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Intracellular reactive oxygen species scavenging effect of fucosterol isolated from the brown alga *Sargassum crassifolium* in Vietnam

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Abstract
Sargassum is a widely distributed marine brown algal genus in Vietnam and has been considered a source of diverse bioactive metabolites. In this study, *S. crassifolium* collected from South Central region of Vietnam was chemically studied and bioactively evaluated. Fucosterol was isolated and identified from the methanolic extract of the alga by means of chemical fractionation and spectral analysis and showed no cytotoxic effect in Hep-G2 cells at the observed concentrations. In vitro assay for intracellular reactive oxygen species by dichlorofluorescein method revealed a potent scavenging effect of the isolated compound. Accordingly, the level of intracellular reactive oxygen species induced by hydrogen peroxide (H_2O_2) was reduced by 71.66% with the treatment of fucosterol at 10 µg.ml^{-1}. The results indicated the ability of algal fucosterol in diffusing into cells and preventing the production of different ROS compounds and further suggested the therapeutic potential against diseases caused by oxidative stress of natural metabolites from *S. crassifolium* in Vietnam.

Keywords: *Sargassum crassifolium*, alga, fucosterol, ROS, antioxidant, Hep-G2 cells.

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INTRODUCTION

With approximately 3,300 km long coastline spanning over 15 degrees of latitude and varying climatic zones [1], Vietnam has appropriated conditions for a diverse and prosperous marine algal flora [2]. According to a recent checklist of marine algae, a total number of 827 species belonging to phyla Rhodophyta (412 species), Chlorophyta (180 species), Phaeophyceae (147 species) and Cyanobacteria (88 species) were recorded in different regions of Vietnam [3]. These data contribute to highlighting the potential of exploiting natural products with therapeutic potential and health benefits from algae in Vietnam.

Sargassum is a genus of brown algae (Fucales, Phaeophyceae) containing approximately 400 species worldwide [4] that have been reported to produce bioactive metabolites such as polysaccharides, polyphenols, terpenoids, sargaquinoic acids, sargachromenol, plastoquinones, steroids, glycerides,... [5, 6]. Unlike other species within the class Phaeophyceae distributed predominantly in cold-water areas, algae of the genus *Sargassum* are commonly found in temperate and tropical waters [7]. In 2013, 72 algal species of *Sargassum* in Vietnam were listed [3], with several chemically characterized and bioactively well studied species such as *S. mcclurei*, *S. polycystum*, *S. oligocystum*, *S. serratum*, *S. crassifolium*, *S. denticarpum* and *S. crassifolium* [8–12].

From the prospective of biological activities, there have been a great number of successful attempts to elucidate antioxidant properties of metabolites extracted from *Sargassum* species, such as phlorotannins from the extract of *S. ringgoldianum* [13], sulfated polysaccharides from *S. Fulvellum* [14], phenolic compounds from *S. hemiphyllum* [15], thunbergols (tetraprenyltoluquinols) and sargothunbergol (chromene) isolated from *S. Thunbergii* [16], sargachromanols (meroterpenoids) isolated from *S. siliquastrum* [17, 18] and plastoquinones isolated from *S. micracanthum* [19, 20]. Antioxidant activities of *Sargassum* algae and their metabolites have been determined by various methods, most commonly in vitro methods such as 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging and 2,2'-azinobis-3-ethylbenzothizoline-6-sulfonate (ABTS) radical scavenging assays [6].

Our study focused on *S. crassifolium*, one of the most abundant tropical brown algal species in South Central region of Vietnam. To our knowledge there had been no report on the intracellular reactive oxygen species (ROS) scavenging effect of its methanolic extract. The purpose of this study was to isolate and structurally elucidate the antioxidant component in dichlorofluorescein assay from *S. Crassifolium* methanolic extract.

