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Comparison of fucosterol content in algae using high-performance liquid chromatography

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Abstract

Background: Fucosterol is a compound commonly found in algae that has various biological activities. The purpose of this study was to develop a high-performance liquid chromatography (HPLC) validation method for fucosterol and to compare the fucosterol contents of 11 algal species from Ulleungdo, Korea.

Method: In this study, we successfully isolated and identified fucosterol from a 70% EtOH extract of *Sargassum miyabei*, and subsequently conducted specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision analyses for development of an HPLC validation method. Fucosterol contents were compared using the established HPLC validation conditions.

Results: We successfully isolated fucosterol from a 70% EtOH extract of *S. miyabei* and identified it based on spectroscopic analysis. On the basis of HPLC validation using the fucosterol isolated from *S. miyabei*, we confirmed specificity (8.5 min), linearity ($R^2 = 0.9998$), LOD ($3.20 \mu\text{g mL}^{-1}$), LOQ ($9.77 \mu\text{g mL}^{-1}$), accuracy (intra-day and inter-day variation, 90–110%), and precision (RSD, 1.07%). Fucosterol contents in the 11 assessed algal species ranged from 0.22 to 81.67 mg g⁻¹, with the highest content being recorded in a 70% EtOH extract of *Desmarestia tabacoides* (81.67 mg g⁻¹), followed by that of *Agarum clathratum* (78.70 mg g⁻¹).

Conclusions: The results indicate that 70% EtOH extracts of *D. tabacoides* and *A. clathratum* containing fucosterol with various effects can be potential alternative sources of fucosterol.

Keywords: Alga, Fucosterol, High-performance liquid chromatography, Validation

Background

Algae are classified into three main classes, namely, Chlorophyceae, Phaeophyceae, and Rhodophyceae, and there are approximately 6000 algal species worldwide, of which approximately 150 species are used as food (Devi et al. 2011; Meenakshi et al. 2011). Algae are known to be a rich source of bioactive substances such as carotenoids, dietary fiber, proteins, minerals, vitamins, polyphenols, and low-calorie polyunsaturated fatty acids. Research and development of health foods, cosmetics, and medicines using algae have been

increasing globally (Lee et al. 2011). As aquatic algae can inhabit extreme environments, unlike terrestrial organisms, they are known to produce various bioactive substances that enable them to adapt to such environments (Jeon et al. 2012). Owing to the presence of these bioactive substances, algae have been reported to possess anticancer, antioxidant, antibacterial, antitrypanosomal, antiangiogenic, and anti-HIV activities (Synytsya et al. 2010; Veiga-Santos et al. 2010; Souza et al. 2012; Guerra Dore et al. 2013; Shanmugam et al. 2014; Thuy et al. 2015).

Several bioactive substances, including fucoidan (polysaccharide), fucoxanthin, chlorophyll, xanthophyll (carotenoid), phlorotannin (tannin), and fucosterol (sterol) have been isolated from algae (Hosokawa et al. 2004). Fucosterol is

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commonly found in algae and has been reported to lower cholesterol levels and possess various physiological activities, such as antidiabetic, anticancer, and antioxidant activities (Ikeda et al. 1988; Tang et al. 2002; Lee et al. 2004; Ham et al. 2010).

Currently, fucosterol is mainly isolated from *Sargassum* species. In this study, we isolated fucosterol from *Sargassum miyabei* and developed a high-performance liquid chromatography (HPLC) validation method for this compound. Furthermore, we compared the content of fucosterol in 11 algal species from Ulleungdo, Korea, using the established HPLC validation conditions.

