

Testing the *Halophila Stipulacea* Environmental Threat as A Potential Antioxidant Agent

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Research

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Abstract

Marine biotechnology is promising to provide new solutions to society and humans, such as for health protection or natural treatments against diseases. In this framework, different strategies are explored to select marine organisms to initiate the pipeline, hopefully ended by the product development. Organisms with fast growth rates and great biological adaptive capacity are of biotechnological interests, since competitiveness might rely on increased physiological or biochemical processes' capability seeking to protect, defend on repair intracellular damages. This study focussed on the fast-growing and invasive seagrass *Halophila stipulacea*, a non-indigenous species widespread in the Mediterranean Sea to investigate its antioxidant activity, both in scavenging reactive oxygen species and in repairing damages from oxidative stress on human fibroblast cells. Results indicated that the ethanol/water extracts of the leaves of *H. stipulacea* were able to scavenge reactive oxygen species and in repair damages from oxidative stress. Moreover, greater bioactivities were observed in old leaves compared to young ones, paralleled with a higher content in carotenoids and carbohydrate contribution to organic matter content. Our results suggest a potential of the (old) leaves of *H. stipulacea* as skin-protective and/or oxidative stress damage repair agents. This study paves the way to transmute the invasive *H. stipulacea* environmental threat in goods for human health.

Introduction

Among the injuries relied on the production of reactive oxygen species (ROS) in humans, chronic exposure of human skin to pro-oxidant agents is a critical factor causing several deleterious short and long-term damages, e.g. photo-aging (Holick 2016). Antioxidants have a key role in counteracting free radicals, preventing or limiting damages in living organisms (Pandel et al. 2013). Exogeneous antioxidants are requested to enhance the protection or repairing processes, with a special regard on natural antioxidative compounds, generally more active (Schieber and Chandel 2014) and eco-friendly produced. Photosynthetic organisms represent the most important source of natural products, providing health benefits (McChesney et al. 2007), with plant-derived compounds used in clinical practise as anticancer, anti-inflammatory, antibiotic, or other medicinal drugs (McChesney et al. 2007; Cragg et al. 2009). Despite the high genetic and phenotypic diversity of algae and seagrasses, and their increasing biotechnological interests (Zidorn 2016; Galasso et al. 2018; Rocha et al. 2018; Sansone et al. 2018), marine photosynthetic organisms are less exploited compared to terrestrial ones. Conversely to terrestrial plants and marine algae (Thomas et al. 2013), the bioactive profile of seagrasses is still almost unknown; recent studies demonstrated antitumor activity, increase of fibroblast growth rate and collagen production of ethanolic extracts of *Posidonia oceanica* leaves (Cornara et al. 2018; Leri et al. 2018).

It is not conceivable using living seagrasses/plants harvested from their habitats as a biotechnological resource, but in case of invasive species the paradigm becomes not even more true.

Halophila stipulacea (Forsskål and Niebuhr) Ascherson (order Alismatales, family Hydrocharitaceae) is a marine seagrass, increasingly invasive in the Mediterranean where it replaced *Cymodocea nodosa*

(Sghaier et al. 2011), in parallel with the ongoing “tropicalization” of the Mediterranean basin (Borghini et al. 2014). It is noteworthy that *H. stipulacea* was included in the “100 Worst Invasive Alien Species in the Mediterranean” (Lowe et al. 2000). In the Mediterranean, *H. stipulacea* populations vary (Chiquillo et al. 2019), with meadows shrinking in winter, and expanding in summer (Manh Nguyen et al. 2020; Winters et al. 2020).

The potential bioactivity and biological properties of *Halophila stipulacea* has been reported (Mabrouk et al. 2020). Organic extracts of *H. stipulacea* obtained from leaves and stems were evaluated on human cell lines as antiobesity agent and on microfouling and macrofouling organisms as antifouling product (Mabrouk et al. 2020). Also, ethanolic leaf extracts have shown antioxidant activity as well as antibacterial activities (Kannan Rengasamy et al. 2012).

This study aims to investigate the potential biotechnological interest of the invasive *H. stipulacea*. *Halophila stipulacea* was sampled in the Sicily coast (Mediterranean Sea) while the target human cell line was the WI38 lung epithelial cells. This cell line representing a well-established experimental tool since they have a limited replicative potential, which brings to cease of *in vitro* growth in short time (Finkel and Holbrook 2000; Toussaint et al. 2000; Seo et al. 2016), is frequently used as an *in vitro* model for studying toxicity, oxidative stress and aging-related disorders (Wolf et al. 2002).

In this study, we determined the bioactive interest of *H. stipulacea* as antioxidant and in scavenging and/or repairing oxidative stress in the human cell line, using biochemical and molecular approaches. Macromolecular composition and pigments content of the extract were also characterized in both old and young leaves of *H. stipulacea*. It is noteworthy that the antioxidant activity of plant extracts might increase from early vegetative stage to maturation, as it occurs in fruits (Lisiewska et al. 2006; Habermann et al. 2016; Salami et al. 2017), in relation with the changes in secondary metabolites production during aging (Figueiredo et al. 2008). Indeed, old leaves displayed a significantly higher antioxidant capacity and ability to scavenge and repair epithelial cells from oxidative stress, probably at least in part relied to the much higher content of carotenoids, mainly xanthophylls known as photoprotective and/or antioxidant compounds in photosynthetic organisms. These results are discussed for their biotechnological meaning and in the optics of transmuting an environmental threat in a societal good. The exploitation of *H. stipulacea* as a source of wellness, however, still requires technological development and strategy for the control and removal of this invasive species (Hyytiäinen et al. 2013; van Tussenbroek et al. 2016).

