hz, H-26), 0.66 (3H, s, H-18); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (ppm): 146.9 (C-24), 140.7 (C-5), 121.7 (C-6), 115.5 (C-28), 71.8 (C-3), 56.7 (C-14), 55.7 (C-17), 50.1 (C-9), 42.3 (C-13),42.2 (C-4), 39.7 (C-12), 37.2 (C-1), 36.5 (C-10), 36.4 (C-20), 35.2 (C-22), 34.7 (C-25), 31.9 (C-7,8), 31.6 (C-2), 28.2 (C-16), 25.6 (C-23), 24.3 (C-15), 22.2 (C-26), 22.1(C-27), 21.0 (C-11), 19.4 (C-19), 18.7 (C-21), 13.1 (C-29),11.8 (C-18).

Compound 2 sargaquinoic acid: Brown viscous oil, ESI-MS/MS: negative mode m/z 424.06 $(M-H)^-$, 379.3 $(M-COOH)^-$: ^{1}H, ^{13}C NMR Table 1.

Compound 3 sargachromenol: Brown viscous oil, ESI-MS/MS: negative mode m/z 424.03 $(M-H)^-$, 379.2 $(M-COOH)^-$: ^{1}H, ^{13}C NMR Table 1.

Three compounds were then used in further experiments with an ESR spectrometer. The ESR spin trapping provides a sensitive, direct and accurate means of monitoring reactive species (Guo *et al*., 1999). Therefore, this study used ESR to compare the DPPH and hydroxyl radical scavenging abilities of the sargaquinoic acid, sargachromenol and fucosterol (Fig. 1A) isolated from *S. micracanthum* 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 sargachromenol (SC) and sargaquinoic acid (SQ) towards DPPH free radicals is shown in Fig. 1B, where, 78.85 and 69.82% of the DPPH free radicals were scavenged at 250 μM, plus the radical scavenging occurred in a dose-dependent manner. Generally, hydroxyl radicals are generated from hydrogen peroxide and the scavenge hydroxyl radicals passed through the so-called Fenton reaction (Huang *et al*., 2002; Heo *et al*., 2008). Therefore, to scavenge hydroxyl radicals, the following *in vitro* reaction was generated: $Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+{}^{-}OH+{}^{\bullet}OH$. As shown in Fig. 1B, the sargaquinoic acid, sargachromenol and fucosterol was not exhibited a decrease in the amount of DMPO-OH adducts by the ESR signals. In conclusion, as shown in Fig. 1C, sargachromenol is relatively strong DPPH antioxidant activity in three constituents. However, the sargachromenol exhibited relatively lower levels of DPPH free radical activity (IC_{50} values, 49.3 μM) than the commercial antioxidant ascorbic acid (IC_{50} values, 19.9 μM). Natural antioxidants can protect the human body from serious cellular or molecular damage by free radicals and retard the progress of many chronic diseases in food and cosmetic industry. Thus, owing to growing health concerns, interest has significantly increased in

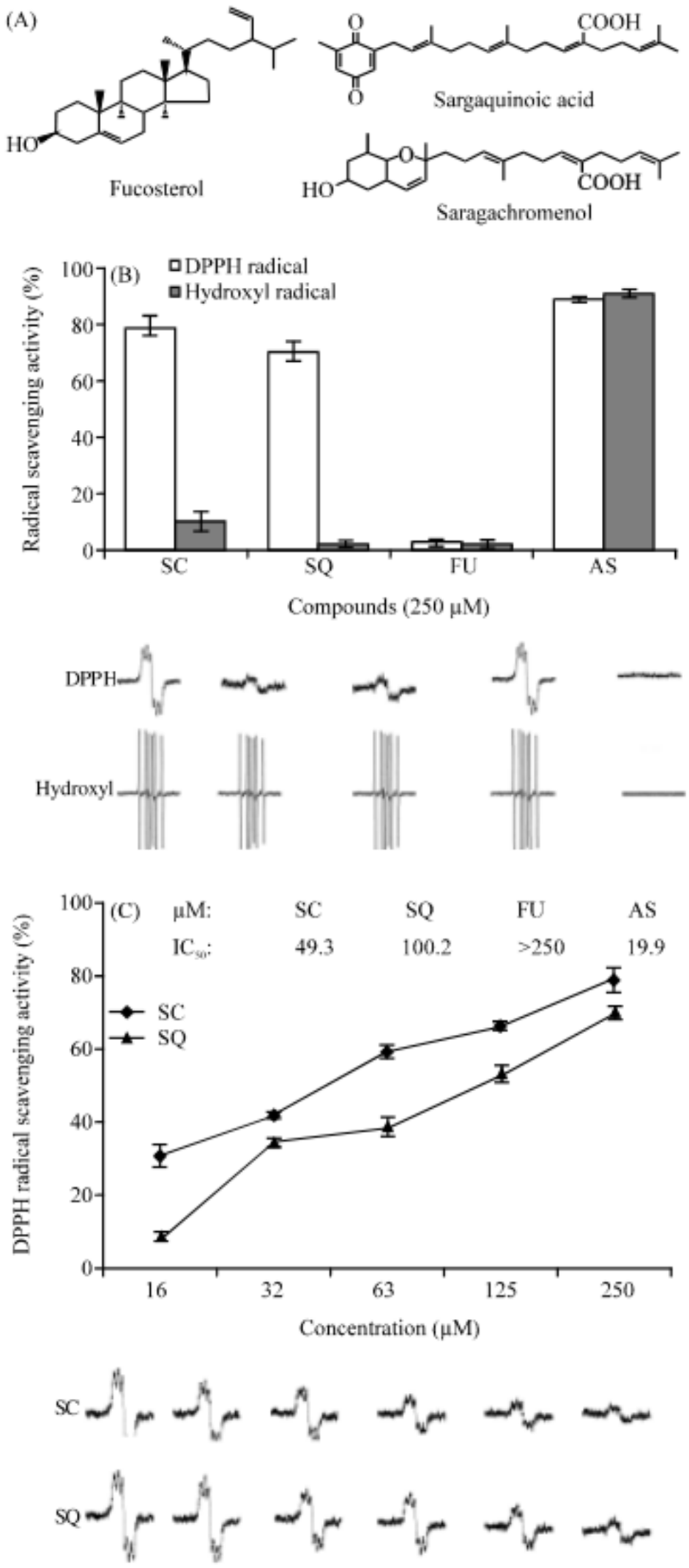

Fig. 1: Chemical structures and antioxidant activities of fucosterol, sargachromenol and sargaquinoic acid. (A) Chemical structures of fucosterol (FU), sargachromenol (SC) and sargaquinoic acid (SQ). (B) DPPH and hydroxyl radicals scavenging activity of FU, SC and SQ. ESR spectrum obtained in an ethanol solution of 60 μmol L^{-1} DPPH of three compounds and ascorbic acid. ESR spectrum obtained in Fenton reaction system of three compound and ascorbic acid. (C) DPPH Radical scavenging activities of SC and SQ via the ESR system. SC: Sargachromenol, SQ: Sargaquinoic acid, FU: Fucosterol, AS: Ascorbic acid

finding natural antioxidants for use in food, cosmetic and medicinal materials to replace synthetic antioxidants with harmful effects. Even if, sargachromenol and sargaquinoic acid possessed relatively lower antioxidant activities than

synthetic chemicals, therefore they may be a useful natural radical scavenger and a possible supplement for the food, pharmaceutical and cosmetic industries.

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