Methods

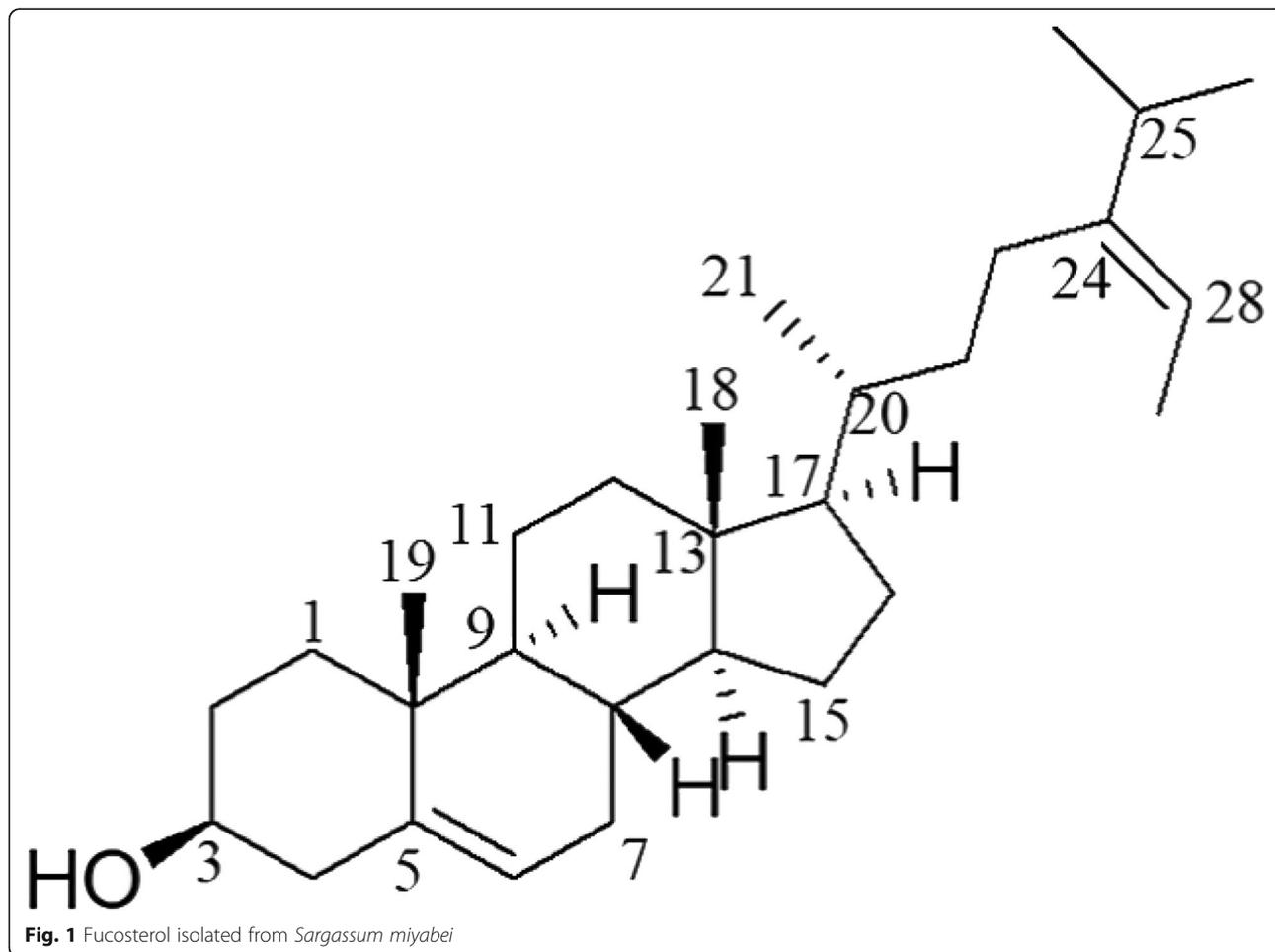
Samples of algae

In 2015, eleven species of algae (*Agarum clathratum*, *Caulerpa okamurae*, *Codium fragile*, *Desmarestia tabacoides*, *Dictyopteris divaricata*, *Ecklonia cava*, *Eisenia bicyclis*, *Myagropsis myagroides*, *Sargassum horneri*, *S. serratifolium*, and *Sporochnus radiciformis*) were collected in Ulleungdo Island, Korea, for comparison of

fucosterol contents. In the same year, *S. miyabei* was collected in Pohang-si, Korea, for isolation of fucosterol. The specimens have been deposited in the Marine Biodiversity Institute of Korea (MABIK). All algal samples were lyophilized, pulverized, and extracted with 70% EtOH using a sonicator (WUC-N30H; DAIHAN Scientific Co., Ltd., Wonju, Korea).