Materials And Methods

Halophila stipulacea collection

Rhizomes of *H. stipulacea* were collected in July 2016 in the harbour of Castellammare del Golfo in the Mediterranean Sea (38°01'45.2"N, 12°52'51.8"E) at 0.5 m depth. The seagrass formed a dense patch of c.a. 1.5 m diameter on muddy sand bottom, exposed to direct sunlight, in an area characterised by urban

run-off. After collection, the fronds were gently rinsed in seawater to remove sediment particles and associated epibionts and transported in seawater tanks to the laboratory for the subsequent analyses.

Preparation of ethanol/water extracts from *Halophila stipulacea* leaves

Extracts for pigment analysis and biological assays were obtained from freeze-dried young and old leaves of *H. stipulacea*, according to Sansone et al. (Sansone et al. 2017) at room temperature, under dark and N₂ atmosphere to avoid oxidation during procedure. One g of leaves was placed in 1 ml of ethanol/water mixture (3/1; v/v) for 30 min in dark condition and mechanically disrupted with a pestle. The mixture was separated by centrifugation at 4500 *g* for 15 min, at 4°C, and the upper layer was transferred to a clean tube. Pellets were resuspended in 1 ml of ethanol/water mixture to repeat the procedure, maximising the extraction yield. Hydroalcoholic extracts were dried in a rotary vacuum evaporator (Buchi rotavapor R-114) and the dried extracts were stored at -20°C.

Pigment HPLC analysis

Pigment analysis was conducted by High Performance Liquid Chromatography (HPLC), according to method described in Smerilli et al. (Smerilli et al. 2019). Prior to injection into the HPLC, 250 µl of an Ion Pairing Agent (ammonium acetate 1 mol l⁻¹, final concentration of 0.33 mol l⁻¹) was added to 0.5 ml of the lyophilised extracts and incubated for 5 min in the dark at 4°C. These samples were then injected in the 50 µl loop of the Hewlett Packard series 1100 HPLC (Hewlett Packard, Wilmington, NC, USA), equipped with a reversed-phase column (C8 Kinetex column; 50 mm × 4.6 mm; 2.6 µm particle size, Phenomenex®, USA). The temperature of the column was steadily maintained at 20°C and the flow rate of the mobile phase was set up at 1.7 ml min⁻¹. The mobile phase was composed of two solvent mixtures: “A” - methanol/aqueous ammonium acetate (70/30, v/v); “B” - methanol. During the elution (12 min), a gradient between the solvents was programmed: 75% A (0 min), 50% A (1 min), 0% A (8 min) isocratic for 3 min. Chlorophylls and carotenoids were detected by diode-array spectroscopy (spectrum data collected in the range 350 - 750 nm) using a Hewlett Packard photodiode array detector, model DAD series 1100 and absorbance chromatogram was reported at 440 nm. Chlorophylls, and their degradation products were also detected by fluorescence using a Hewlett Packard standard FLD cell series 1100 with excitation and emission wavelengths set at 407 nm and 665 nm, respectively. Identification and quantification of pigments were carried out using pigment standards from the D.H.I. Water & Environment (Horsholm, Denmark).

Biochemical characterization of lipids, proteins and carbohydrates

The concentration of proteins, carbohydrates and lipids was determined on young and old leaves, previously freeze dried. Total protein concentrations were determined according to Lowry et al. (LOWRY et al. 1951) as modified by Hartree (Hartree 1972) and Rice (Rice 1982) to compensate for phenol interference and are expressed as bovine serum albumin (BSA) equivalents. Total carbohydrate concentrations, expressed in glucose equivalents, were obtained according to the Gerchakov and Hatcher (Gerchakov and Hatcher 1972) protocol based on the phenol and concentrated sulfuric acid reaction with

saccharides. Total lipids were extracted in chloroform: methanol (1:1, vol:vol) (Bligh and Dyer 1959), and the resulting fraction, after evaporation in a dry hot bath at 80 to 100°C for 20 min, was quantified according to the sulfuric acid carbonisation procedure (Marsh and Weinstein 1966). Total lipids were expressed in tripalmitine equivalents. Protein, carbohydrate and lipid concentrations were expressed as mg g⁻¹ of dry weight.

Scavenging activity against DPPH radical

2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) was used for the radical scavenger assay (Sigma Aldrich, cat. 257621). Various concentrations of extract from *H. stipulacea* were mixed with a final concentration of DPPH of 0.1 mM in methanol, allowed to react for 30 min in the dark, and absorbance was measured at 517 nm using a microplate reader

Cell culture and treatments of human cells

WI-38 cell line was purchased from the American Type Culture Collection (ATCC® CCL-75™) and grown in MEM (Eagle's minimal essential medium) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units ml⁻¹ penicillin, 100 units ml⁻¹ streptomycin, 2mM of L-glutamine and non-essential amino acids (NEAA, 2 mM) in a 5% CO₂ atmosphere at 37°C. WI-38 cells (2 x 10³ cells well⁻¹) were seeded in a 96-well plates and kept overnight for attachment. The two ethanol/water dried extracts, dissolved in dimethyl sulfoxide (DMSO, final concentration 0.5 %), was used for cell treatments. Seventy percent confluent cells were treated with the two ethanol/water extracts (young and old leaves) at 1, 10 and 100 µg ml⁻¹ for 48 hours, in order to assess cytotoxicity. In addition, cells were pre-treated with the two extracts at 1, 10 and 100 µg ml⁻¹ for 2 hours and then injured with 10 mM of H₂O₂ for 48 hours. This experiment can reveal the potential scavenging activity of samples tested; positive control was obtained injuring cells with only 10 mM of H₂O₂ for 48 hours. Moreover, WI-38 cells were pre-injured for 2 hours with 10 mM of H₂O₂ and then recovered with the extracts at 1, 10 and 100 µg ml⁻¹ for 48 hours. This experimental approach examines the potential repair activity of samples after cell oxidative damage; positive control was obtained pre-injuring cells for 2 hours with 10 mM of H₂O₂ and then recovered with only culture medium.

