Alpha-amylase inhibitory activity and sterol composition of the marine algae, *Sargassum glaucescens*

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Submitted: 15-10-2014 Revised: 10-12-2014 Published: 21-10-2015

ABSTRACT

Background: *Sargassum* species (phaeophyceae) are economically important brown algae in southern parts of Iran. *Sargassum* is mainly harvested as a row material in alginate production industries and is a source of plant foods or plant bio-stimulants even a component of animal foods. **Objective:** In this study, *Sargassum glaucescens*, collected from the seashore of Chabahar, was employed for phytochemical and biological evaluations. **Materials and Methods:** For that purpose, the dried algae was extracted by methanol and subjected to different chromatographic separation methods. **Results:** Six sterols, fucosterol (1), 24(*S*)-hydroxy-24-vinylcholesterol (2), 24(R)-hydroxy-24-vinylcholesterol (3), stigmasterol (4), *β*-sitosterol (5) and cholesterol (6) were identified by spectroscopic methods including ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy. *In vitro* alpha-amylase inhibitory test was performed on the methanolic extract and the results revealed a potent inhibition (IC_{50} = 8.9 ± 2.4 mg/mL) of the enzyme compared to acarbose as a positive control. **Conclusion:** Various biological activities and distribution of sterols in *Sargassum* genus have been critically reviewed here. The results concluded that these algae are a good candidate for further anti-diabetic investigations in animals and human.

Key words: Alpha-amylase inhibitor, Brown algae, *Sargassum* glaucescens, Sterol

Access this article online

Website: www.phcogres.com

DOI: 10.4103/0974-8490.167893

Quick Response Code:

INTRODUCTION

Sargassum (Phaeophyceae family) is a genus of brown algae, well-known as macroalgae or seaweeds, distributed throughout the temperate and tropical oceans in the world. Moreover, this genus is known for its planktonic species. Interestingly, *Sargassum* species are found to grow in temperate water opposite of other species belonging to Phaeophyceae that are predominantly cold water organisms.[1] These algae are generally observed in brown or dark green colors and commonly grow sub-tidally attached to the corals, rocks or shells in moderately exposed or sheltered rocky areas. *Sargassum* is mainly harvested as a row material in alginate production industries and is a source of plant foods or plant bio-stimulants even a component of animal foods. These are also employed as nutraceuticals and pharmaceuticals due to the presence of fucoidan and other bioactive compounds in their extracts.[2,3]

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A bibliography revealed that *Sargassum* may be the source of several bioactive compounds including, steroids,[4,5] flavonoids,[4-6] polysaccharides,[7,8] terpenoids,[9,10] fatty acids,[11] plastoquinones[12] and tannins.[13] Among them, sterols are the major bioactive secondary metabolites in these algae. Recently, several sterols have been isolated and identified from brown algae, of which fucosterol and saringosterol were separated from *S. pallidum*.[5] Various Δ^5-3 β-sterols possessing carbon numbers ranged from C19-C23 to C26-C30 have been reported from the extract of *S. muticum*.[14] Furthermore, *S. oligocystum* has been reported for isolation and identification of 22-dehydrocholesterol, cholesterol, fucosterol, 29-hydroperoxystigmasta-5,24 (28)-dien-3 β-ol, 24-hydroperoxy-24-vinylcholesterol, 24(S) and 24(R)-hydroxy-24-vinylcholesterol, as well as ostreasterol.[15] A number of sterols reported from various species of *Sargassum* are exhibited in Table 1.

