Egyptian red sea seagrass as a source of biologically active secondary metabolites

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Background and objective
The Red Sea seagrass *Halophila stipulacea* (Forsskål) Ascherson and *Thalassodendron ciliatum* (Forsskål) den Hartog were poorly investigated either for their biological activities or for chemical constituents. This study aims to investigate the phytochemical constituent of both grass, along with studying the different biological activities (osteoclastogenesis, antioxidant activity, and anticancer activity) of the crude extract as well as purified compounds.
Materials and methods
The present study used three different in-vitro bioassay methods to screen the fractions and/or isolated compounds of both seagrass, to assess their possible biological activity. Osteoclastogenesis assay, antioxidant activity, and anticancer activity were carried out using different assays of the different anticancer mechanism of action.
Results and conclusion
Ten secondary metabolites were isolated and identified for the first time from Red Sea seagrass *H. stipulacea* (Forsskål) Ascherson: (1) *p*-hydroxybenzoic acid, (2) bis(2-ethyl hexyl) phthalate, (3) benzoic acid, (4) *p*-hydroxybenzaldehyde, (5) thymidine, (6) stigmasterol, (7) oleic acid, (8) linoleic acid, (9) linoleic methyl ester, and (10) apigenin. Furthermore, a new dihydrochalcone and a known flavonoid were isolated and identified from Red Sea seagrass *T. ciliatum* (Forsskål) den Hartog: (11) 2,4′ dihydroxy-4-methoxy-6-glucosyl dihydrochalcone (Thalassodendron B) and (12) rutin. Fractions of both seagrass showed promising biological activities.

Keywords:
anticancer, antioxidant, *Halophila stipulacea* (Forsskål) Ascherson, osteoclastogenesis, red sea seagrass, *Thalassodendron ciliatum* (Forsskål) den Hartog

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Introduction

Seagrass are a rich source of secondary metabolites, which are economically important in the field of food additives, nutraceutical, and drugs and possess a variety of biological activities, such as antibacterial, antifungal, anticancer, and anti-inflammatory [1–5]. The Red Sea encompasses 24 seagrass species on reef flats and in deep water, more than any other region [6]. Along the Egyptian Red Sea coast, there are 10 seagrass species that belong to two families, Cymodoceaceae and Hydrocharitaceae [7–9]. *Halophila stipulacea* (Forsskål) Ascherson (family Hydrocharitaceae) is the most common in the Sinai Coast of the Red Sea when compared with that of Asian Coast [10]. *Thalassodendron ciliatum* (Forsskål) den Hartog (family Cymodoceaceae) is the most common seagrass meadow in the Northern Red Sea coast, with long, woody vertical stems and extensive woody rhizomes [11].

In the sequence of our continuing exploration of bioactive secondary metabolites from Red Sea seagrass [12,13], herein we report the phytochemical and biological investigation of two Egyptian Red Sea seagrass: *H. stipulacea* (Forsskål) Ascherson and *T. ciliatum* (Forsskål) den Hartog. To the best of our knowledge, this is the first report on the chemistry and biological activity of Red Sea seagrass, *H. stipulacea* (Forsskål) Ascherson, and our third report on *T. ciliatum* (Forsskål) den Hartog.

Materials and methods

General experimental procedures

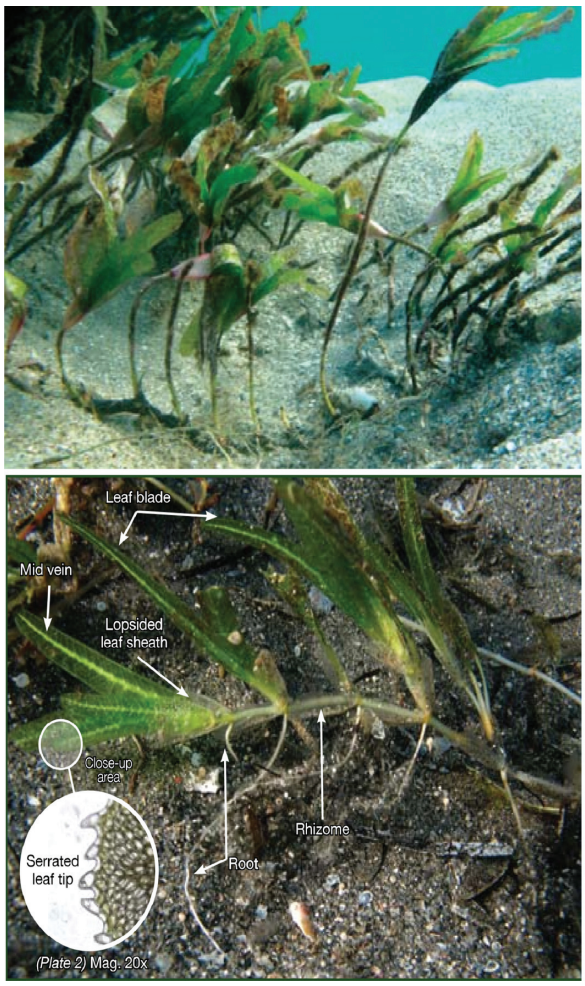
Compounds' structures were elucidated on the basis of NMR and mass spectroscopic analysis. All biological assays carried were in-vitro no vivo tests performed. Assignment of the compounds was achieved by means of 2D NMR spectra. Chemical shifts are referenced to the deuterated solvent used: CD_3OD signal at δH 3.30

and δC 49.00, $CDCl_3$ signals at δH 7.24 and δC 77.23, or DMSO, d_6, signals at δH 2.50 and δC 39.50 in ^{1}H and ^{13}C-NMR, respectively. High-resolution mass spectra (High-resolution MALDI-MS) were recorded on JEOL JMS-700N for electron ionization or on JEOL JMS-T100 TD for electrospray ionization. Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and/or silica gel 60 (0.063–0.2 mm; Merck, Darmstadt, Germany) were used for column chromatography. For thin-layer chromatography (TLC), aluminium sheet silica gel 60 F254 precoated plates (Merck) were used. High-performance liquid chromatography (Younglin; Youngling Cooperation, Seoul, South Korea) was used for purification, and the apparatus was adjusted for the selected program: flow rate (2 or 4 ml/min), ultraviolet (UV) detector, appropriate column, and appropriate elution system.

Plant material

Fresh seagrass materials of *H. stipulacea* and *T. ciliatum* (Fig. 1) [14] were collected from Hurghada, Red Sea, Egypt, and were identified by Professor Dr Wafaa Amer and Professor Dr Mounir Abd-El Ghaney, Department of Botany, Herbarium, Faculty of Science, Cairo University.

Figure 1



Photograph of (a) *Thalassodendron ciliatum* (b) *Halophila stipulacea*.

Extraction and isolation

Fresh wet *H. stipulacea* (22 kg) and/or *T. ciliatum* (800 g) were extracted according to the procedure already reported [12]. In brief, the seagrass was blended in an electric blender with methanol; the process was repeated till exhaustion. The methanolic extract was filtered off and concentrated under reduced pressure at 45°C. The obtained crude extract was suspended in distilled water and partitioned against ethyl acetate (EtOAc) several times. The combined EtOAc fraction was separated, washed with distilled water, and evaporated under reduced pressure at 45°C. The ethyl acetate layer of the methanolic extract yielded dark green residue: H-1, 22.6 g, and/or T-1, 10.6 g, respectively. The remaining aqueous fraction was frozen at –80°C for 24 h and lyophilized, yielding yellow powder (H-2, 10 g) and/or reddish-brown powder (T-2, 6.6 g), correspondingly.