Instrument and reagents

Medium-pressure liquid chromatography (MPLC) and HPLC were performed using the Buchi Sepacore Flash system (Flawil, Switzerland) consisting of a UV photometer C-640 and Agilent 1260 Infinity system (Tokyo, Japan) equipped with a diode array detector (DAD). Mass spectrometry (MS) was conducted using a JEOL JMS-600-W Spectrometer (Tokyo, Japan), and nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AVANCE 500 NMR spectrometer (Rheinstetten, Germany) in CDCl₃ with tetramethylsilane used as the internal standard. Chemical shifts are reported in parts per million (δ) and the coupling constant (J) is expressed in Hertz (Hz). All reagents used were of analytical grade.



Isolation of fucosterol

A dried sample of *S. miyabei* (1.2 kg) was powdered and extracted with 70% EtOH (12 L \times 3) using a sonicator and then evaporated under vacuum. The 70% EtOH extract (91.7 g) was suspended in water and partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. A portion of the *n*-hexane fraction (15.6 g) was subjected to chromatographic analysis using an MPLC system and eluted in a gradient solvent system (100% *n*-hexane and up to 100% ethyl acetate) to yield eight subfractions (H1–H8). Subfraction H5 (8.1 g, *n*-hexane:ethyl acetate = 60:40) was re-chromatographed in an MPLC system and eluted in a gradient solvent system (70% *n*-hexane and up to 100% ethyl acetate) to yield five subfractions (H5-1 to H5-5). H5-3 was recrystallized with MeOH to obtain fucosterol (Fig. 1).

Fucosterol was obtained as a white powder; EI-MS *m/z*: 412 [M]⁺ (13.0), 314 (100), 299 (19.2), 271 (11.3), 229 (15.5); ¹H-NMR (500 MHz, CDCl₃, δ_{H}) 5.35 (1H, d, *J* = 5.5 Hz, H-6), 5.18 (1H, q, *J* = 6.5, 13.5 Hz, H-28), 3.53 (1H, m, H-3), 1.58 (3H, d, *J* = 7.5 Hz, H-29), 1.02 (3H, s, H-19), 1.00 (3H, d, *J* = 7.0 Hz, H-21), 0.98 (3H, d, *J* = 6.5 Hz, H-27), 0.97 (3H, d, *J* = 7.0 Hz, H-26), 0.69 (3H, s, H-18); ¹³C-NMR (125 MHz, CDCl₃, δ_{C}) 147.2 (C-24), 141.0 (C-5), 121.9 (C-6), 115.8 (C-28), 72.0 (C-3), 56.9 (C-14), 56.0 (C-17), 50.3 (C-9), 42.5 (C-13), 42.4 (C-4), 39.9 (C-12), 37.5 (C-1), 36.7 (C-10), 36.6 (C-20), 35.4 (C-22), 35.0 (C-25), 32.1 (C-7,8), 31.9 (C-2), 28.4 (C-16), 25.9 (C-23), 24.5 (C-15), 22.4 (C-26), 22.3 (C-27), 21.3 (C-11), 19.6 (C-19), 19.0 (C-21), 13.4 (C-29), 12.1 (C-18).

Sample preparation and HPLC condition

A stock solution of fucosterol (1 mg) was dissolved in 1 mL of acetonitrile and was diluted to give the desired concentrations (3.91, 7.82, 15.63, 31.25, 62.50, and 125.00 $\mu\text{g mL}^{-1}$). To analyze fucosterol content, samples of the 11 aforementioned algal species were extracted with 70% EtOH for 1 h by sonication (three times) and evaporated under vacuum. The residues were dissolved in 1 mL of 50% MeOH and filtered using a 0.45- μm syringe filter. The resulting solution was subjected to HPLC analysis, using a Kinetex C18 (4.6 mm \times 100 mm, 2.6 μm) column, with a mobile phase comprising a solution of MeOH (solvent A) and 0.1% acetic acid (solvent B). The gradient solvent system was as follows: from 50 to 0% B for 5 min, and then retained at 0% B for 10 min. The injection volume was 10 μL and the flow rate was 1 mL min⁻¹. The column temperature was maintained at 25 °C and detection wavelength was 210 nm. All injections were performed in triplicate.

Validation

The HPLC method was validated for specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision according to the International Conference on Harmonization (ICH; 1997, 2005) guidelines.