Table 1: Distribution of sterols in *Sargassum* genus

Algae	Isolated sterols	Reference
Sargassum asperifolium	Saringosterol Saringosterone	[9]
Sargassum fluitans	Fucosterol Cholesterol 22-dehydrocholesterol Ostreasterol	[16]
Sargassum fusiforme	Fucosterol Saringosterol 24-hydroperoxy-24-vinylcholesterol 24, 28-epoxy-24-ethylcholesterol 29-hydroperoxystigmasta-5, 24 (28)-dien-3 β-ol	[17,18]
Sargassum hemiphyllum	Fucosterol Saringosterol 24-hydroperoxy-24-vinylcholesterol Ergosterol peroxide Ergosta-7, 22-diene-3, 5, 6-triol	[19]
Sargassum henslowianum	Saringosterol	[20]
Sargassum horneri	Fucosterol Saringosterol β-stigmasterol Ergosterol peroxide	[21]
Sargassum linifolium	Fucosterol β-sitosterol	[22]
Sargassum micracanthum	Fucosterol	[23]
Sargassum muticum	Fucosterol Cholesterol 22-dehydrocholesterol Isofucosterol Cholesta-5, 22-dien-3-ol, 24-propyl	[15]
Sargassum oligocystum	Fucosterol Cholesterol 22-dehydrocholesterol Saringosterol Ostreasterol 24-hydroperoxy-24-vinylcholesterol 29-hydroperoxystigmasta-5, 24 (28)-dien-3 β-ol	[16]
Sargassum pallidum	Fucosterol Saringosterol 24-hydroperoxy-24-vinylcholesterol Ergosterol peroxide	[4,5]
Sargassum parvivesiculosum	Stigmasta-4,24 (28)-dien-3 β-ol Stigmasta-5, 23-diene-3,28-diol Hydroperoxide, (24*E*)-stigmasta-5, 28-dien-24-yl	[24]
Sargassum polycystum	Stigmasta-5,23,25-triene-3-ol 24-methylcholesta-3 β,5α,6 β,25-tetrol-25-acetate	[25]
Sargassum ringoldianum	Saringosterol	[26]
Sargassum thunbergii	Fucosterol 24-vinyloxycholesta-5, 23-dien-3 β-ol	[11,27,28]
Sargassum vulgare	Fucosterol Cholesterol	[29]

Here in this study, we focused on *S. glaucescens* as one of the most abundant brown algae distributed in Persian Gulf and Oman Sea. As far as we could ascertain there is no report on its sterol composition. Therefore, we aimed to report the isolation and structural elucidation of the sterols from *S. glaucescens* methanolic extract for the first time.

MATERIALS AND METHODS

Instruments and materials

All the chemicals used in the biochemical assay were purchased from Sigma-Aldrich Chemie Gmbh (Germany) and Merck (Germany) companies. The chemicals were of analytical grade. The enzyme (EC 3.2.1.1) was purchased from Sigma (Germany) that extracted from soy bean source. α-amylase activity was determined by measuring the absorbance of the mixtures at 540 nm in Elisa stat fax 2100 (Awarness Technology Inc., FL, USA). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Brucker Avance 500 DRX spectrometer® (Germany) with tetramethylsilane as an internal standard and chemical shifts are given in δ (ppm). Multiple-pulse experiments (HSQC, HMBC, and

H-H COSY) were performed using the standard Bruker® programs. Silica gel 60 F_{254} and Silica gel 60 RP-18 $F_{254}S$ pre-coated plates (Merck®, Germany) were used for thin layer chromatography. The spots were detected by spraying with anisaldehyde-H_2SO_4 reagent (Mahestan Shimi, Iran), followed by heating.

Plant materials

The whole parts of *Sargassum glaucescens* were collected from the seashore of Chabahar, Sistan and Baluchestan Province in the Southeast of Iran in 2011, washed with distilled water, dried at room temperature and Identified by Mr. B. M. Gharanjik. A voucher specimen (No. *P* 50-16) was deposited at the Research Center of Persian Gulf Biotechnology (Qeshm Island, Iran).