Another method was adopted only for the fresh *H. stipulacea* (Forss) Asch. (16 g), according to the methodology stated by Gavagnin *et al.* [15]. The seagrass was extracted by acetone at room temperature in electric blender several times till exhaustion. The combined extract was filtered off, concentrated under reduced pressure at 45°C and partitioned using diethyl ether (Et_2O) followed with *n*-butanol (*n*-BuOH) several times for each. Both Et_2O and *n*-BuOH layers were separated and evaporated under reduced pressure at 45°C), giving compounds H-E (dark green oily fraction, 5 ml) and H-B (yellow residue, traces), correspondingly.

Halophila stipulacea (Forss) Asch. fractions

Ethyl acetate fraction (H-1), 22.6 g. was purified using Sephadex LH-20 column (60×4 cm.) eluted with chloroform/methanol (50%) followed by step gradient elution of methanol/dist. water (30%) with an increasing percentage of methanol till 100%. Fractions of 250 ml each were collected, concentrated, and monitored by TLC. The plates were examined under UV, before and after spraying with a vanillin-sulfuric acid reagent. Fractions with similar TLC profile were combined. Subfractions were consequently fractionated on Sephadex LH-20 and/or silica gel columns with different elution systems to allow the purification and identification of *p*-hydroxybenzoic acid (1), bis (2-ethyl hexyl)

phthalate (2), benzoic acid (3), and P-hydroxybenzaldehyde (4).

The lyophilized remaining aqueous fraction (H-2), 10 g., was dissolved in the least amount of distilled water and partitioned against saturated *n*-butanol several times. The *n*-butanol fraction was filtered off and evaporated yielding 6.5 g of residue. The obtained residue was chromatographed on a Sephadex LH-20 column (60×4 cm) with chloroform/methanol (50%). Fractions of 250 ml were collected and monitored by TLC analysis. Fractions with similar TLC profiles were combined. Subfractions were purified on HPLC [column; silica (Zobrex Rx Sil, 5 µm), flow rate; 4 ml/min, UV detector; λ=210 and 254 nm, eluting solvent; hexane: ethyl acetate (2 : 1, v/v)], allowing the purification and identification of nucleotide thymidine (5).

The oily diethyl ether fraction (H-E), 5 ml, was chromatographed on silica gel 60 column (40×3 cm). Gradient elution was adopted starting with hexane and increasing polarity with acetone. Fractions 250 ml each were collected and monitored by TLC analysis. Fractions with similar TLC profiles were combined. Subfractions were re-fractionated on silica gel column and purified on HPLC [silica gel column (Zobrex Rx Sil, 5 µm), flow rate; 3 ml/min, eluting system; hexane/ethyl acetate (3 : 2, v/v) and UV detector; λ=254, 280 nm], allowing the purification and identification of sterol, three fatty acids, and flavonoid: stigmasterol (6), oleic acid (7), linoleic acid (8), linoleic methyl ester (9), and apigenin (10), respectively.

Thalassodendron ciliatum (Forss) den Hartog fractions

The ethyl acetate fraction (T-1), 10.6 g, and/or lyophilized remaining aqueous (T-2), 6.6 g, was chromatographed on Sephadex LH-20 column (60×4 cm) with step gradient elution starting with 30% ethanol/dist. water and increasing the percentage of ethanol till 100%. Fractions of 250 ml each were collected, concentrated, and monitored by TLC. The plates were examined under UV, before and after spraying with a vanillin-sulfuric acid reagent. Fractions with similar TLC profile was combined. Subfractions were subsequently fractionated on Sephadex LH-20 columns with different elution systems to allow the purification and identification of new dihydrochalcone, 2,4′-dihydroxy-4-methoxy-6-glucosyl dihydrochalcone (Thalassodendron B))11) and rutin (12), respectively.

Biological activities

Osteoclastogenesis assay

Osteoclastogenesis was performed on the fractions only and was determined using tartrate-resistant acid phosphatase (TRAP) assay as previously described by Tsukamoto et al [16]. The murine cell line (RAW264) was seeded on a 96-well plate (1000 cells/well), cultured for 1 day, then treated with receptor activator of nuclear factor κ-B ligand (RANKL) (a soluble form of RANKL; oriental yeast; 50 ng/ml) and test samples (100 µg/ml; DMSO, control), and then were allowed to differentiate for 4 days. The differentiated cells were washed with PBS and treated with 4% paraformaldehyde solution for 10 min at room temperature. After washing with PBS, the cells were stained with TRAP-staining solution. TRAP-positive cells that stained red and contained three or more nuclei were determined to be multinuclear osteoclasts. Quercetin was used as a positive control and treated under same condition as the tested sample.

Antioxidant activity

Antioxidant activity was determined using 1,1-diphenyl-2-picrylhydrazyl free radical scavenging percentage method, as previously described by Zhao *et al.* [17].

Cytotoxic activity

The activity of the prepared fractions of the two seagrass was determined using the different in-vitro bioassays of different anticancer mechanism of action: proteasome assay [18], Ubc13–Uev1A interaction inhibition assay [19], P53-Mdm2 interaction inhibition assay [18] and Sulforhodamine-B (SRB) protein stain assay [20].

Proteasome used in this study was partially purified from rat liver. The prepared proteasome-enriched fraction in a mixture (0.1 ml) that contained 50 mmol/l Tris-HCl, pH 7.8, 1 mmol/l dithiothreitol, and 5 mmol/l ethylene diamine tetraacetic acid (EDTA) was preincubated with each test sample (20 µg/ml) at 30°C for 10 min. Then, 0.05 mmol/l succinyl-leucyl-leucyl-valyl-tyrosine-4-methylcoumaryl-7-amide (MCA) fluorogenic substrate, used as a substrate for chymotrypsin-like activity of the proteasome (Peptide Institute Inc., Osaka, Japan), was added to the mixture and further incubated at 30°C for 1 h. The reaction was stopped by adding 0.1 ml of 10% SDS, and the fluorescence intensity owing to 7-amino-4-methylcoumarin was measured (excitation, 360 nm; emission, 460 nm). Results were expressed in terms of the inhibition

percent of the chymotrypsin-like activity of proteasome as compared with the negative control. The positive control MG132 (specific, potent, reversible, and cell permeable proteasome inhibitor) was used. *Escherichia coli* BL21 cells transformed with pGEX-6P-1-Ubc13 or GEX-6P-1-FLAG Uev1A were precultured overnight at 37°C in LB medium supplemented with 100 µg/ml ampicillin, transferred to a 20-fold volume of the same medium, and cultured at 37°C for 1.5 h 11, 20, isopropyl-1-thio-*β*-d-galactoside was then added at a final concentration of 0.1 mmol/l, and the cells were further cultured at 25°C for 6 h. Two glutathione S-transferase (GST)-fused proteins were purified by using glutathione immobilized agarose beads, and the GST tag of GST-Ubc13 or GST-FLAG-Uev1A was removed by cleavage with PreScission protease (GE Healthcare, USA).

The inhibition of the Ubc13–Uev1A interaction was tested by enzyme-linked immunosorbent assay (ELISA) with a 96-well plate (Maxisorp, Nunc, Roskilde, Denmark). Human Ubc13 diluted in PBS was coated onto a 96-well plate and incubated at 4°C overnight. The wells were extensively washed with 0.05% Tween 20 in PBS (PBST) and incubated with 5% bovine serum albumin (BSA) (Sigma) in PBS at 37°C for 1.5 h. After washing with PBST, the wells were incubated for 1.5 h with a mixture of FLAG-Uev1A and a test sample diluted in PBS that had been previously incubated at 37°C for 15 min. The wells were thoroughly washed with PBST and incubated with anti-FLAG M2 monoclonal antibody (Sigma) in 5% BSA in PBST for 1.5 h, followed by the second antibody mouse IgG-HRP (Amersham) in 5% BSA in PBST for 1.5 h. After washing with PBST and then citrate-phosphate buffer (pH 5.0), a mixture of *ortho*-phenylene diamine and 0.007% H_2O_2 in the citrate-phosphate buffer was added to the wells, and the wells were incubated at 37°C for 30 min. Finally, 2 M H_2SO_4 was added to the wells, and the optical density at 490 nm was measured on a microplate reader. NSC (anticancer drug) was used as the positive control.