Viability assays

Cytotoxicity, scavenging and repair activity of the ethanol/water extracts were tested using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Applichem) on the WI-38 cell line (Mosmann 1983). After 48 hours of treatment, WI-38 cells were incubated with 10 µl (5 mg ml⁻¹) of MTT and incubated for 3 hours at 37°C with 5% CO₂. The resulting formazan crystals produced by only viable cells were dissolved with 100 µl of isopropyl alcohol. The absorbance was recorded on a microplate reader at a wavelength of 570 nm. The effects of the extracts were evaluated as percent of cell viability estimated as the ratio between mean absorbance of each sample and mean absorbance of control (untreated cells).

RNA extraction and Real-Time qPCR

WI-38 cells (2×10^6 cells), used for RNA extraction and analysis, were seeded in Petri dishes (100 mm diameter) to obtain several types of samples. For gene expression studies, we chose the most active concentration ($10 \mu\text{g ml}^{-1}$) of old leaf extract that showed strong repair and scavenging activities, without any cytotoxic effect, and 10 mM H_2O_2 as cellular injury. Controls encompassed negative (cells without any treatments, collected after 3 hours) and positive ones (cells treated with H_2O_2 for 1 hour and then with fresh medium for 2 hours). The three types of analysed samples correspond to cells treated with the leaf extract (3 hours); cells treated with the leaf extract (2 hours) and then injured with H_2O_2 (1 hour) and cells injured with H_2O_2 (1 hour) and recovered with the leaf extract (2 hours). Gene expression was analysed after 3 hours of treatment to observe activation of detoxification and cell repair pathways occurring after this time. Once the treatment was over, WI-38 cells were washed in the Petri dish by adding cold Phosphate-Buffered Saline (PBS) and rocking gently. Cells were lysed in the Petri dish by adding 1 ml of Trisure Reagent (Bioline) and RNA was isolated according to the manufacturer's protocol. RNA concentration and purity were assessed using the nanophotometer Nanodrop. The reverse transcription reaction was carried out with the RT² first strand kit (Qiagen), and Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) was performed using the RT² Profiler PCR Arrays kit (Cat. No. PAHS-065Y, Qiagen). Plates were run on a ViiA7, Standard Fast PCR Cycling protocol with 10 μl reaction volumes. Cycling conditions were 1 cycle initiation at 95°C for 10 min followed by amplification for 40 cycles at 95°C for 15 s and 60°C for 1 min. Amplification data were collected via ViiA 7 RUO Software (Applied Biosystems). The cycle threshold (Ct)-values were analysed with PCR array data analysis online software (GeneGlobe Data Analysis Center <http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>, Qiagen). Real time data were expressed as fold expression, describing the changes in gene expression between treated cells and untreated cells (control). The expression of 84 genes involved in oxidative stress was analysed (Table S1). Control genes for real-time qPCR were actin-beta (ACTB), beta-2-microglobulin (B2M), hypoxanthine phosphoribosyl transferase (HPRT1) and ribosomal protein large P0 (RPLP0), the expression of which remained constant.

Results

Pigments

The main pigments found in *H. stipulacea* belong into the two photoprotective cycles, violaxanthin, antheraxanthin and zeaxanthin (xanthophyll cycle), and the siphonein-siphonaxanthin cycle, together with other carotenoids neoxanthin, lutein, α - and β -carotenes and the chlorophylls *a* and *b* (chl. *a* and chl. *b*). Pigment composition was identical in old and young leaves, but their concentration significantly varied with leaf aging (Fig. 1A). Indeed, the concentrations of xanthophyll-cycling pigments (sum of violaxanthin, antheraxanthin and zeaxanthin), as well as the sum of siphonein and siphonaxanthin pigments, were significantly higher in old leaves than in the young ones ($p < 0.001$). The sum of α - and β -carotenes was similar in the two types of leaf (Fig. 1A), while chl. *a* concentration was significantly higher in young than in old leaves ($p < 0.01$).

The ratio between accessory pigments and chl.*a* was used as an index of change in photosystems' structure in old *vs* young leaves (Fig. 1B). Except for chlorophyll.*b* and the α - and β -carotenes, all the accessory pigments *vs* chl. *a* ratios were significantly higher in old leaves compared to young ones ($p<0.001$). Among the xanthophyll-cycling pigments, the two photoprotective antheraxanthin and zeaxanthin displayed the greatest increase with leaf aging (Fig. 1B). In synthesis, carotenoids with known role in photoprotection and/or protection against reactive oxygen species enhanced with aging (Fig. 1 A, B).