Extraction and isolation process

Dried algae, *Sargassum glaucescens*, were cut into small pieces (2 kg) and extracted with methanol at room temperature by percolation method for 48 h and 3 times. The solvent was evaporated by rotary evaporator. The methanolic extract (60 g) was fractionated by silica gel column chromatography (CC) with hexane: chloroform (2:8), chloroform: ethyl acetate (8:2, 5:5) and ethyl acetate, respectively, to yield seven fractions (A-G). Fraction C (6.5 g) were chosen and subjected to silica gel CC with chloroform: ethyl acetate (19:1, 8:2 and 0:1) to obtain six main fractions (C1-C6). Fraction C2 (1100 mg) was submitted to silica gel CC with chloroform: ethyl acetate (19:1) to yield compound 1 (22 mg). Fraction C4 (610 mg) was submitted to silica gel CC with chloroform: ethyl acetate (9:1) to result five fractions. Third fraction (52 mg) was subjected to sephadex LH_{20} to yield three other main fractions. First fraction (26 mg) was fractionated on silica gel (chloroform: ethyl acetate, 8:2) to gain compound 2 and 3 (8 and 5 mg, respectively). Fraction D (330 mg) was submitted to silica gel CC with chloroform: methanol (98:2) to result four parts (D_1-D_4). D_2 (83 mg) was fractionated on sephadex LH_{20} with methanol to purify compound 4 and 5. From Fraction E (710 mg) after chromatographing on silica gel eluted with chloroform: ethyl acetate (8:2, 6:4), five parts resulted. Compound 6 (12 mg) was purified from a third part (69 mg) after loading on sephadex LH_{20} CC eluted with methanol.

Alpha-Amylase inhibitory assay

The α-amylase inhibition assay was performed by some modification in the method proposed by Giancarlo *et al*.[30] The starch solution (1% w/v) was obtained by boiling and stirring 1 g of potato starch in 100 mL of sodium phosphate buffer for 30 min. The enzyme (EC 3.2.1.1) solution (50 U/1 mL) was prepared by mixing 0.01 g of α-amylase in 10 mL of sodium phosphate buffer (PH 6.9) containing 0.0006 mM sodium chloride. The extracts were dissolved in dimethyl sulfoxide (DMSO) to give concentrations from 5 to 15 mg/mL (5, 10 and 15 mg/mL). The color reagent was a solution containing 0.1 g of 3, 5-dinitrosalicylic acid plus 2.99 g sodium potassium tartrate in 0.16 g sodium hydroxide and phosphate buffer (10 mL).

Totally, 50 microliter of each algae extract, and 150 μL of starch solution, as well as 10 μL of enzyme, were mixed in a 96 well plate and incubated at 37°C for 30 min. Then, 20 μL of sodium hydroxide and 20 μL of color reagent were added, and the closed plate placed into a 100°C water bath. After 20 min, the reaction mixture was removed from the water bath and cooled, thereafter α-amylase activity was determined by measuring the absorbance of the mixture at 540 nm using Elisa. Blank samples were used to correct the absorption of the mixture in which the enzyme was replaced with buffer solution. Furthermore, a control reaction was used, in which the algae extract was replaced with 50 μ of DMSO, and the maximum enzyme activity was determined. Acarbose solution at the concentrations (5, 10, 15 mg/mL) was used as a positive standard. The inhibition percentage of α-amylase was assessed by the following formula:

$$I\ \alpha\text{–Amylase}\ \% = 100 \times (\Delta A_{control} - \Delta A_{sample})/\Delta A_{control}$$

$$\Delta A_{control} = A_{test} - A_{Blank}$$

$$\Delta A_{sample} = A_{test} - A_{Blank}$$

I: Inhibitory activity; A: Absorbance at 540 nm.

Statistical analysis

Statistical analysis was performed using the SPSS version 21.0 (IBM Corporation, 2012). The IC_{50} values were estimated by nonlinear curve and presented as their respective 95% confidence limits. Probit analysis of variance was used to assess the presence of significant differences ($P < 0.05$) between the extracts.