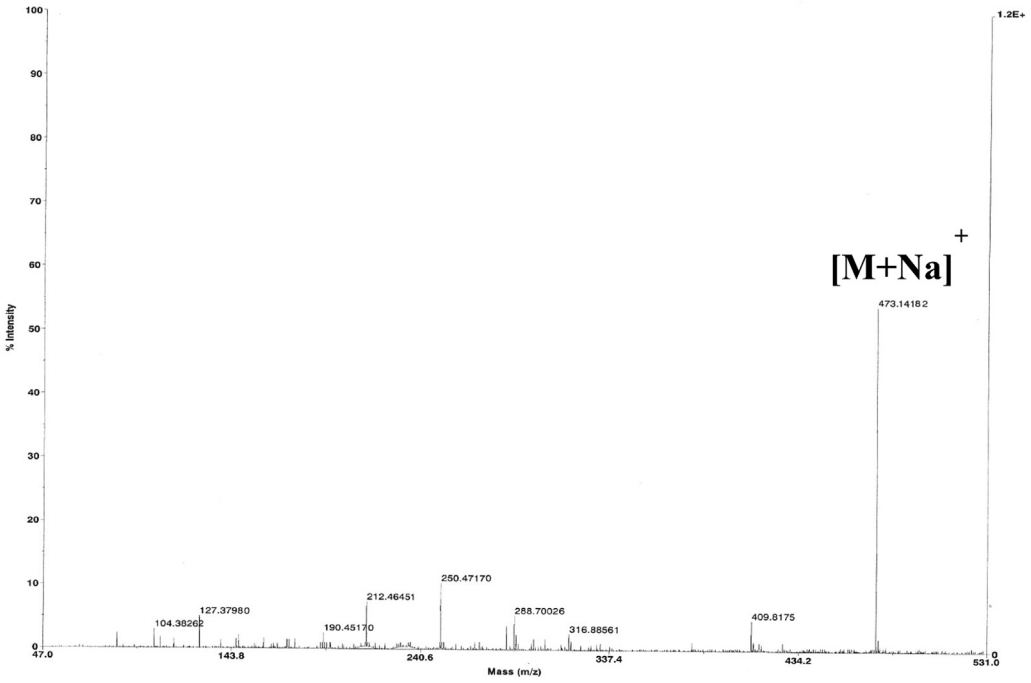
The inhibition of p53-Mdm2 interaction was tested by ELISA according to the standard procedure using purified recombinant p53 and HDM2 (human double minute 2) proteins and the following primary anti-MDM2 (mouse double minute 2) antibody (SMP14; Santa Cruz). *E. coli* BL21 cells transformed with pGEX6P1-p53 or pGEX6P1-HDM2 were precultured overnight at 37°C in LB medium supplemented with 100 µg/ml ampicillin, transferred to a 100-fold volume of the same medium, and cultured at 37°C for 2 h. Isopropyl-1-thio-*β*-d-galactoside was then added at the final concentration of 0.1 mmol/l, and the cells were further cultured at 30°C for 6 h. Two GST-fusion proteins were purified by using glutathione immobilized agarose beads and the GST tag of GST–p53 or GST–MDM2 was removed by cleavage with PreScission protease (Amersham). Standard 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 µl of P53 in PBS. Plates were subsequently washed three times with washing buffer PBS containing 0.05% v/v tween 20. Excess binding sites were blocked for 90 min at 37°C using 150 µl of a solution of 5% bovine serum albumin (BSA) (A7906; Sigma). Wells were washed, and 110 µl of MDM2 was added. Wells were washed again with PBS and then 50 µl of the first antibody (anti-MDM2; SMP14, Sc-965) was added. The wells were washed again with PBS. A volume of 50 µl of anti IgG-HRB (streptavidin) was added and incubated for 90 min at 37°C. Plates were washed with washing buffer. Then 100 µl of the mixture containing *ortho*-phenphenylene diamine (OPD) solution (8 mg/20 ml citrate phosphate buffer pH 6.8) and 30% hydrogen peroxide was added to each well. The 96-well microtiter plate was kept in the dark at 37°C for 30 min. The reaction was stopped by adding 50 µl 2 M H_2SO_4 per well, and the absorbance in each well was measured at 490 nm in a microtiter plate reader. Nutelin was used as a positive control.

In the SRB assay, human cancer cell lines were used when 90% confluence was reached. Adherent cell lines were harvested with 0.025% trypsin. Viability of cells were determined by trypan blue exclusion. Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the tested samples to allow attachment of cell to the wall of the plate. Different concentrations of the test sample (5, 12.5, 25, and 50 µg/well) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the test sample for 48 h at 37°C and in atmosphere of 5% carbon dioxide. After 48 h, cells were fixed with cold 50% trichloroacetic acid for 1 h at 4°C. Wells then were washed with distilled water and stained with 0.4% sulforhodamine-B stain dissolved in 1% acetic acid for 30 min at room temperature. Excess stain was washed with 1% acetic acid, and attached stain was recovered with Tris EDTA buffer. Absorbance at 570 nm was measured in an ELISA reader.

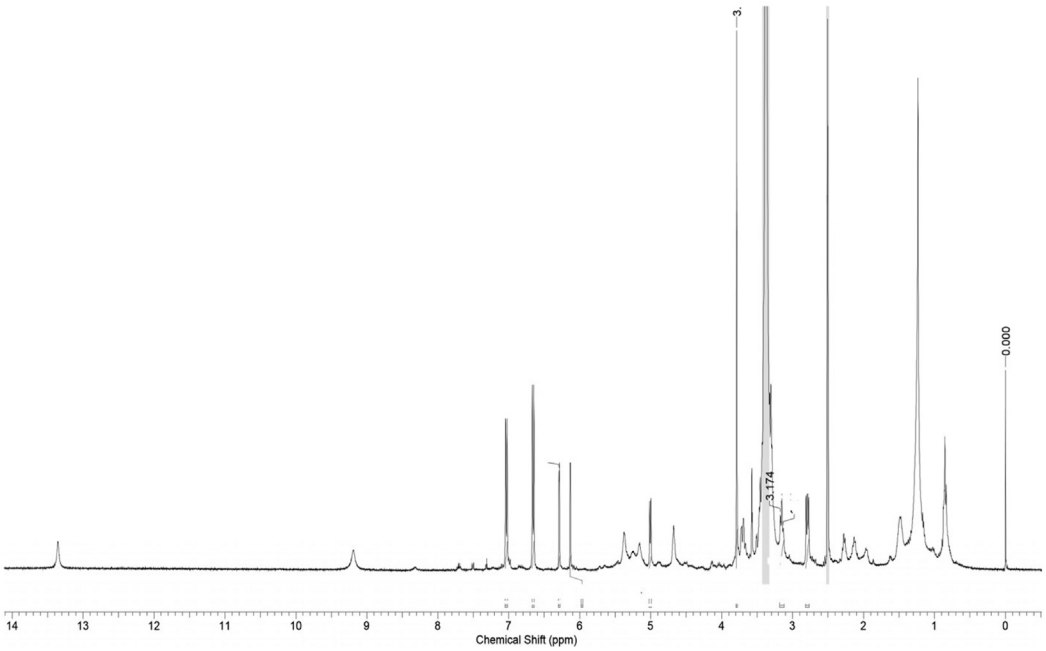
Result and discussion

The study revealed isolation of ten compounds from *H. stipulacea* (Forss.) Ascherson, comprising *P*-hydroxybenzoic acid (4 mg, **1**) [21], Bis (2-ethyl hexyl) phthalate (50 mg, **2**) [22], Benzoic acid (27 mg, **3**) [23], *P*-Hydroxybenzaldehyde (20 mg, **4**) [21], Thymidine (20 mg, **5**) [24], Stigmasterol (5 mg, **6**) [25], Oleic acid (0.5 ml, **7**) [26], Linoleic acid (0.5 ml, **8**) [26], Linoleic methyl ester (5 mg, **9**)