Macromolecular composition

Protein, carbohydrate and lipid concentrations in young leaves were significantly higher than in old ones ($p<0.05$; Table 1). In particular, protein concentrations decreased from 21.3 ± 0.5 to 8.2 ± 0.6 , carbohydrates from 19 ± 0.6 to 10.9 ± 1.1 and lipids from 5.8 ± 0.1 to 1.6 ± 0.3 mg·g⁻¹ in young and old leaves, respectively (Table 1).

In young leaves, proteins and carbohydrates represented the main macromolecular components, contributing for 46.2 % and 41.2 % to the total organic matter pool, respectively, followed by lipids (12.6 %). Conversely, in old leaves carbohydrates represented the dominant component (accounting for 52.6% of the total organic matter pool) followed by proteins and lipids (39.6 % and 7.8 %, respectively).

Table 1 Macromolecular composition. Protein, carbohydrate and lipid concentrations (mg·g⁻¹ dry leaf) of young and old leaves of *Halophila stipulacea*. Values are reported as mean $\pm$ S.D.

	Proteins	Carbohydrates	Lipids
Young leaves	21.3±0.5	19.0±0.6	5.8±0.1
Old leaves	8.2±0.6	10.9±1.1	1.6±0.3

Antioxidant activity

Old leaf extracts provided a significantly ($p<0.001$) much higher antioxidant power than young leaves (Fig. 2). Indeed, at the two low concentrations, young leaf extracts did not exert any antioxidant function, while old leaf extract was already bioactive.

Cytotoxicity, scavenging and repair effects of the leaf extracts

The old leaf extract did not induce any cytotoxicity on WI-38 cells for none of the three tested concentrations (Fig. 3A). Conversely, the young leaf extract displayed a slight cytotoxicity on WI-38 cells (Fig. 3A), with a cell viability decreasing from 83% (at 1 μ g ml⁻¹) to 67% (at 100 μ g ml⁻¹).

For the scavenging assay, positive control, corresponding to WI-38 cells injured by 10 mM of H₂O₂ for 48 hours, resulted in very low cell viability (38%). When WI-38 cells were pre-treated with young leaf extract

before H₂O₂ injury, cell viability enhanced up to 64%, 71% and 81% at 1, 10 and 100 µg ml⁻¹, respectively (Fig. 3B). Using old leaf extract as pre-treatment, a further enhancement of cell viability was noticed up to 82%, 96% and 113% at 1, 10 and 100 µg ml⁻¹, respectively (Fig. 3B).

For the repair assay, the positive control represented by WI-38 cells injured with 10 mM of H₂O₂ for 1 hour, displayed 52% of viable cells. Recovery treatment using 1, 10 and 100 µg ml⁻¹ of young leaf extract increased cell viability up to 75%, 79% and 86%, respectively (Fig. 3C). Using the old leaf extract, cell viability became 62%, 94% and 108%, respectively (Fig. 3C).

Oxidative stress gene expression in WI-38 cells treated with old leaf extracts

WI-38 cells treated with old leaf extract upregulated MBL2 and MPO (17.1 and 31.1 in fold regulation, respectively) and downregulated EPHX2 and GSR (-18.2 and -17.1 in fold regulation, respectively) (Tables S1, S2 and Fig. 4). Conversely, the WI-38 cells pre-treated with old leaf extract, before the prooxidant treatment with 10 mM of H₂O₂ induced an upregulation of all genes involved in antioxidant activity analysed (e.g., CCL5, GPX5, GSR, KRT1, LPO, MBL2, MPO, MT3, NOX5, TPO), with the exception of EPHX2 (-9.9 in fold regulation, Fig. 4, Tables S1, S2).

The prooxidant treatment alone of the WI-38 cell line with 10 mM of H₂O₂ induced an upregulation of the genes MBL2, MPO and SPINK1 (7.0, 8.8 and 33.7 in fold regulation, respectively) and the downregulation of the GSR gene (-2.7 in fold regulation) (Tables S1, S2 and Fig. 4).

Recovery treatment with old leaf extract after the injury with H₂O₂ revealed by the upregulation of all genes analysed (e.g., CCL5, EPHX2, GPX5, KRT1, LPO, MBL2, MT3, NOX5, TPO) with the exception of the GSR gene (Fig. 4 and Tables S1, S2).

Discussion

The seagrass *H. stipulacea* was selected as target in our study due to its capacity to thrive in a wide range of environmental conditions and to out-compete native species in the Mediterranean Sea, where it is invasive (Beer and Waisel 1982; Schwarz and Hellblom 2002; Sharon et al. 2009; Mejia et al. 2016). Indeed, opportunism and/or competitiveness is linked to a high ability in coping with environmental variations to expand their ecological niche, profiting of new favourable environmental conditions, probably based on physiological and biochemical plasticity for maintaining high growth rate. This implies relevant biochemical energy conveyed towards growth as well as efficient processes to protect cells and repair damages in case of non-optimal environmental conditions. Those characteristics are putative promoters for biotechnological investigations looking for active natural compounds/extracts for human health benefits, such as antioxidants.

Indeed, results obtained in this study highlight the high potential of the seagrass species *H. stipulacea* as source of bioactive extracts able to counteract and repair human epithelial cells from induced-oxidative stress. While young leaf extracts are not attractive, old leaf ones are, conversely, biotechnologically

appealing. Organismal ageing induces a senescence process that in turn accentuates the modulation of physiology, biochemistry and functioning of biological machinery (Pushpa Bharathi et al. 2016). Senescence is regulated by many processes (Pushpa Bharathi et al. 2016) and by ROS, which act as signalling for production of secondary metabolites involved in several defence and protective mechanisms (Wingler et al. 2006, 2009). Even though concentration of carotenoids might remain stable during ageing/senescence in some plants (Van Doorn 2008), growth stage of a photosynthetic organism notably affects the bioactive compounds content and antioxidant activity often increasing in aged biological material (Raniello et al. 2004, 2006; Sánchez-Quiles and Tovar-Sánchez 2014; Sugawara et al. 2014; Uragami et al. 2014).