MATERIALS AND METHODS

Algal sampling

Thalli of *S. crassifolium* were collected from Nha Trang bay in Khanh Hoa province (12°26'63"42 N, 109°20'67"60 E) in February 2018 and stored with sea water in 50 l portable tanks. Samples were then transferred to Nha Trang Institute of Technology Research and Application (NITRA) to be taxonomically confirmed and washed with tap water, followed by drying in ovens (50°C) until moisture content less than 5%. The dried samples were then stored in a sealed plastic bag and transferred to Institute of Natural Products Chemistry (INPC) for further studies.

Extraction and isolation processes

The dried algal samples (15 kg) were ground and extracted with methanol (Xilong Scientific, China) by ultra-sonication assisted extraction method at 40°C for 48 hours and replicated thrice, followed by rotary evaporation (Eyela, Japan). The methanolic extract was then liquid-liquid extracted with different solvents of increasing polarity (*n*-hexan, ethyl acetate and water), yielding A, T and W fractions, respectively.

The ethyl acetate fraction (T) (90 g) of algal methanolic extract was chosen to be subjected to column chromatography on silica gel C-18 (Merck, 0.063–0.2 mm, Φ = 10 cm), eluted with *n*-hexan:ethyl acetate (1:0, 20:1, 10:1, 5:1, 1:1 and 0:1, v/v) to obtain 8 sub-fractions (T1-T8). Owing to similarities in thin-layer chromatography (TLC) results, T3 and T4 sub-fractions were accumulated and then chromatographed on silica gel C-18 (Merck,

0.04–0.063 mm, Φ = 3 cm), eluted with *n*-hexan:ethyl acetate (10:1, v/v), followed by loading on Sephadex LH-20 column (Merck, Germany) with petroleum ether/chloroform/methanol (2:1:1, v/v/v) elution to obtain compound S1 (45 mg).

Structural characterization

Nuclear magnetic resonance (NMR) spectra were measured on a Bruker AVANCE (Bruker, USA) spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C using tetramethylsilane as an internal standard. Chemical shifts were expressed in δ (ppm) and coupling constants (*J*) in Hz. Spectra of heteronuclear multiple quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) were further recorded (Bruker, USA) to support data for structure elucidation of compound S1.

Cell culture

Cells of human hepatocellular carcinoma line (Hep-G2) originated from the American Type Culture Collection (HB8065, USA) were grown in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS), streptomycin (100 µg/ml), and penicillin (100 U/ml), followed by incubation (37^{o}C, 5% CO_2) with medium change twice a week.

Determination of cytotoxicity

The cytotoxicity of test compound (S1) was determined in Hep-G2 cells by sulforhodamine B (SRB) colorimetric assay [21]. Cancer cells were seeded at $5*10^3$ cells per well in 96-well microtiter plates and incubated with test compound at 37^{o}C (72 h, 5% CO_2). Trichloroacetic acid solution was then added to wells to fix cells, followed by staining with 0.4% (w/v) SRB for 30 minutes. The absorbency of each well was determined at 564 nm in plate reader (Tecan, Switzerland) and the percentage of cell survival was calculated.

Dichlorofluorescein assay for measurement of intracellular ROS

The quantification of intracellular ROS was measured in 96-well plates as the method described previously by Wang & Joseph (1999) [22] and Sohn et al., (2005) [23]. In brief, cells were trypsinized (Trypsin-EDTA, Sigma-Aldich) and seeded at concentration of $5*10^4$ cells/ml. After 24 h, the cells in the plates were washed with phosphate buffered saline (PBS) buffer and incubated with various concentrations of test sample (37^{o}C, 30 min). After being washed by PBS buffer, the cells were added with 1 mM H_2O_2, and then incubated at 37^{o}C for additional 30 min to induce intracellular ROS. Eventually, 2',7'-dichlorofuofescein diacetate (DCFH-DA, Sigma-Aldrich) was loaded to the culture plate at the final concentration of 5 µM and incubated in darkness (37^{o}C, 96 h). The oxidation of DCFH to DCF (2',7'-dichlorofuofescein) was detected by quantifying the intensity of DCF fluorescence with emission wavelength at 530 nm and excitation wavelength at 485 nm using Spark® Cyto multi-well fluorescence plate reader (Tecan, Switzerland) at Institute of Natural Products Chemistry. Data points were exported to Excel (Microsoft, USA) spreadsheet software for analysis.