Figure 2



(a) MALDI mass spectrum

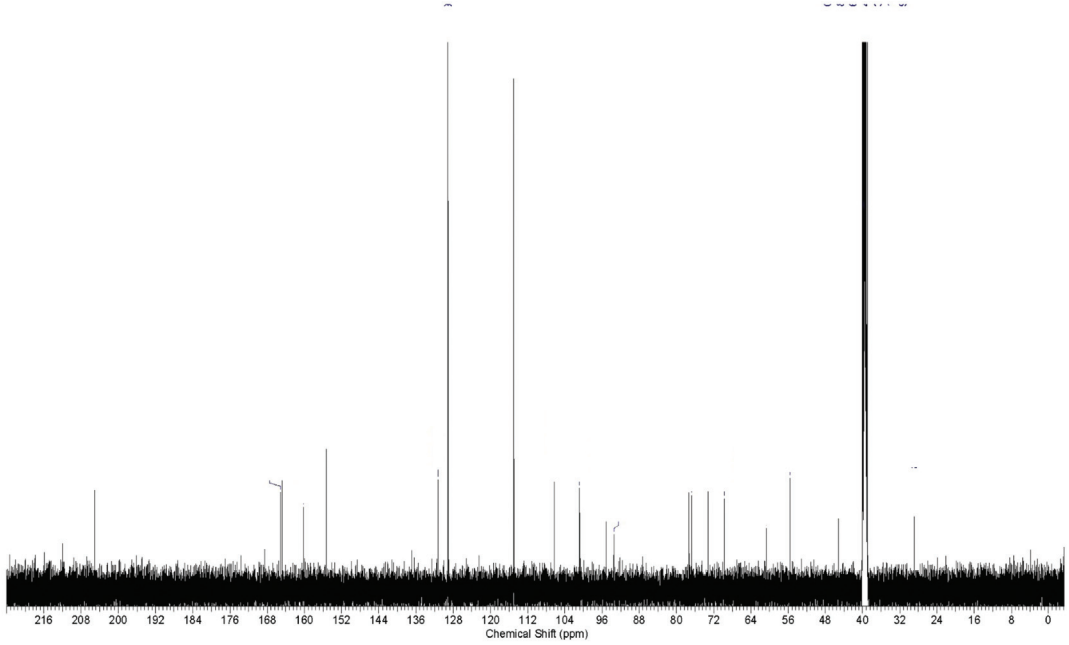


(b) ^{1}H-NMR spectrum, DMSO, d_6, 400Hz.

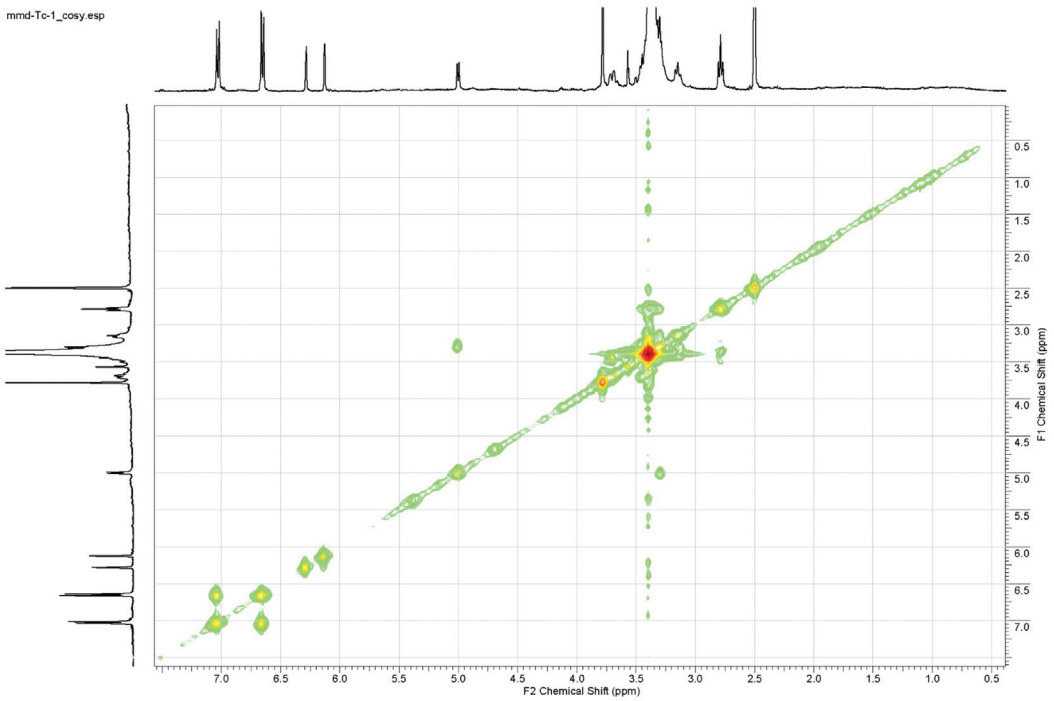
Spectral data and chemical structure of 2,4'-dihdroxy-4-methoxy-6-glucosyl dihydrochalcone and thalassodendron B. (a) MALDI mass spectrum. (b) ^{1}H-NMR spectrum, DMSO, d_6, 400Hz. (c) ^{13}C-NMR spectrum, DMSO, d_6, 400Hz. (d) COSY spectrum, DMSO-d_6, 400 Hz. (e) HSQC spectrum, DMSO-d_6, 400 Hz. (f) HMBC spectrum, DMSO-d_6, 400 Hz.

[27] and Apigenin (6 mg, **10**) [28] and two compounds from *T. ciliatum* (Forss.) den Hartog, comprising new dihydrochalcone; 2,4′-dihydroxy-4-methoxy-6-glucosyl dihydrochalcone (Thalassodendron B, 25 mg, 11; Fig. 2) and rutin (15 mg, 12) [12].

Figure 2



(c) ^{13}C-NMR spectrum, DMSO, d_6, 400Hz.