The linearity of the method was established using triplicate injections at six concentrations in the range 3.91–125.00 $\mu\text{g mL}^{-1}$. A calibration curve for fucosterol was constructed using peak area (*Y*), concentration (*X*, $\mu\text{g mL}^{-1}$), and mean (*n* = 3) \pm standard deviation values. Correlation coefficient values (*R*²) were determined from the calibration curve. The LOD and LOQ of fucosterol were calculated using the following formulae: LOD = 3.3 \times σ /*S* and LOQ = 10 \times σ /*S*; where, σ = the deviation and *S* = the slope of the calibration curve. The accuracy of the method was determined based on intra- and inter-day variations. Intra-day variation was determined by analyzing triplicate samples at three different concentrations (15.63, 31.25, 62.50 $\mu\text{g mL}^{-1}$) in a single day. Inter-day variation was determined by analyzing a single sample at three different concentrations (15.63–62.50 $\mu\text{g mL}^{-1}$) for 3 days. Variations are expressed as percentage recoveries. Precision was assessed based on repeatability. Repeatability data were obtained from six injections of samples at a concentration of 125.00 $\mu\text{g mL}^{-1}$ on the same day. Repeatability is expressed as the percentage relative standard deviation (%RSD).

Results and discussion

Isolation of fucosterol

The fucosterol extracted from *S. miyabei* was obtained as a white powder with a molecular formula of C₂₉H₄₈O based on EI-MS analysis. In the ¹H-NMR spectrum of fucosterol, we detected two olefinic proton signals [δ_{H} 5.35 (1H, d, *J* = 5.5 Hz, H-6), 5.18 (1H, q, *J* = 6.5, 13.5 Hz, H-28)], one oxygenated methine proton signal [δ_{H} 3.53 (1H, m, H-3)], and six methyl groups [δ_{H} 1.58 (3H, d, *J* = 7.5 Hz, H-29), 1.02 (3H, s, H-19), 1.00 (3H, d, *J* = 7.0 Hz, H-21), 0.98 (3H, d, *J* = 6.5 Hz, H-27), 0.97 (3H, d, *J* = 7.0 Hz, H-26), 0.69 (3H, s, H-18)]. In the ¹³C-NMR spectrum of fucosterol, we observed 29 carbon signals, including two olefin quaternary carbons [δ_{C} 147.2 (C-24), 141.0 (C-5)], two olefin methine carbons [δ_{C} 121.9 (C-6), 115.8 (C-28)], one oxygenated methine carbon [δ_{C} 72.0 (C-3)], and six methyl carbons [δ_{C} 22.4 (C-26), 22.3 (C-27), 19.6 (C-19), 19.0 (C-21), 13.4 (C-29), 12.1 (C-18)]. Fucosterol was elucidated by comparison of the EI-MS and NMR (¹H-NMR, ¹³C-NMR, DEPT 45, 90, 135, ¹H-¹H COSY,