Statistical analysis

Each experiment was performed in triplicate. Statistical analysis was performed using analysis of variance (ANOVA) with subsequent post hoc comparisons by t-test using SPSS (version 17.0; SPSS Inc., USA). *P* less than 0.05 was considered to have statistical significance.

RESULTS AND DISCUSSION

Structure of compound S1

Compound S1 was isolated as a white solid. The ^{1}H-NMR, ^{13}C-NMR and DEPT spectra revealed the typical steroid structure of S1. The ^{1}H-NMR spectrum of S1 (table 1) revealed 6 methyl groups at δ_H 0.68 (3H, s, CH_3-18), 0.86 (3H, s, CH_3-19), 0.89 (3H, s, CH_3-21), 0.97 (3H, s, CH_3-26), 0.97 (3H, s, CH_3-27), and 1.56 (3H, s, CH_3-29), an olefinic proton at δ_H 5.27 (1H, d, *J* = 5.0 Hz, H-6) and an oximethine group at δ_H 3.27 (1H, m, H-3).

The ^{13}C-NMR and DEPT spectrum disclosed the signal of 29 carbons, including 6 methyl groups (δ_C 11.24, 18.67, 18.23, 21.66, 21.55, 12.37), 10 methylenes (δ_C 36.63, 31.00, 41.60, 31.09, 20.26, 39.00, 23.50, 27.23, 34.38, 25.06), 9 methines (δ_C 119.80, 114.87, 69.70, 31.20, 49.46, 55.90, 55.06, 35.28, 33.70) and 4 quaternary carbons (δ_C 146.06, 141.06, 35.77, 41.89).

The correlation of olefinic proton at δ_H 5.27 (1H, d, J = 5 Hz, H-6) with carbon at δ_C 119.80 ppm, and that of oximethine proton at δ_H 3.27 (1H, m, H-3) with carbon at δ_C 69.70 ppm were observed in HSQC spectrum. Further data by HMBC uncovered the interactions between H-6 (δ_H 5.27) and carbons C-4 (δ_C 41.60), C-7 (δ_C 31.09), C-8 (δ_C 31.20) and C-10 (δ_C 35.77); between the proton of methyl group CH_3-18 (δ_H 0.68) and carbon C-12 (δ_C 39.00) and C-13 (δ_C 41.89), between the proton of methyl group CH_3-19 (δ_H 0.86) and carbons at C-1 (δ_C 36.63), C-9 (δ_C 49.46) and C-10 (δ_C 35.77). The spectrum also revealed the correlations between the proton of methyl group CH_3-21 (δ_H 0.89) and carbons C-22 (δ_C 34.38) and C-17 (δ_C 55.06), between the protons of methyl group CH_3-27 (δ_H 0.97) and carbon C-24 (δ_C 146.06), and between the protons of methyl group CH_3-29 (δ_H 1.56) and carbons C-24 (δ_C 146.06) and C-28 (δ_C 114.87).

By analyzing and comparing the obtained data to literature [24] (Data shown in table 1), the structure of compound S1 was determined as fucosterol (fig. 1).