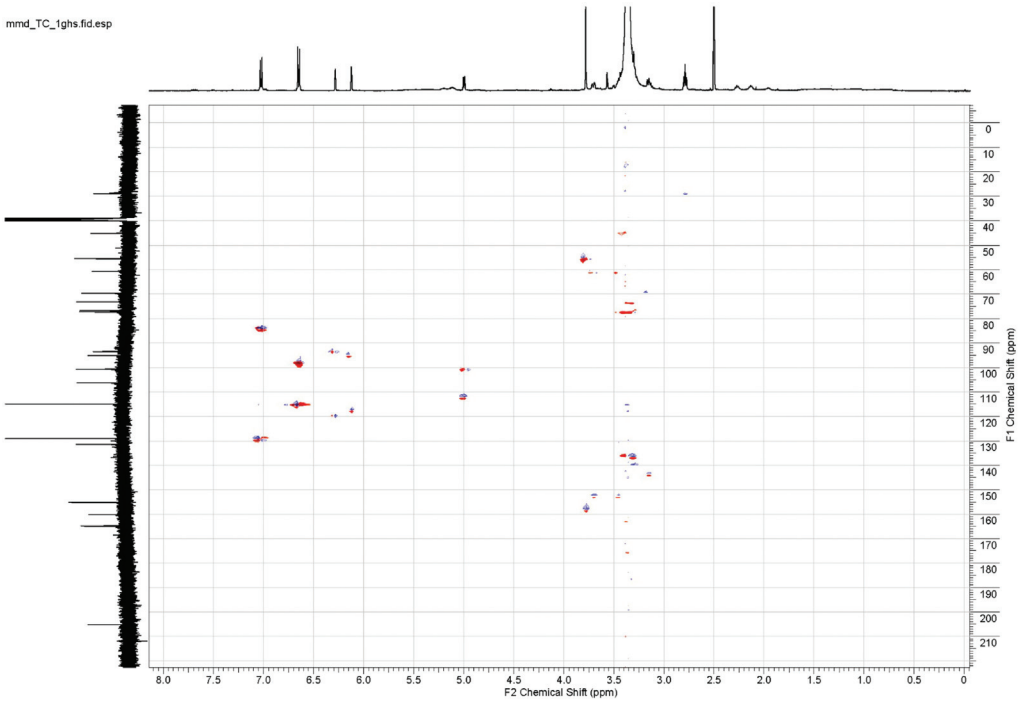


(d) COSY spectrum, DMSO-d_6, 400 Hz.

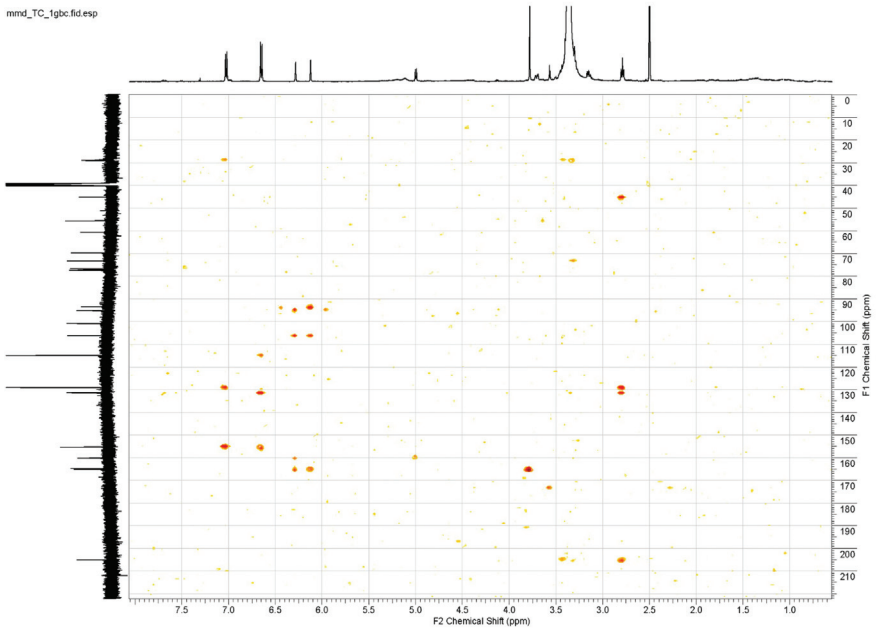
(Continued)

To the best of our knowledge, this is the first report on Red Sea seagrass *H. stipulacea* (Forss.) Ascherson. Bis (2-ethyl hexyl) phthalate (2) was recently detected by GC/MS in the same species of the Mediterranean Sea [29]. Thymidine (5) is the first nucleotide isolated from a seagrass. Moreover, this is the second report for isolation of apigenin (10) from the grass, because it has been previously isolated from

Figure 2



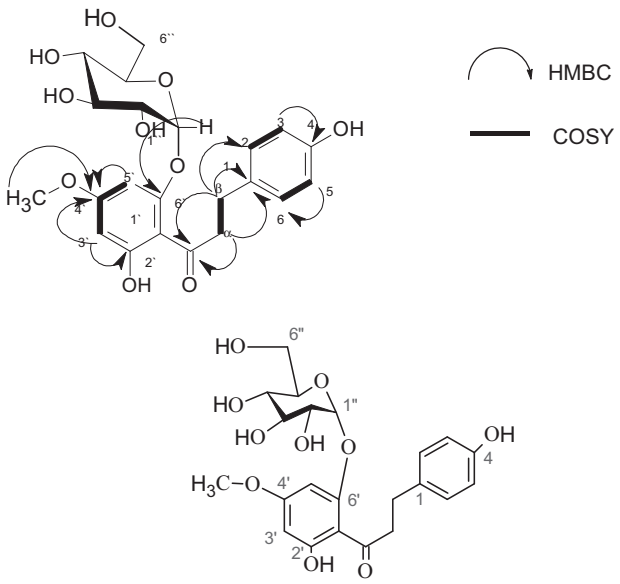
(e) HSQC spectrum, DMSO-d_6, 400 Hz.



(f) HMBC spectrum, DMSO-d_6, 400 Hz.

(Continued)

Figure 2



(Continued).

Mediterranean Sea *H. stipulacea* (Forss.) Ascherson by Ernesto *et al.* [30].

2,4'-dihydroxy-4-methoxy-6-glucosyl dihydrochalcone (Thalassodendron B, 11) was isolated from *T. ciliatum* (Forss.) den Hartog, which is a new compound first to be isolated from nature. It was isolated as yellow amorphous powder, with molecular formula $C_{22}H_{26}O_{10}$ as determined by high-resolution MALDI-MS (Fig. 2) ($[M+Na]^+$ at *m/z* 473.14182). The combination of ^{13}C-NMR and ^{1}H-^{13}C HSQC spectra Fig. 2 revealed the presence of 1 methyl, 3 methylene, 11 methene, and 7 quaternary carbons. The ^{1}H-NMR spectrum (Fig. 2) showed one anomeric proton signal of a hexose sugar appeared at δ_H 5.00 (1 H, d, *J*=6 Hz, H-1′), which was confirmed to be glucose from ^{13}C-NMR data (Fig. 2). Its attachment position was suggested to be at C-6' based on the long-range correlation between H-1′ at δ_H 5.00 and C-6′ at δ_C 160.18 as appeared in HMBC spectrum (Fig. 2). Furthermore, two correlated meta-coupled doublets appeared at δ_H 6.13 (1 H, d, *J*=2 Hz, H-3′) and at δ_H 6.28 (1 H, d, *J*=2 Hz, H-5′). Several correlated multiplets appeared between δ_H 3.15–3.72 (6 H, m), corresponding to the remaining sugar protons. Sharp singlet signal appeared at δ_H 3.78 (3 H, s, OCH_3), characteristic for an aromatic methoxyl, confirmed by HMBC to be at C-4'. Two triplets characteristic for the dihydrochalcones appeared at δ_H 2.79 (2 H, t, *J*=5.6 Hz, CH_2-β) and δ_H 3.45 (2 H, t, *J*=5.6 Hz, CH_2-α). The AA′BB′ system was confirmed by the presence of the two *ortho*-coupled doublets at δ_H 7.02 (2 H, d, *J*=6.8 Hz, H-2 and H-6) and at δ_H 6.65 (2 H, d, *J*=6.8 Hz, H-3 and H-5), indicating an oxygen substitution at the 4-position.