In *H. stipulacea* the enhancement of antioxidant ability with leaf aging is paralleled an almost all carotenoids increase and with the decrease in chl.*a* content. The decrease in the chlorophyll concentration in old leaves, as already observed (Socaci et al. 2017) might induce a lowering of light energy transfer in the cells, that in turn would explain the lower organic matter content in aged leaves compared to young leaves. The higher contribution of carbohydrates into the organic matter content is a common feature in plants during ageing, potentially promoting or triggering senescence (Drew 1978; Pourtau et al. 2006; Gullón et al. 2018; Santhanam et al. 2018; Woo et al. 2018).

The significant increase in carotenoids with age might – at least in part -explain the enhanced antioxidant power of the extracts. Indeed, carotenoids, such as violaxanthin, neoxanthin, antheraxanthin, zeaxanthin, lutein are epidemiologically correlated with a lower risk for several diseases and to have beneficial effects on eye health (Stahl and Sies 2005; Ma and Lin 2010; Galasso et al. 2017). Additionally, *H. stipulacea* contains the keto-carotenoids siphonein and siphonaxanthin, exclusively present in aquatic photosynthetic organisms, as already found in green macroalgae such as *Codium fragile*, *Caulerpa lentillifera* and *Umbraulva japonica* (Ricketts 1971). These pigments fill the role of maximizing the absorption of the green and blue-green wavelengths (Raniello et al. 2004). Moreover, they are known to act as defence against light stress (Raniello et al. 2004), being active in photoprotection processes (Raniello et al. 2006; Uragami et al. 2014). The two carotenoids siphonein and siphonaxanthin have been reported as bioactive compounds (Ganesan et al. 2010, 2011). Siphonoxathin is also known to induce apoptosis in leukemia cells (Ganesan et al. 2011), with additional anti-angiogenic or anti-diabetic role (Ganesan et al. 2010; Li et al. 2015).

The bioactivity induced on human fibroblast cells by the extracts involves a molecular response in cells towards the defence and detoxification from peroxidative cell damages. Among the targeted genes, CCL5, LPO, KRT1, LPO, MBL2, MT3, NOX5 and TPO are known to activate intracellular detoxification pathways in response to oxidative stress, with the involvement of the peroxisome's machinery (Izumi and Ishida 2011). Yet, the activation of GPX5 is responsible for the protection from lipid oxidation and DNA mutation induced by high level of intracellular ROS (Jajic et al. 2015).

All these results confirm the pro-active effect of the *H. stipulacea* extracts against damaged epithelial human cells or protecting them, thanks to an induced-gene activation cascading effect. Advanced

chemical investigations might further help in relating the displayed bioactivity to the compounds' diversity harvested in the extract, even though the role of carotenoids – in terms of quantity and diversity, the latter enhancing the bioactive ability of the extract – is well-known. The high content and diversity in carotenoids, mainly those involved in photoprotective processes, is also promising for developing further studies investigating the role of these extracts on human skin cell line to counteract UV damages.

Conclusion

Our study lays the foundation for a potential biotechnological future of the invasive seagrass *H. stipulacea* that might fill the role of natural provider of antioxidant compounds acting against oxidative stress in human epithelial cells. This species being a threat for coastal marine ecosystems due to its invasiveness might represent a novel valuable source of human health benefits natural products. It is important to consider that such opportunity, even if not unique, is very rare, since it is not thinkable to harvest from the sea species for biotechnological outputs without causing detrimental effects to the environment. In this case, a careful harvesting of *H. stipulacea* from nature could bring up a synergy between the commercial exploitation for human wellbeing of the invader in areas where the species is not native and benefits for the conservation of the natural capital and ecosystem health.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors agreed with this publication

Availability of data and materials

The datasets generated for this study are available on request to the corresponding author

Competing interests

There are no competing interests

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Author Contributions

C.S., L.M. and T.V.F. conceived the experiments. C.S. and C.B. designed the experiments. L.M. and TVF sampled *H. stipulacea*. C.S. and C.G. carried out the experiments with cell culture and real-time-PCR. M.L.M. and A.d.A. performed the biochemical composition analysis. C.B. analyzed the HPLC results. C.S., C.G., A.d.A., C.B., A.B., D.M.N. and A.A. analyzed the data. All authors wrote the paper.