RESULTS AND DISCUSSION

Methanolic extract of marine algae, *S. glaucescens*, was used for the separation of sterols. Isolation and purification of the main compounds were carried out on silica gel and sephadex LH_{20} CC to obtain six pure compounds. Structural elucidation of these compounds was based on the data obtained from ^{1}H-NMR, ^{13}C-NMR studies. Separated compounds from *S. glaucescens* were identified as fucosterol (1),[31] a mixture of 24(S)-hydroxy-24-vinylcholesterol (2) and 24(R)-hydroxy-24-vinylcholesterol (3),[15] stigmasterol (4),[32,33] β-sitosterol (5)[34,35] and cholesterol (6)[36] compared to the spectral data reported in the literatures [Figure 1]. ^{13}C NMR data of sterols isolated from *S. glaucescens* are summarized in Table 2.

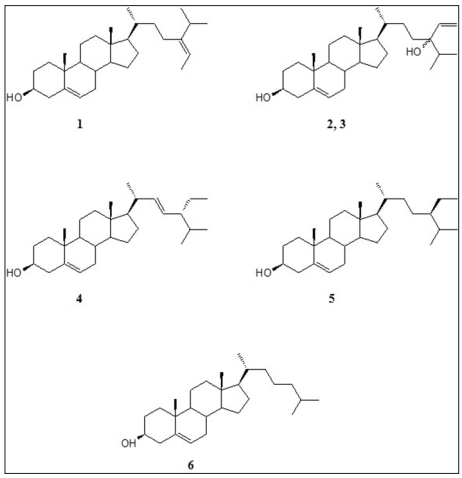

Figure 1: Chemical structures of isolated sterols from *Sargassum glaucescens*

Besides cholesterol and fucosterol as the main and characteristic sterols in brown algae, hydroperoxy sterols like 24-hydroperoxy 24-vinyl cholesterol are predominantly found in those algae.[36-38] A literature review demonstrates that different species of *Sargassum* exhibited various biological activities, which are summarized in Table 3.

Among the isolated compounds from the algae, sitosterol is reported to possess several biological activities including anti-inflammatory, hypocholesterolemic, analgesic, chemoprotective, immunomodulatory, and anthelmintic activities. The most important effects on Benign prostatic hyperplasia and prostatic cancer treatment, as well as anti-diabetic and antioxidant activities, are well-documented.[69] Furthermore, another compound stigmasterol has been frequently reported for its anti-inflammatory, hypocholesterolemic, ameliorating, antiperoxidative, thyroid inhibitory, and hypoglycemic properties.[69] In addition, fucosterol is a major compound of this algae and well-known for its anti-diabetic, antioxidant and hepatoprotective effects as well as histamine and acetylcholine esterase inhibitory activities.[69]

As far as we could ascertain, 24-hydroxy-24-vinylcholesterol (saringasterol), the most aboundant sterol in *Sargassum* species, are reported for a variety of biological activities. For instance, 24 *R*-saringasterol exhibited the proliferation activity (under 1000 nM after 24 h incubation) and concentration-dependent proliferation activity (under 300 nM after 72 h incubation) significantly. Furthermore, this compound could have inhibitory activity against bone-resorbent metabolic bone disorders including osteoporosis and periodontitis.[70] Saringasterol is also evaluated for its probable cytotoxic activity and showed weak inhibitory effects on LNCaP cells (IC_{50} = 41.60 ± 4.26 μM) and was inactive on DU145 and PC3 cells (IC_{50} > 50 μM).[71] In addition, saringasterol possesses *in vitro* antitrypanosomal activity with IC_{50} value of 3.2 ± 1.2 μM.[72] Finally, inhibition of *Mycobacterium tuberculosis* growth has been reported by saringosterol while no significant toxicity was observed from this compound against Vero cells.[73]

In the present study, the results of biochemical assay showed that this algal species exhibited a potent inhibitory activity on α-amylase enzyme (IC_{50} = 8.9 ± 2.4 mg/mL) compared

Table 2: ^{13}C-NMR data (δ ppm) of isolated sterols from *Sargassum glaucescens* in $CDCl_3$