2,4′-dihydroxy-4-methoxy-6-glucosyl dihydrochalcone (Thalassodendron B))11) was obtained in the form of yellow amorphous powder. MALDI mass spectrum exhibited a molecular ion peak $[M+Na]^+$ at *m/z* 473.14182, which was in agreement with the molecular formula $C_{22}H_{26}O_{10}$ -^{1}H-NMR (δ ppm, DMSO-d6, 400 Hz); 6.13 (1H, d, J = 2.0 Hz, H-3′), 6.28 (1H,d,J = 2.0 Hz, H-5′), 7.02 (2H,d, J = 6.8 Hz, H-2 and H-6), 6.65 (2H, d, J = 6.8 Hz, H-3, H-5), 2.79 (2H, t, J = 5.6 Hz, CH_2-b),3.45 (2H, t, J = 5.6 Hz, CH_2-a), 3.78 (3H, s, H3CO-4′), 5.00 (1H, d, J = 6 Hz, H-1″), 3.15–3.72 (5H,m, CH 2″, 3″, 4″, 5″, 6″) -^{13}C-NMR (δ ppm, DMSO-d6, 400 Hz); 106.30 (C-1′, C),164.77 (C-2′, C), 93.5 (C-3′, CH), 165.1 (C-4′, C), 95.19 (C-5′,CH), 160.18 (C-6′, C), 131.33 (C-1, C), 129.09 (C-2 and -6, CH), 114.98 (C-3 and -5, CH), 155.28 (C-4, C), 28.90 (CH_2-b), 45.11 (CH_2-a), 205.07 (C=O), 55.56 (H3CO-4′), 100.81 (C-1″,CH), 73.21(C-2″, CH), 76.73 (C-3″, CH), 69.69 (C-4″, CH), 77.35 (C-5″, CH), 60.69 (CH2-6″).

Osteoclastogenesis assay

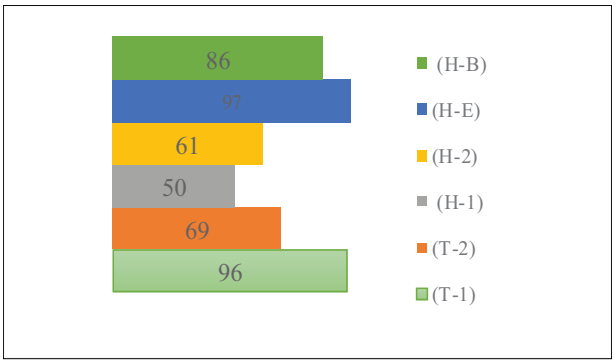
Inhibition percentage of osteoclastogenesis by main fractions at concentration of 100 µg/ml is illustrated in Fig. 3 (Table 1). The inhibition of osteoclastogenesis is a promising target for treatments that can improve osteoclast-related diseases such as osteoporosis, rheumatoid arthritis, and bone metastasis [31].

According to the published research studies, this is the first study on the osteoclastogenesis inhibition percentage of different fractions of alcoholic extract of *H. stipulacea* (Forss.) Ascherson and *T. ciliatum* (Forss.) den Hartog.

H-E followed by H-B showed the highest inhibition percentage even higher than positive control quercetin. *Thalassodendron* fractions, T-1 and T-2, were the same and had nearly one-third activity of that of positive control.

H-E exhibited the highest antiosteoclastogenic activity, which is related to its several antiosteoclastogenic phytochemicals' content. Phytosterols and fatty acids inhibit osteoclastogenesis. Unsaturated fatty acids have a beneficial effect on bone, omega 3 and oleic acid was reported to inhibit osteoclast formation and enhanced osteoblast [32]. Stigmasterol has been reported to exhibit a potent anti-osteoarthritic activity and anti-osteoclastogenic activity [33,34].

Figure 3



Inhibition percentage of osteoclastogenesis by seagrass fractions.

Table 1 Inhibition percentage of osteoclastogenesis by seagrass fractions (100 µg/ml)

Sample code	Inhibition %
Halophila ethyl acetate main fraction (H-1)	17
Halophila remaining aqueous main fraction (H-2)	29
Halophila diethyl ether main fraction (H-E)	117
Halophila butanol main fraction (H-B)	114
Thalassodendron ethyl acetate main fraction (T-1)	35
Thalassodendron remaining aqueous main fraction (T-2)	32
Quercetin (control)	90

Table 2 1,1-Diphenyl-2-picrylhydrazyl scavenger percentage of isolated compounds

Compounds	Scavenger %
P-Hydroxybenzoic acid 1	2.0
Bis (2-ethyl hexyl) phthalate 2	24.0
Benzoic acid 3	4.8
P-Hydroxybenzaldehyde 4	11.4
Thymidine 5	1.6
Stigmasterol 6	5.5
Oleic acid 7	4.1
Linoleic acid 8	3.1
Linoleic methyl ester 9	9.4
Apigenin 10	6.1
Thalassodendron B 11	92.4
Rutin 12	82.7
Ascorbic acid	93.4
Negative control	0.0

This is the first preliminary study to evaluate the possible use of seagrass as a novel natural resource in the treatment of osteoclastogenesis-related disease. Results suggest the beneficial use of these extracts as a herbal drug, especially H-B, which was obtained in traces not enough for its chemical analysis. Thus, future studies are required and should focus on the isolation of the active component and the safety.

Antioxidant activity

The scavenging activity percentages of the identified compounds are calculated and illustrated in Table 2. Compounds thalassodendron B (11) and rutin (12) showed the highest antioxidant activity and were found to be nearly the same as that of positive control ascorbic acid. The remaining compounds showed low activity in comparison with that of the control. *T. ciliatum* (Forss.) den Hartog ethyl acetate fraction was previously reported to show potent antioxidant activity [12].

The antioxidant activity of the tested compounds was in agreement with that reported for phenolic compounds and their structure-activity relationship (SAR) [35,36]. Rutin (12) was the most potent antioxidants, as it has all the structural features that induced the antioxidant activity [12]. The antioxidant activity of flavonoids resides in the aromatic hydroxyl groups [37], whereas the hydroxyl groups of the sugar moiety have no role in the activity [38]. Glycosylation or methoxylation decreases the activity, as it blocks the active free hydroxyl group and even C-glycosides as the sugar substitute reduces the antioxidant activity of adjacent hydroxyl groups owing to steric hindrance [39]. Therefore, aglycones are more potent than their corresponding glycoside [40]. Ring-A substitution is slightly linked with the antioxidant activity. The activity was enhanced by an electron donating effect of the hydroxyl groups at positions 5 and 7. Ring B, either catechol (C-3′, C-4′) and/or pyrogallol (C-3′, C-4′, C-5′) structure form intramolecular hydrogen bonds with the C-3 hydroxyl group (flavonol), which maintain the coplanarity of the moiety, and so its electron delocalization capacity promotes the antioxidant activity. The influence of a C-3 hydroxyl group is potentiated by the presence of a catechol group. Ring-C ketone group at C-4 is not essential for the antioxidant activity, and saturation of C-2–C-3 bond decreases the activity [41]. Apigenin (10), although is a flavonoid in nature, has very weak activity, which was confirmed by previously reported [42,43]. This is because of the absence of the main antioxidant pharmacophore: hydroxyl group at C-3 and the catechol group (C-3′, C-4′) in ring-B.

Regarding the dihydrochalcones, hydroxyl group at the C2, C4, and C-2′ position of the Ring-A and the orthogonal configuration of it to the carbonyl group is an essential pharmacophore for antioxidant activity [44–46]. Thalassodendron B 11 showed a potent activity owing to the presence of the hydroxyl group at the C-2′ position.