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Figures

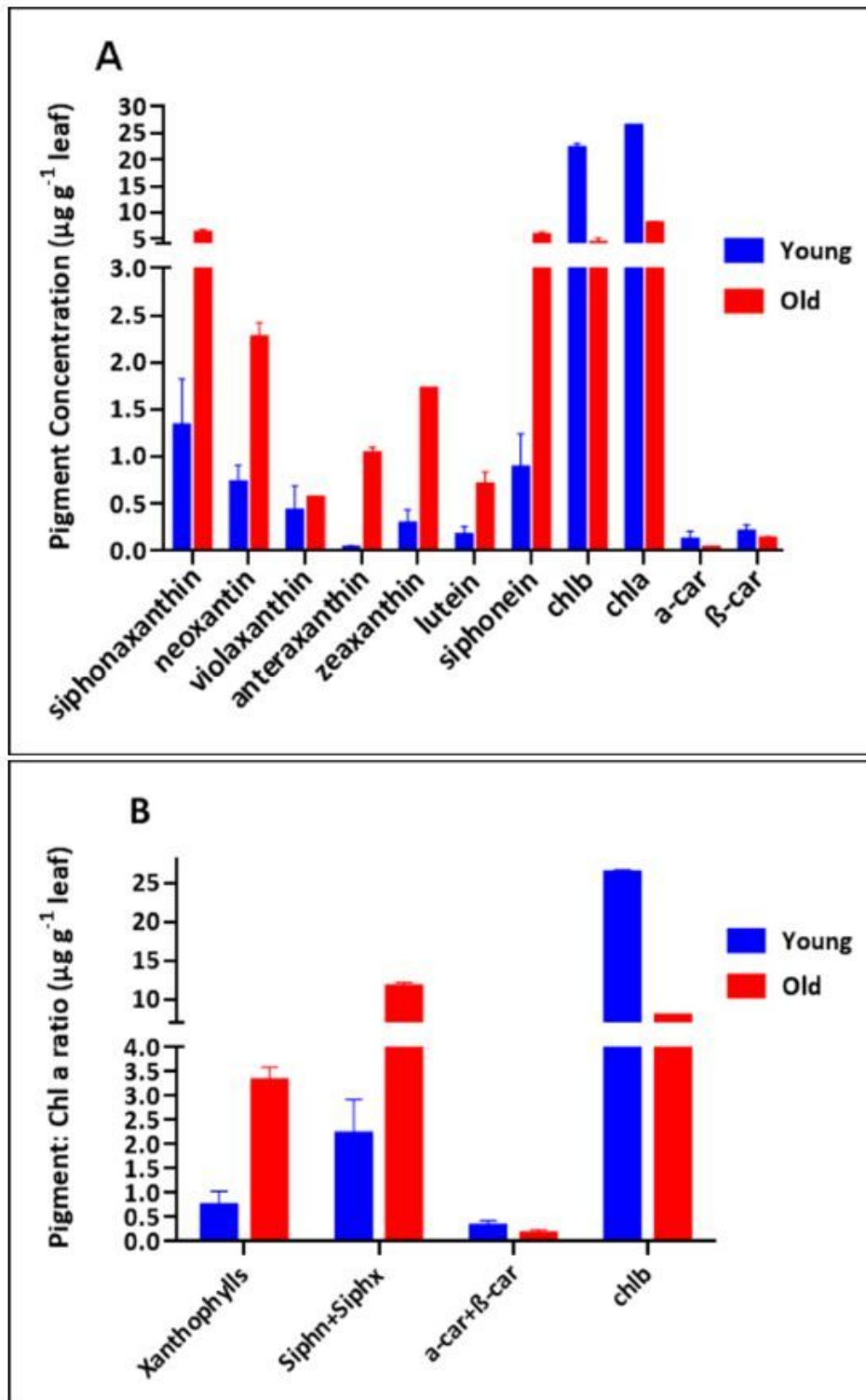


Figure 1

Pigment analysis. (A) Concentrations ($\mu\text{g.g}^{-1}$ of wet leaf) of chlorophylls a and b, the xanthophyll cycling pigments (violaxanthin, antheraxanthin and zeaxanthin), neoxanthin, lutein, siphonein, siphonaxanthin, α - and β -carotenes. (B) Ratio of the xanthophyll cycling pigments (violaxanthin+antheraxanthin+zeaxanthin), the sum of siphonein+siphonaxanthin and α + β -carotenes vs

chlorophyll a ($\mu\text{g}.\mu\text{g}^{-1}$) in young and old leaves of *H. stipulacea*. Values are reported as means of triplicates with standard deviation.