Table 1. ^{1}H-, ^{13}C-NMR and DEPT assignments for compound S1

No.	$^{[*]}\delta_C$	$\delta_C^{a,b}$	DEPT	$\delta_H^{a,c}$ mult. (J in Hz)
1	37.2	36.63	CH_2	1.78 (1H, m), 1.10 (1H, m)
2	31.6	31.0	CH_2	1.52 (1H, m), 1.38 (1H, m)
3	71.7	69.70	CH	3.27 (1H, m)
4	42.3	41.60	CH_2	2.16 (1H, m), 2.08 (1H, m)
5	140.6	141.06	C	-
6	121.5	119.8	CH	5.27 (1H, d, 5.0 Hz)
7	31.9	31.09	CH_2	1.92 (1H, m), 1.70 (1H, m)
8	31.9	31.2	CH	1.42 (1H, m)
9	50.1	49.46	CH	0.90 (1H, m)
10	36.4	35.77	C	-
11	21.1	20.26	CH_2	1.50 (1H, m), 1.42 (1H, m)
12	39.8	39.0	CH_2	1.96 (1H, m), 1.16 (1H, m)
13	42.3	41.89	C	-
14	56.7	55.9	CH	1.00 (1H, m)
15	24.3	23.5	CH_2	1.57 (1H, m), 1.08 (1H, m)
16	28.2	27.23	CH_2	1.82 (1H, m), 1.28 (1H, m)
17	55.7	55.06	CH	1.15 (1H, m)
18	11.9	11.24	CH_3	0.68 (3H, s)
19	19.4	18.67	CH_3	0.86 (3H, s)
20	36.4	35.28	CH	1.40 (1H, m)
21	18.7	18.23	CH_3	0.89 (3H, s)
22	35.2	34.38	CH_2	1.41 (1H, m), 1.09 (1H, m)
23	25.7	25.06	CH_2	2.04 (1H, m), 1.90 (1H, m)
24	146.7	146.06	C	-
25	31.8	33.7	CH	2.20 (1H, m)
26	22.2	21.66	CH_3	0.97 (3H, s)
27	22.1	21.55	CH_3	0.97 (3H, s)
28	115.4	114.87	CH	5.17 (1H, dd, 6.5/13.5)
29	13.1	12.37	CH_3	1.56 (3H, s)

Notes: [*]: All spectra recorded in $CDCl_3$, a: Spectra recorded in DMSO, b: 125 MHz, c: 500MHz.

Fucosterol has been known as the predominant sterol in brown seaweeds [25] and has been considered a major bioactive metabolite of algae belonging to genus *Sargassum*, such as *S. pallidum* [26], *S. glaucescens* [27] and *S. fusiforme* [28]. Recently, fucosterol was isolated from *S. binderi* [29], a synonym of *S. crassifolium* [30]. However, result in the present study corresponds the first time to the compound isolated from alga *S. crassifolium* in Vietnam. Interestingly, the sterol has been proved to possess numerous biological activities, such as cytotoxic [31], anti-oxidant [32],

antidiabetic [33], anti-inflammatory [34]. Fucosterol obtained from the extract of *S. crassifolium* in this study was subjected to bioassays to study their cytotoxic and antioxidant properties against intracellular reactive oxygen species.

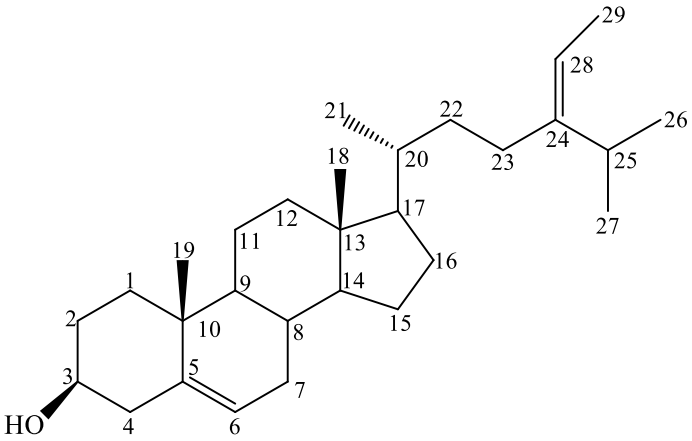

Figure 1. Chemical structure of fucosterol

Cytotoxictity in Hep-G2 cells

The cytotoxicity of fucosterol in Hep-G2 cells was evaluated by SRB method with concentrations ranging from 1 µg/ml to 10 µg/ml. After 72 h of incubation, fucosterol exhibited no significant cytotoxic effect at observed doses (Fig. 2). The percentages of cell survival at 10, 5 and 2.5 µg.ml^{-1} were 89.25 ± 1.32, 97.56 ± 2.01 and 99.05 ± 1.25, respectively. These dosages of compound were thus chosen to proceed intracellular ROS scavenging assayi.