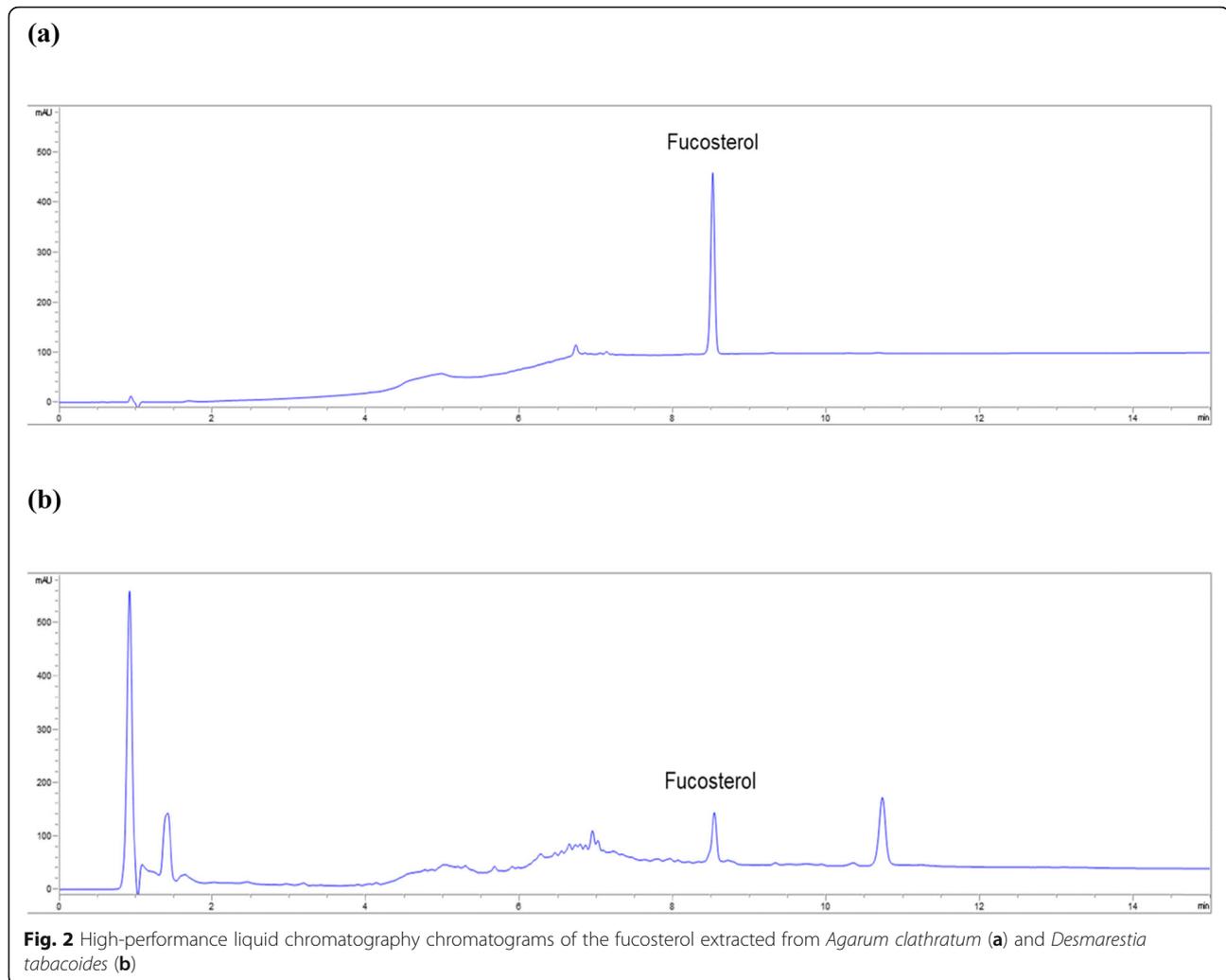


Fig. 2 High-performance liquid chromatography chromatograms of the fucosterol extracted from *Agarum clathratum* (a) and *Desmarestia tabacooides* (b)

HSQC, HMBC) spectral data with those previously published (Bang et al. 2011).

Validation

In the HPLC data, fucosterol showed a single peak unaffected by solvent and other components at 8.5 min (Fig. 2a). Good resolution of fucosterol and specificity of the method were confirmed. In the calibration curve at six different concentrations (3.91–125.00 $\mu\text{g mL}^{-1}$) of fucosterol for determining linearity, $y = 5.70x - 23.90$ and R^2 was 0.9998. The R^2 of

fucosterol was greater than 0.9995, thereby verifying the linearity of fucosterol. The LOD and LOQ of fucosterol were 3.20 and 9.77 $\mu\text{g mL}^{-1}$, respectively (Table 1). The intra- and inter-day accuracy ranges were 95.76–103.21% and 96.49–101.46%, respectively. The intra- and inter-day variations were within the range of 90–110%, which confirmed the accuracy of the method (Table 2). The precision of the HPLC

Table 1 High-performance liquid chromatography calibration data for fucosterol from *S. miyabei*

Linear range ($\mu\text{g mL}^{-1}$)	Regression equation ^a	Correlation coefficient (R^2)	LOD ($\mu\text{g mL}^{-1}$) ^b	LOQ ($\mu\text{g mL}^{-1}$) ^c
3.91–125.00	$y = 5.7x + 23.9$	0.9998	3.20	9.77

Triplicate injections at six concentrations

^aRegression equation is $y = Ax + B$ [y , the peak area; x , concentration ($\mu\text{g mL}^{-1}$)]