However, for the phenolic acids, the number of aromatic hydroxyl groups, length of side carbon chain between the ring and the carboxylic group, and its degree of saturation facilitate the electron delocalization by resonance between the aromatic ring and conjugated side chain [47]. P-hydroxybenzoic acid **1** and P-hydroxybenzaldehyde **4**, are one of the most abundant phenolic acid/aldehydes in seagrass, and along with benzoic acid, they exhibit a very weak antioxidant activity, which was in agreement with previously reported data [48–50] and with the SAR.

Zwolak has recently stated that stigmasterol has improved superoxide dismutase, hepatic lipid peroxidation, and the glutathione activity, predicting its antioxidant activity [51], which is in contrary to our finding, which shows the weak activity of stigmasterol. On the contrary, Omisore *et al.* [52] have ensured our finding, reporting the weak antioxidant activity of stigmasterol using 1,1-diphenyl-2-picrylhydrazyl assay.

Table 3 Proteasome inhibition percentage of seagrass fractions (20 µg/ml)

Samples	Inhibition %
Halophila ethyl acetate fraction (H-1)	50
Halophila remaining aqueous fraction (H-2)	61
Halophila diethyl ether fraction (H-E)	97
Halophila butanol fraction (H-B)	86
Thalassodendron ethyl acetate fraction (T-1)	96
Thalassodendron remaining aqueous fraction (T-2)	69

Table 4 Ubc13–Uev1A interaction inhibition percentage of seagrass fractions (10 µg/ml)

Samples	Inhibition %
Halophila ethyl acetate fraction (H-1)	55
Halophila remaining aqueous fraction (H-2)	62
Halophila diethyl ether fraction (H-E)	62
Halophila butanol fraction (H-B)	56
Thalassodendron ethyl acetate fraction (T-1)	72
Thalassodendron remaining aqueous fraction (T-2)	58
NSC anticancer drug (10 µmol/l) (positive control)	42

Table 5 Binding percentage of seagrass fractions (20 µg/ml)

Sample code	Binding %
Halophila ethyl acetate fraction (H-1)	32
Halophila remaining aqueous fraction (H-2)	57
Halophila diethyl ether fraction (H-E)	67
Halophila butanol fraction (H-B)	67
Thalassodendron ethyl acetate fraction (T-1)	43
Thalassodendron remaining aqueous fraction (T-2)	0
Nutelin (positive control) (810 nmol/l)	43

Cytotoxic activity

Cancer nowadays is one of the most common diseases that lead to death among older individuals and even babies. The treatment protocol is usually chemotherapy and/or radiotherapy, but unfortunately, some cancer cells show resistance to these treatments and/or shows severe adverse effects. Thus, searching for a new line in cancer treatment is required. According to WHO, ~80% of the world's population depends on phytochemicals for their primary health care. Nutraceuticals have great probability to be developed into anticancer drugs because of their diverse mechanisms and low adverse effects. Plants rich in polyphenolic compounds might be used as potential chemoprevention agents [53].

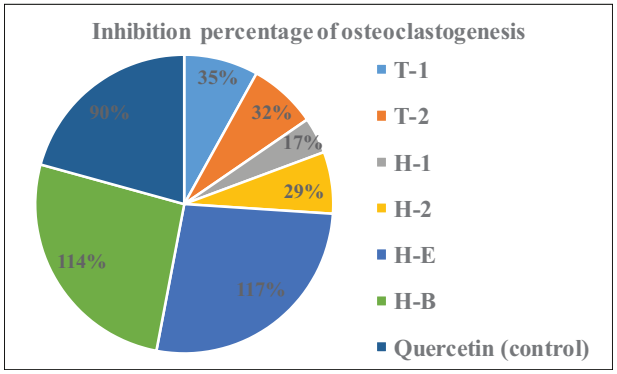
The recent successful use of anticancer drug Velcade (bortezomib), a synthetic proteasome inhibitor, has developed a new trend in the treatment and triggered the discovery of a new line of treatment using drugs targeting the ubiquitin–proteasome pathway [54]. To date, various synthetic and neutraceuticals have been reported to inhibit different targets in the ubiquitin–proteasome pathway. Several compounds are now undergoing preclinical and clinical trials for cancer treatment [55].

Thus, herein we investigated the bioavailability of seagrass in different organic fractions on the ubiquitin–proteasome pathway as a new line in cancer treatment, as shown in Tables 3–5.

Proteasome assay

Proteasome inhibitors showed unexplained mechanism of antitumor activity against different cancer cells [54,56]. *Halophila* diethyl ether fraction (H-E) followed by *Thalassodendron* ethyl acetate fraction (T-1) and *Halophila n*-butanol fraction (H-B) shows the highest activity, respectively. The other fractions exhibit moderate activity, as shown in Fig. 4 (Table 3).

Figure 4



Proteasome inhibition percentage of seagrass fractions.

Tsukamoto *et al.* [57] isolated polyhydroxysterols from a marine sponge, *Acanthodendrilla* spp. and Yang *et al.* [58] isolated a steroidal lactone from 'Indian Winter Cherry' which acts as proteasome inhibitors. Moreover, oleic and linoleic acids were previously reported to inhibit proteasome activity [59], which may speculate the activity of H-E.

T-1 fraction is rich with phenolic compounds [12], which may attribute to its high activity. Polyphenols, flavonoids, and phenolic acids exhibit proteasome inhibition activity [60–62].

Ubc13–Uev1A interaction inhibition assay

Both seagrass show promising activity (Fig. 5, Table 4). *Thalassodendron* ethyl acetate fraction (T-1) exhibited a higher activity followed by *Halophila* remaining aqueous fraction (H-2) and *Halophila* diethyl ether fraction (H-E). Ubc13 inhibitor (preventing the formation of the Ubc13–Uev1A complex) would be a lead candidate for an anticancer agent [19]. Published research studies on inhibitors of the ubiquitin–proteasome system from natural sources are very limited. Tsukamoto *et al.* [63] and Dalisay *et al.* [64] isolated sphingolipid Leucettamol A as an inhibitor of Ubc13–Uev1A interaction from a marine sponge, *Leucetta aff. microrhaphis* and reported the activity of its *n*-BuOH fraction. Ushiyama *et al.* [65] studied the activity of different organic solvent fractions of marine sponge *Lissodendryx fibrosa* where the residual H_2O and *n*-BuOH fractions showed higher inhibitory activity against Ubc13–Uev1A interaction and they isolated active sulfonated sterol dimers, manadosterols A and B.

P53-Mdm2 interaction inhibition assay

The binding percentage of P53-Mdm2 is inversely proportional to the activity (Fig. 6, Table 5). Thus, *Thalassodendron* remaining aqueous fraction (T-2) exhibited a higher activity followed by *Halophila* ethyl acetate fraction (H-1) and *Thalassodendron* ethyl acetate fraction (T-1).

P53 is a gene that expresses the protein that regulates the cell cycle, and is a tumor suppressor [66]. It is the most frequently mutated gene (>50%) in human cancer. p53 has many mechanisms as tumor suppressor. In normal cell, p53 undergoes continuous degradation through a protein called Mdm2 (also called HDM2 in humans, a key regulator for p53 stability), which binds to p53, preventing its action and tagging it for degradation by the proteasome. Thus, any treatment that prevents this protein–protein interaction, that is, prevent mdm2 binding to p53, will release active p53 which in sequence stops tumor growth through cell cycle arrest and/or apoptosis.