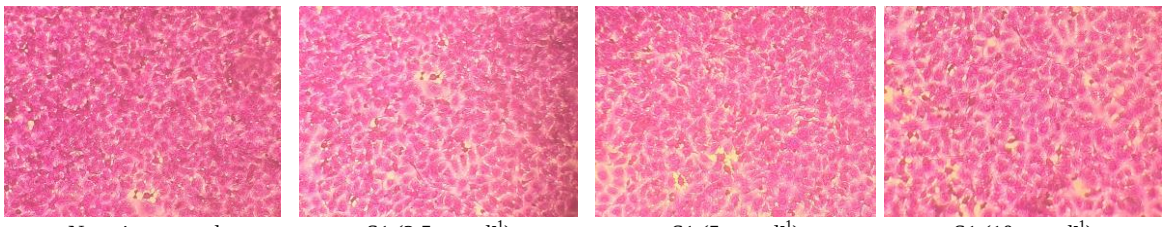

Figure 2. Microscopic images of fucosterol incubated Hep-G2 cell cultures in comparison to negative control (x40)

Intracellular ROS scavenging activity

The intracellular ROS scavenging activity of test compound was evaluated by the fluorescence intensity resulting from converting reaction of DCFH-DA to fluorescent compound DCF. The effect of fucosterol on ROS level in H_2O_2 treated cells in comparison to negative control (DMSO 10%) was observed under fluorescent microscope (Olympus IX83, Japan). Figure 3 shows dose-dependent scavenging effect of fucosterol at concentrations ranging from 2.5 µg.ml^{-1} to 10 µg.ml^{-1}. While the DCF fluorescence intensity caused by H_2O_2 in negative control (PBS instead of test compound) was recorded at 739.62 ± 18.43 AU, decreased intensity was observed in cells treated with 10 µg.ml^{-1} of ascorbic acid (positive control, 125.87 ± 35.11 AU) and with 10 µg.ml^{-1} of fucosterol (209.62 ± 22.15 AU). The result also showed that fucosterol has scavenging effect on intracellular ROS level at observed concentrations ranging from 2.5 µg.ml^{-1} to 10 µg.ml^{-1}.

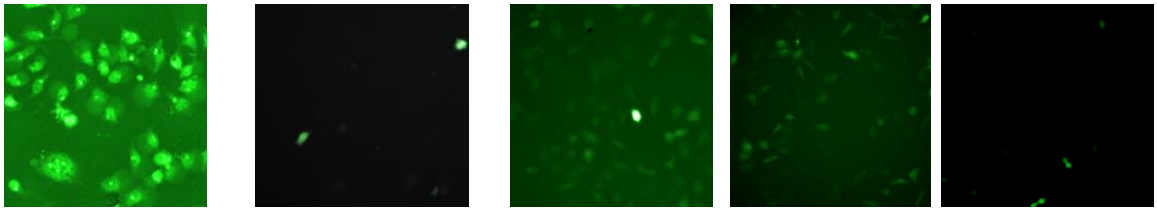

Figure 3. Dose-dependent ROS scavenging effect of fucosterol (S1) in comparison to negative control and ascorbic acid captured by fluorescent microscope (x60)

CONCLUSION

In the study we isolated and structurally elucidated fucosterol from marine brown alga *S. crassifolium* in Vietnam, as well as investigated its intracellular ROS scavenging property. The dichlorofluorescein assay revealed apparent reduced DCF fluorescence intensity of 209.62 ± 22.15 AU in the presence of fucosterol at concentration of 10 µg.ml^{-1}, accounting for 71.66% of diminution in comparison to negative control (739.62 ± 18.43 AU). To our knowledge, this is the first report on the protective effect against ROS of fucosterol isolated from a brown alga of *Sargassum* in Vietnam.

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