Number	1	2, 3[a]	4	5	6
1	37.2	37.2	37.2	37.2	37.2
2	31.5	31.6	31.6	31.6	31.6
3	71.8	71.7	71.7	71.7	71.8
4	42.3	42.2	42.2	42.2	42.2
5	140.6	140.7	140.7	140.7	140.7
6	121.7	121.6	121.7	121.7	121.7
7	31.8	31.8	31.8	31.8	31.9
8	31.8	31.8	31.8	31.8	31.9
9	50	50	50.1	50.1	50.1
10	36.4	36.4	36.4	36.4	36.5
11	21	21	21	21	21
12	39.7	39.7	39.7	39.6	39.7
13	42.2	42.2	42.2	42.2	42.2
14	56.7	56.7	56.7	56.8	56.7
15	24.3	24.2	24.2	24.3	24.2
16	28.2	28.1	28.2	28.9	28.2
17	55.7	55.7	56	55.9	56.1
18	11.8	11.8	11.8	12	11.8
19	19.3	19.3	19.3	19.3	19.3
20	36.4	35.8	36.1	40.5	35.7
21	18.7	18.7	18.7	21.2	18.7
22	35.2	29	33.8	138.3	36.1
23	25.6	34.5,34.7	25.9	129.2	23.8
24	146.9	77.7	45.7	51.2	39.5
25	34.7	36	29	31.8	28
26	22.1	16.4	19.8	21	22.8
27	22.2	17.5	18.9	18.9	22.5
28	115.5	142.4,142.5	23	25.4	
29	13.1	112.8,112.9	11.9	12.2	

[a]Assignments for S and R epimers. NMR=Nuclear magnetic resonance

Table 3: A number of biological activities reported from various species of *Sargassum*

Biological activity	Algae	Reference
Antioxidant activity	*Sargassum filipendula*	[39]
	Sargassum fusiforme	[40]
	Sargassum kjellmanianum	[41]
	Sargassum micracanthum	[23]
	Sargassum pallidum	[42]
	Sargassum plagiophyllum	[43]
	Sargassum siliquastrum	[44]
	Sargassum thunbergii	[45]
	Sargassum vulgare	[8]
	Sargassum wightii	[46]
Anti-tumor and antiproliferative activity	*Sargassum filipendula*	[39]
	Sargassum fusiforme	[47]
	Sargassum kjellmanianum	[48]
	Sargassum pallidum	[6]
	Sargassum plagiophyllum	[43]
	Sargassum thunbergii	[7]
	Sargassum vulgare	[49]
Antihyperlipidemic activity	*Sargassum fluitans*	[50]
	Sargassum fusiforme	[47]
	Sargassum henslowianum	[51]
	Sargassum natans	[50]
	Sargassum thunbergii	[52]
Anti-inflammatory activity	*Sargassum hemiphyllum*	[53]
	Sargassum sagamianum	[54]
	Sargassum siliquastrum	[55]
	Sargassum vulgare	[8]
Anti-herpes activity	*Sargassum horneri*	[56]
	Sargassum naozhounse	[57]
	Sargassum patens	[58]
	Sargassum tenerrimum	[59]
Anticoagulant activity	*Sargassum fulvellum*	[60]
	Sargassum thunbergii	[13]
	Sargassum vulgare	[8]
Antimicrobial activity	*Sargassum johnstonii*	[61]
	Sargassum pallidum	[62]
	Sargassum wightii	[63]
Immunological activity	*Sargassum fusiforme*	[47]
	Sargassum kjellmanianum	[48]
	Sargassum pallidum	[42]
Anti-HIV activity	*Sargassum fusiforme*	[64]
Sedative and hypnotic activity	*Sargassum pallidum*	[65]
Anti-diabetic activity	*Sargassum fusiforme*	[47]
Tyrosinase inhibitory activity	*Sargassum siliquastrum*	[66]
Anti-cholinesterase activity	*Sargassum wightii*	[46]
Adipogenesis regulatory activity	*Sargassum yezoense*	[67]
Insecticidal effect	*Sargassum dentifolium*	[68]

the positive standard, acarbose (IC_{50} = 6.6 ± 2.1 mg/mL) [Table 4]. A concentration dependent inhibition was observed for various concentrations of this algal extract. The highest inhibitory activity for *S. glaucescens* was found to be 77.8 ± 1.9 (at 15 mg/mL) while the percentage inhibitory activity for acarbose at the same concentration was observed as 69.2 ± 3.9.