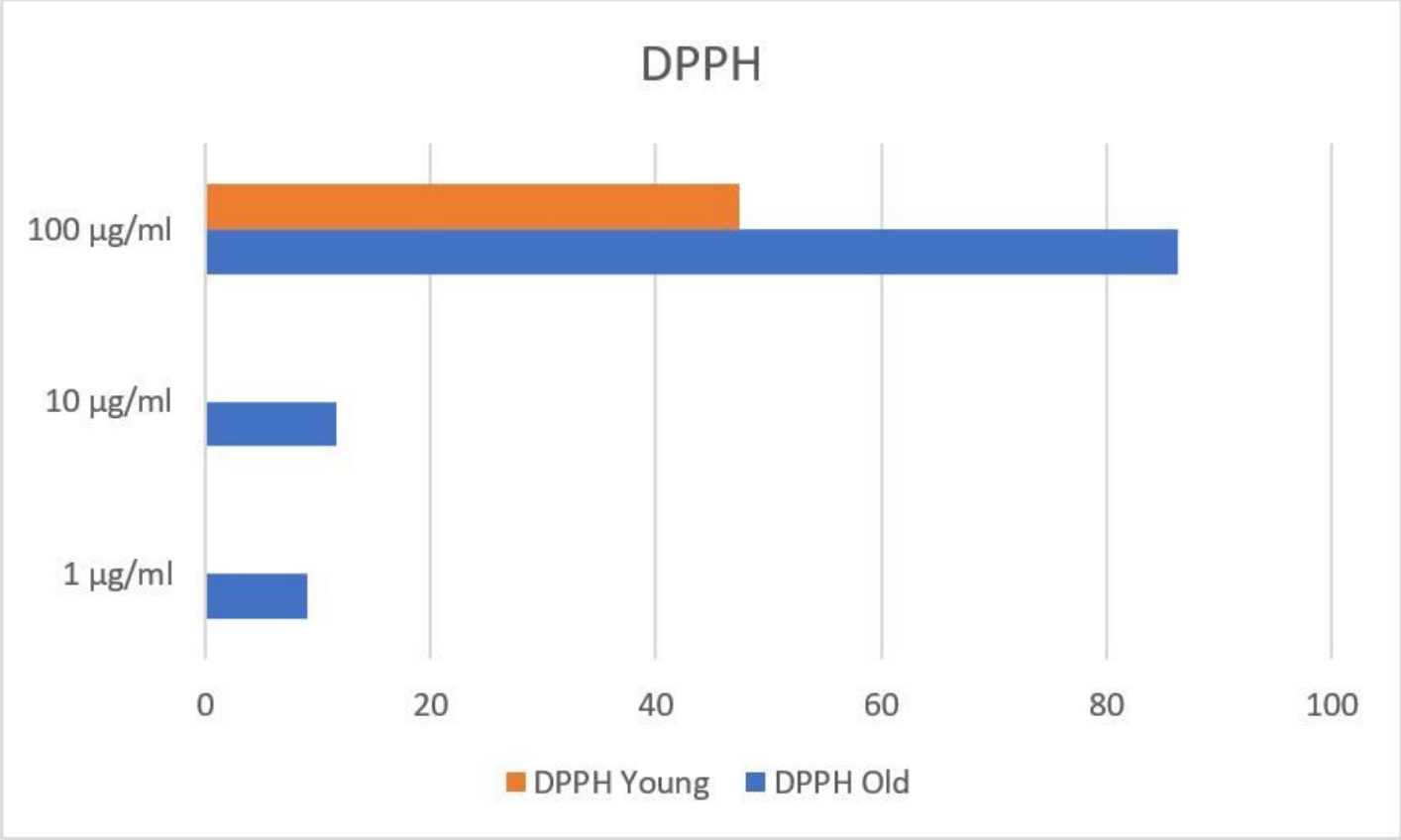


Figure 2
Antioxidant capacity of the leaf extracts of *H. stipulacea*.

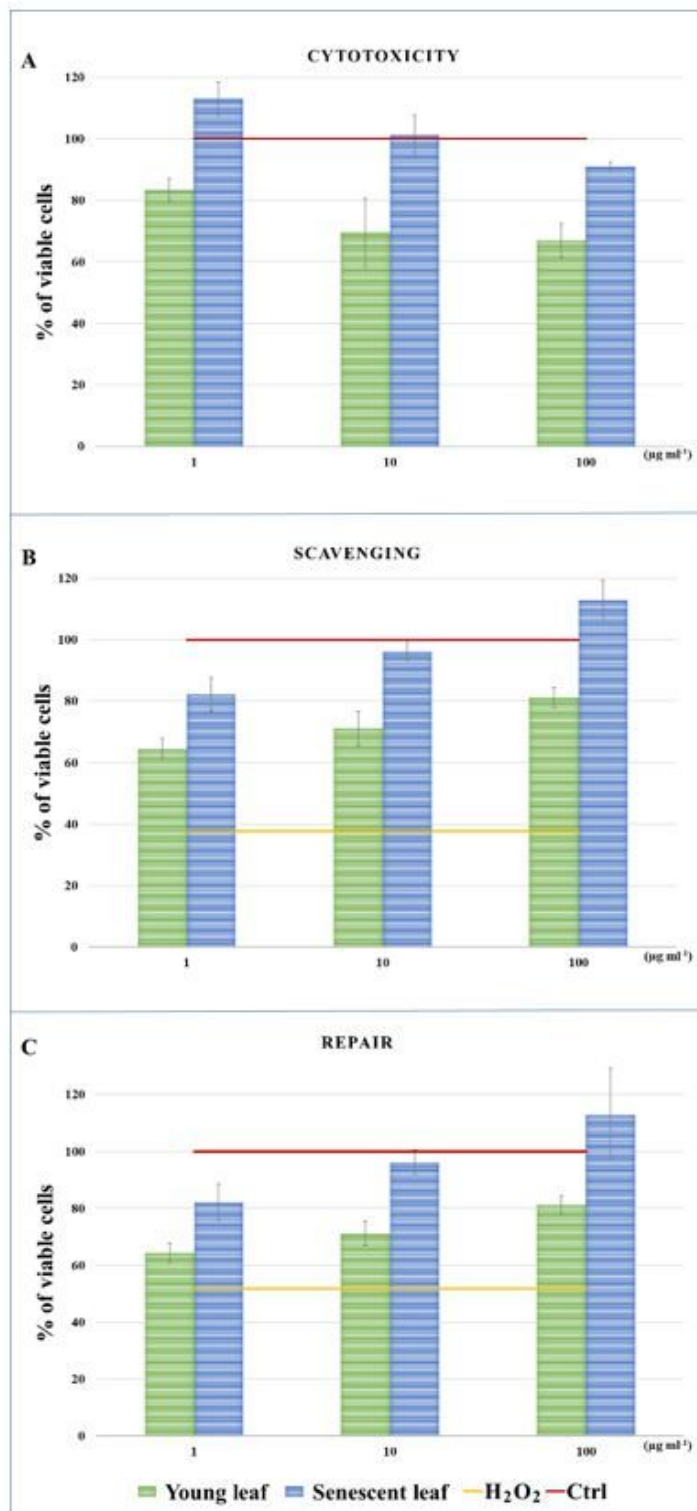


Figure 3

Cytotoxicity (A), scavenging (B) and repair activity (C) of ethanol/water extracts (of young and old leaf) of *Halophila stipulacea* on human normal lung fibroblasts (WI-38). (A) WI-38 cells treated with 1, 10 and 100 µg ml⁻¹ of the two extracts for 48 hours. (B) WI-38 cells pre-treated with 1, 10 and 100 µg ml⁻¹ of the two extracts and, after 2 hours, injured with 10 mM of H₂O₂ for 48 hours. (C) WI-38 cells pre-injured with 10 mM of H₂O₂ for 2 hours and then recovered with 1, 10 and 100 µg ml⁻¹ of the two extracts for 48