^bLimit of detection

^cLimit of quantification

Table 2 Accuracy of the high-performance liquid chromatography validation method for fucosterol from *S. miyabei*

Concentration ($\mu\text{g mL}^{-1}$)	Intra-day variation ^a		Inter-day variation ^b	
	Recovery (%)	SD (%)	Recovery (%)	SD (%)
62.50	101.05	0.71	101.46	0.59
31.25	103.22	0.13	99.55	0.28
15.63	95.76	2.25	96.49	0.97

^aTriplicate injections at three different concentrations in a single day

^bOne injection at three different concentration for 3 days

Table 3 Precision of the high-performance liquid chromatography validation method for fucosterol from *S. miyabei*

Concentration ($\mu\text{g mL}^{-1}$)	Number of measurements	Area	Mean	RSD (%)
125.00	1	682.8	685.4	1.05
	2	674.5		
	3	681.7		
	4	688.0		
	5	692.0		
	6	693.6		

method, calculated as %RSD, was evaluated for repeatability ($125.00 \mu\text{g mL}^{-1}$). The RSD was less than 2.0% (1.07%), which confirmed the precision of the method (Table 3).

Determination of fucosterol contents

The fucosterol contents in the 11 assessed algal species ranged from 0.22 to 81.67 mg g^{-1} (Table 4). Specifically, the content of fucosterol in *A. clathratum*, *D. tabacoides*, *E. cava*, and *S. radiformis* was approximately four times higher than that in *Sargassum* species. The highest content of fucosterol was detected in the 70% EtOH extract of *D. tabacoides* (81.67 mg g^{-1} , Fig. 2b), followed by that of *A. clathratum* (78.70 mg g^{-1}).

Fucosterol has been reported to have various effect such as antioxidant, antidepressant, anticonvulsant, antipredator, and antiplasmodial activities, and it has been isolated from the *Sargassum* species *S. micracanthum*, *S. tenerrimum*, *S. fusiforme*, *S. horneri*, and *S. linearifolium* (Ham et al. 2010;

Table 4 Content of fucosterol in 70% EtOH extracts of 11 algal species

Sample	Content of fucosterol (mg g^{-1})
<i>Agarum clathratum</i>	78.70 ± 0.01^a
<i>Caulerpa okamurae</i>	0.22 ± 0.02
<i>Codium fragile</i>	N.D. ^b
<i>Desmarestia tabacoides</i>	81.67 ± 0.07
<i>Dictyopteris divaricata</i>	Trace
<i>Ecklonia cava</i>	48.03 ± 0.14
<i>Eisenia bicyclis</i>	13.10 ± 0.07
<i>Myagropsis myagroides</i>	3.13 ± 0.01
<i>Sargassum horneri</i>	2.63 ± 0.02
<i>Sargassum serratifolium</i>	19.75 ± 0.04
<i>Sporochnus radiformis</i>	21.13 ± 0.02

^aData are expressed as the mean \pm SD ($n = 3$) in $\mu\text{g mL}^{-1}$

^bNot detected

Terasaki et al. 2009; Zhen et al. 2015; Majik et al. 2015; Perumal et al. 2018). Although *Sargassum* species are a good source of fucosterol, our present study results confirmed that 70% EtOH extracts of *D. tabacoides* and *A. clathratum* could replace *Sargassum* species as a potential source of fucosterol. Therefore, we anticipate that *D. tabacoides* and *A. clathratum*, which have high fucosterol contents with the various aforementioned properties, will have a high industrial value.

Conclusions

We successfully isolated fucosterol from *S. miyabei* and developed an HPLC validation method for this compound for comparison of the fucosterol contents of 11 selected algal species. The recorded fucosterol contents in 70% EtOH extracts of *D. tabacoides* and *A. clathratum* were 81.67 and 78.70 mg g^{-1} , respectively. These results indicate that 70% EtOH extracts of *D. tabacoides* and *A. clathratum* containing high contents of fucosterol with various beneficial properties can be potential alternative sources of fucosterol.

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Authors' contributions

JML isolated the compounds, elucidated the structures, and wrote the manuscript. JHJ performed validations of the HPLC method and investigated the content of fucosterol in the algae. MJY and GC collected algal samples and assisted in the preparation of the manuscript. YGP and MSL contributed to plant material preparation. DSL designed and managed the research program. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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