A literature review shows that fucosterol isolated from *Pelvetia siliquosa* was reported for its anti-diabetic activity. In this study, fucosterol was administered orally (30 mg/kg) in streptozotocin-induced diabetic rats which showed a significant reduction in serum glucose levels and inhibited the sorbitol accumulations in the lenses. It also caused an inhibition of blood glucose level and glycogen degradation at 300 mg/kg in epinephrine-induced diabetic rats. The authors concluded that fucosterol is a main anti-diabetic principle in *P. siliquosa*.[74] Furthermore, the anti-diabetic potential of fucosterol has been reported by evaluating the ability of this compound to inhibit rat lens aldose reductase (RLAR), human recombinant aldose reductase (HRAR), protein tyrosine phosphatase 1B (PTP1B), and α-glucosidase. The investigators revealed that it exhibited a moderate inhibitory activity against RLAR, HRAR, and PTP1B, while a weak or no activity against advanced glycation end formation and α-glucosidase.[75] On the other hand, β-sitosterol is also well-documented for anti-diabetic activity. For instance, in a recent study,

Table 4: α-Amylase inhibitory activities and IC_{50} values of different concentrations of *Sargassum glaucescens* in comparison to acarbose as a positive standard

Compound	Concentration (mg/mL)	Percentage inhibitory (mean±SD)	IC_{50}* (mg/mL)
Acarbose	2.5	26.3±1.8	6.6±2.1
	5	54.5±2.2	
	10	67.3±2.1	
	15	69.2±3.9	
	20	73.9±1.9	
	25	82.1±2.3	
	30	87.1±3.1	
Sargassum glaucescens	2.5	14±1.7	8.9±2.4
	5	27.9±2.1	
	10	64.0±2.2	
	15	77.8±1.9	

*IC_{50} value is the concentration of sample required for 50% inhibition. Each value is expressed as mean±SD (n=3). SD=Standard deviation

three doses of this compound (10, 15 and 20 mg/kg, orally) resulted in decreasing the glycated hemoglobin, serum glucose, and nitric oxide, with concomitant increases in serum insulin levels. In addition, it could increase pancreatic antioxidant levels, with a concomitant decrease in thiobarbituric acid-reactive substances.[76] Moreover, *in silico* studies exhibited the potent inhibition for β-sitosterol on human pancreatic alpha-amylase. The inhibition constant (Ki) for this compound was estimated as 269.35 nmol with two hydrogen bond interactions, although there is no report on the evaluation of fucosterol and β-sitosterol against pancreatic α-amylase activity so far.[77] Taking together, anti-diabetic sterols of this algae may be responsible at least in part for anti-diabetic activity of *Sargassum* species.[46] However, this activity is supposed to be performed via different mechanisms, of which α-amylase inhibitory activity is one of the most critical ones.

CONCLUSION

Taking together, *S. glaucescens* the brown algae from Southern Iran was subjected for isolation and identification of the main sterols resulting in separation of fucosterol, 24(S)-hydroxy-24-vinylcholesterol, 24(R)-hydroxy-24-vinylcholesterol, stigmasterol, β-sitosterol and cholesterol. The α-amylase inhibitory activity of this algae was evaluated compared to acarbose and exhibited a potent inhibition against this enzyme in an *in vitro* assay.

ACKNOWLEDGMENT

This research has been supported by Tehran University of Medical Sciences and Health Services Grant (No. 17619). The authors wish to thank Dr. Bayram Gharanjic for his kind help in algae identification.

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Cite this article as: Payghami N, Jamili S, Rustaiyan A, Saeidnia S, Nikan M, Gohari AR. Alpha-amylase inhibitory activity and sterol composition of the marine algae, *Sargassum glaucescens*. Phcog Res 2015;7:314-21.

Source of Support: Nil, **Conflict of Interest:** None declared.