hours. Yellow and red lines represent the positive and negative controls, respectively. Data are shown as mean $\pm$ S.D.

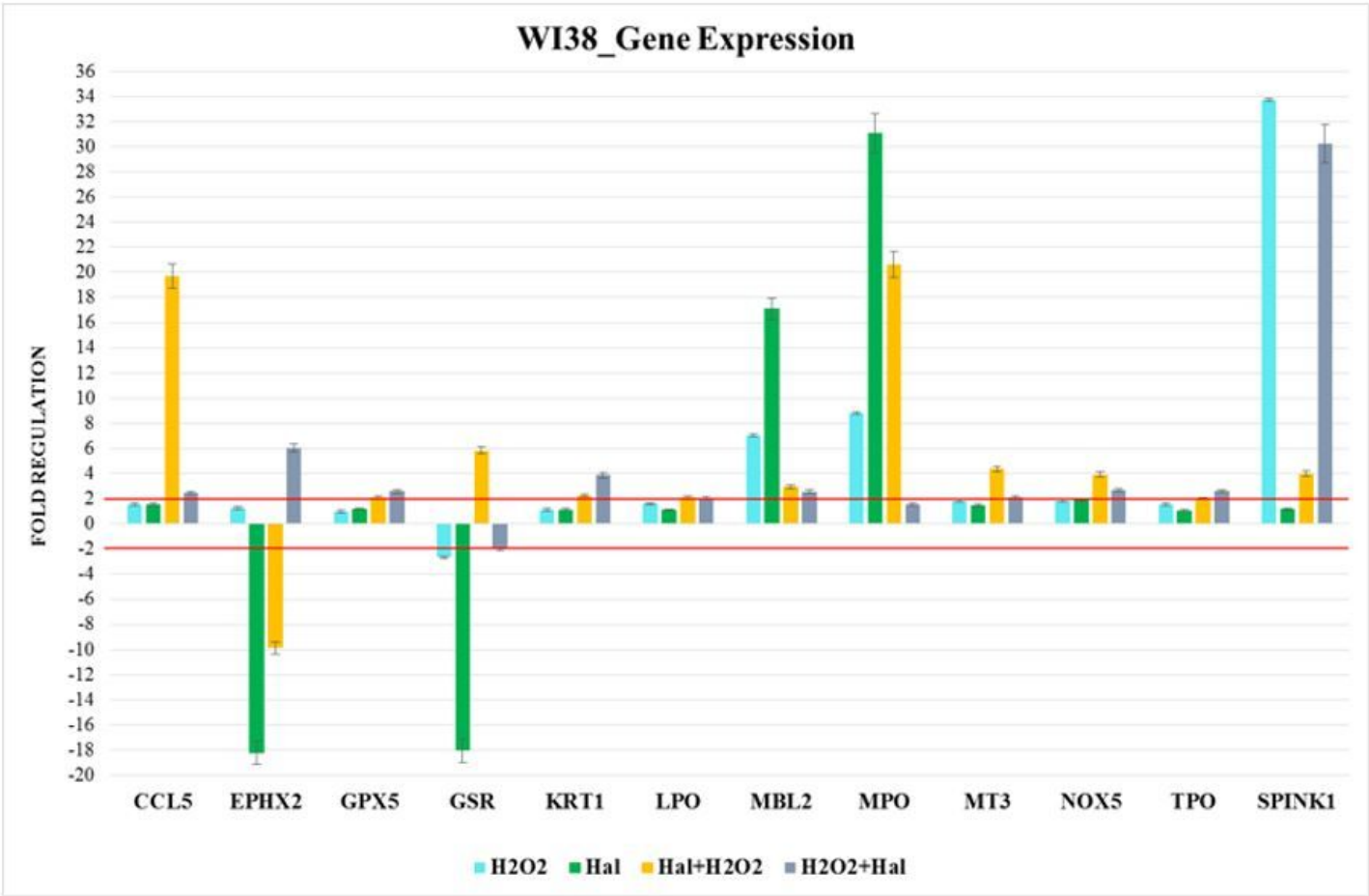


Figure 4

Effect of the old leaf extract (10 μ g ml⁻¹) on oxidative stress gene expression in H₂O₂-treated human normal lung fibroblasts (WI-38). Gene expression values correspond to: WI-38 treated with only H₂O₂ (blue bars), with only the extract (green bars), with the extract for 2 hours and then injured with H₂O₂ for another hour (yellow bars), with H₂O₂ for 1 hour and then recovered with the extract for 2 hours (grey bars). Three independent assays were performed in technical triplicate. Expression values greater or lower than a two-fold difference with respect to the controls were considered significant (red line indicates the $\pm$ 2-fold differences with respect to controls).

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