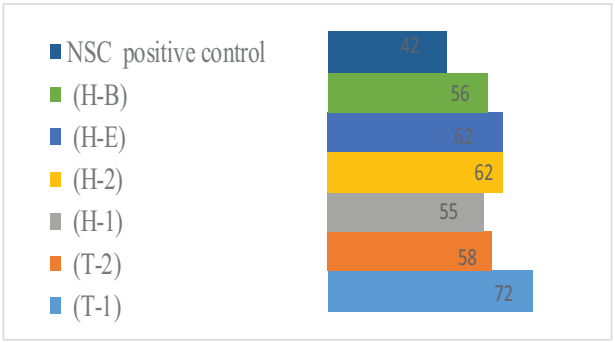
Chlorofusin is the first P53-Mdm2 interaction inhibitor isolated from natural sources, a culture of a *Fusarium* spp. [67]. Different compounds were isolated from marine sources as Hexylitaconic acid was isolated from a marine-derived fungus, Arthrinium and spiladenoserinols A–L a sulfonated serinol derivatives were isolated from a tunicate [68,69].

Recently, Verma *et al.* [70] based on molecular docking and molecular dynamic simulation study stated that polyphenols (flavonoids) exhibited P53-Mdm2 interaction inhibitor activity similar to that of synthetic Nutelin. This finding coincides with our results as compounds isolated from T-1, T-2, and H-1 are phenolic compounds in nature.

Sulforhodamine-B (SRB) protein stain assay

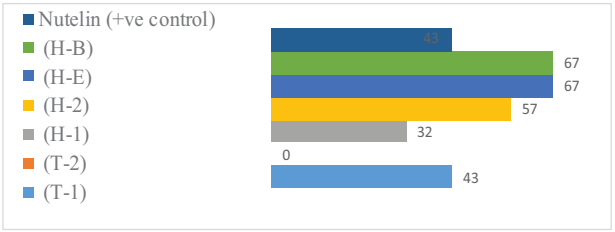
The different organic solvent fractions of both seagrass were evaluated against the following human cancer cell lines: HepG2 (hepatocellular carcinoma), HCT-116 (colon carcinoma), MCF-7 (breast carcinoma), and HeLa (cervical carcinoma) human cancer cell lines. IC50s are calculated and presented in Table 6.

Figure 5



Ubc13–Uev1A interaction inhibition percentage of seagrass fractions.

Figure 6



Binding percentage of seagrass fractions. (a) MALDI mass spectrum.

Table 6 IC_{50} of *Halophila stipulacea* (Forss.) Asch. and *Thalassodendron ciliatum* (Forss.) den Hartog fractions (μg/ml)

Samples	HepG2	HCT-116	MCF-7	HeLa
Halophila ethyl acetate fraction (H-1)	4.73	11.30	10.30	17.50
Halophila diethyl ether fraction (H-E)	3.98	27.10	5.33	NS*
Halophila butanol fraction (H-B)	11.00	NS*	9.98	NS*
Thalassodendron ethyl acetate fraction (T-1)	8.12	4.20	4.12	9.80
Thalassodendron remaining aqueous fraction (T-2)	9.08	28.90	21.50	24.20
Doxorubicin (positive control)	0.60	0.69	0.65	2.01

*Not shown data as according to the protocol of National Cancer Institute (NCI-USA), natural products from plants were considered cytotoxic if IC50 less than 30 μg/ml [71].

Table 7 IC_{50} of the isolated pure compounds (μg/ml)

Compounds	HepG2	Caco-2	Hep-2
P-Hydroxybenzoic acid (1)	21.8	20	NA*
Bis (2-Ethyl Hexyl) Phthalate (2)	18.5	15	NA*
Benzoic acid (3)	19.3	16.8	18.2
P-Hydroxybenzaldehyde (4)	18.3	13	19.2
Thymidine (5)	19	13.2	NS**
Stigmasterol (6)	26.5	25.5	NS**
Oleic acid (7)	NS**	NS**	NA*
Linoleic acid (8)	19.8	8.3	NA*
Apigenin (10)	18.8	13.8	NA*
Thalassodendron B (11)	11.5	8.3	NA*
Rutin (12)	NS**	24	22.6

*IC50 not detected till 50 μg/ml maximum concentration used.
**Not shown data as according to the protocol of National Cancer Institute (NCI-USA), natural products from plants were considered cytotoxic if IC50 less than 30 μg/ml [71].

Halophila diethyl ether fraction (H-E) showed the highest activity against HepG2 cancer cell line followed by *Halophila* ethyl acetate fraction (H-1). *Thalassodendron* ethyl acetate fraction (T-1) showed the highest activity against the other cancer cell lines, MCF-7, HCT-116, and HeLa, respectively, similar to our previous report [12].

T-1 fraction has a high phenolic content [12] and exhibits high activity in the different anticancer assays evaluated as aforementioned, which may speculate its pronounced activity against all human cancer cell lines tested.

Syphonoside previously isolated from H-B fraction was inactive against various cancer cell lines (HeLa, MCF-7, and else) [72]. Moreover, H-B fraction shows only moderate proteasome inhibitor activity and weak activity in other assays carried on, which may postulate its weak anticancer activity against human cancer cells tested.

The isolated compounds were tested against the following human cancer cell lines: HepG2 (hepatocellular carcinoma), Caco-2 (intestinal carcinoma), and Hep-2 (epidermoidal larynx carcinoma) human cancer cell lines. IC_{50} is calculated and presented in Table 7.

Linoleic acid (8) and thalassodendron B (11) showed high activity against Caco-2 cancer cell line. Additionally, the former showed moderate activity against HepG2 cancer cell line, whereas all other compounds showed very weak activity against Hep-2 cancer cell line.

Based on the reported structure-activity relationship for anticancer activity we can postulate that apigenin (10) shows higher activity than rutin (12) because it has only three OH group, and the overall number of hydroxyl groups in the flavonoid moiety is inversely proportion with cytotoxic activity [71,73]. Benzoic acid (3) has higher activity than p-hydroxybenzoic acid (2), as the number of aromatic hydroxyl groups and the length of the side carbon chain between the aromatic ring and the terminal carboxylic group and its degree of saturation affect the anticancer activity [74,75]. Linoleic acid (8) is more active than oleic acid (7), as the activity is related to the length of chain and its degree of saturation, as chain length increases, the activity decreases [76].

Conclusion

Ten compounds were firstly isolated from Red Sea *H. stipulacea*, where compound 2 was previously detected by GC/MS, and compound 10 was previously isolated but from Mediterranean Sea *H. stipulacea*. Moreover, it is the first report for the isolation of compound 5 from seagrass. Two compound were isolated from *T. ciliatum*: a new compound thalassodendron B, and a previously isolated compound 12.

Both seagrass show marvelous bioactivity. *H. stipulacea* fractions showed higher osteoclastogenesis activity than those of *T. ciliatum*. For their anticancer activity, it is the first report on in-vitro anticancer bioassays of different mechanism of action in which H-E shows the highest proteasome inhibitor activity. Both seagrass fractions showed a valuable Ubc13 inhibitor activity ranging from 55 to 72%. T-2 exhibited high P53-Mdm2 interaction inhibition activity. Thus, both seagrass fractions' and isolated compounds' activity against human cancer cell lines were estimated *in vitro*. T-1 showed pronounced

anticancer activity against all cancer cell lines tested, followed by H-E against HepG2 and MCF-7 cancer cell lines.

From the aforementioned data, we can speculate that both seagrass would be a lead candidate for an anticancer agent of natural source, and *H. stipulacea* should be examined *in vivo* for treatment of osteoclast-related diseases such as osteoporosis.

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Conflicts of interest
There are no conflicts of interest.

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