Phytochemical analysis, *in vitro* evaluation of antioxidant and antimicrobial activities of phenolic extracts from *Posidonia oceanica* (L.) Delile leaves

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ABSTRACT

Posidonia oceanica is one of the few marine higher plants endemic to the Mediterranean Sea, considered as an indicator of environment quality in coastal areas. Total phenolic, flavonoid and tannin contents were determined in FP (soluble free phenolic) and BP (insoluble-bound phenolic) extracts obtained from Posidonia oceanica intermediate leaves. Higher phenolic content was obtained in FP (27.71 ± 2.4 GAE/g DW), but higher flavonoid content and tannin contents were detected in BP (19.16 ± 1.8 mg QE/g DW, and 3.34 ± 0.5 mg CE/g DW, respectively). Identification and quantification of phenolic compounds were carried out using RP-HPLC analysis. Protocatechuic, ferulic acids and catechin were the major compounds (constituting 90%) of the identified compounds in FP extract, while in BP extract, quercetin was the main flavonol aglycone (about 95%). Higher DPPH scavenging activity (IC_{50}, 2.5 mg/ml), was measured for BP extract. The study of the antimicrobial activity of FP extract showed that it exhibited potentiating activity of some antibiotics. These findings suggested that Posidonia oceanica could be considered as a natural source for pharmacological products.

Keywords: *Posidonia oceanica*, seagrasses, phenolic compounds, Mediterranean Sea, Phenolic compounds

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INTRODUCTION

Posidonia oceanica (L.) Delile, is a seagrass member of the only group of higher plants living in marine habitats [1]. It is a phanerogam endemic to the Mediterranean Sea, where it forms vast space from the water surface to the depths, up to 40 m, serving as a bio-indicator of the coastal ecosystem stress, like anthropogenic pressures and inter-specific competition, due to its cumulative responses to the environmental changes [2].

Its wide spread surfaces serve as hatcheries and nursery beds for many populations [3,4]. Many human activities are regularly carried out in marine environments, to take advantage of the several benefits provided by these ecosystems [5]. Some studies have dealt with the human impacts, affecting *Posidonia* populations and the consequences depending on it [6].

A number of phytochemical investigations with different focuses have been carried out on *P.oceanica*, their results have been summarized and appraised [7]. The aim of this study is to analyze major metabolites (phenolic and flavonoidal compounds) of the sea-shore collected *P.oceanica* leaves using HPLC analysis, and to determine its

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total phenolics, flavonoids and tannins contents, evaluate its antioxidant and antimicrobial activities in order to determine whether this seagrass can be made used of, as a bioactive source material.

Due to the fact that antibiotics are losing their efficacy facing multidrug resistant bacteria, and considering the vast majority of therapeutic applications from plants, we aimed to evaluate *P. oceanica* for its antibacterial activity against *Staphylococcus aureus* strains, a major pathogen in clinical setting, including efflux pump overexpressing and MRSA strains. Additional studies of synergism between this plant extract and several antibiotics were performed in the quest for new effective co-therapies against this pathogen.

EXPERIMENTAL SECTION

Plant material
P.oceanica leaf shoots were collected at random in early September, off the seashores of North Coast of Egypt. Intermediate, intact leaves or blades measuring 10-15 cm were studied, as they contain more phenolic compounds than sheaths [8]. The plant was authenticated and a voucher specimen was kept in the National Research Center Herbarium under the No.344.

Extraction
Extract of soluble free phenolics (FP) used here was derived from that adapted to marine phanerogams [9]. The plant dry leaves were homogenized in 50% aqueous EtOH in darkness (40°C). The homogenate was filtered, and then slightly adjusted to pH 2. The ethanol was evaporated under reduced pressure, and then the aqueous phase was extracted with EtOAc. The extract was dried using anhydrous Na_2So_4, filtered, then evaporated. The dry residue was re-dissolved in MeOH, ready for quantitative estimation of the total phenolic, flavonoid, tannin contents, and HPLC analysis for extract investigation.

Extract of bound phenolics (BP) was based on the method of Kaundun *et al.* [10]. The residue after the ethanol extraction was suspended in 100 ml of 2M HCl. The solution was heated to 80°C in a water bath with reflux for 90 min. It was left to cool, filtered, and then flavonol aglycones were extracted from the solution by shaking twice with diethyl ether. The ether was evaporated and the residue was re-dissolved in MeOH before HPLC analysis.

Determination of total phenolic content (TPC)
The Folin–Ciocalteu method was used to determine total phenolic content (TPC) [11]. An aliquot (150μl) of 100 μg/ml of each of FP and BP was separately added to 0.5 ml of distilled water and 125 μl of the Folin-Ciocalteu reagent. The mixture was then shaken and allowed to stand for 6 min, prior to adding 1.25 ml of Na_2CO_3 (7%). The solution was then adjusted with distilled water to reach a final volume of 3 ml, mixed thoroughly, and then held in darkness for 90 min at ambient temperature. After incubation, the absorbance was measured at 760 nm. The TPC was determined in triplicate, and gallic acid (Sigma–Aldrich Chemicals Co., St. Louis, MO, USA) was used as a standard (from 50 to 250 mg/L) to produce the calibration curve. The results were expressed as gallic acid equivalents mg/ g of dry plant weight.

Determination of total flavonoid content (TFC)
The total flavonoid content (TFC) was determined according to a colorimetric assay [12]. It is based on the formation of a complex flavonoid –aluminium having the absorptivity maximum at 510 nm, and using a calibration curve with quercetin (Sigma–Aldrich Chemicals Co., St. Louis, MO, USA) as standard. 1ml of diluted sample (FP and BP, separately) was mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixtures was measured. Results were taken in triplicate and expressed as quercetin equivalents mg/g of dry plant weight.

Determination of tannin content (TC)
The tannin content was measured using a colorimetric assay [13]. An aliquot (50 μl) of concentrations 100μg/ml of both extracts and standard solution of (-)-catechin (Sigma–Aldrich Chemicals Co., St. Louis, MO, USA) was prepared in triplicate (for each), mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml of concentrated HCl. The mixture was allowed to stand for 15 min. Absorbance was determined at 510 nm against blank (water). TC was calculated in triplicate as catechin equivalents mg/g of dry plant weight, using a catechin calibration curve (range, 0-400 μg/ml).

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Determination of antioxidant activity
The ability of the corresponding two extracts to donate hydrogen atoms or electrons was determined by the bleaching capacity of purple-colored methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH, Aldrich chem.Co.). The method depends on measuring the decolourizing capacity of each extract against stable DPPH free radical, the colour change being detected spectrophotometrically at 516 nm [14]. Extracts were tested over a range of concentrations to establish the IC_{50} (the concentration reducing DPPH absorbance by 50%). This method follows closely that used by previous workers [15]. DPPH was added to the extracts and Trolox (reference for antioxidant activity) in fixed volumes, and left to stand in dark for 30 min. Any decrease in the absorbance of the purple colour of DPPH solution measured at 516 nm, indicates an antioxidant activity of the extract. A lower IC_{50} value corresponds to a higher antioxidant activity. Tests were carried out in triplicates [16].

$$RSA\% = \frac{Blank_{(abs)} - Sample_{(abs)}}{Blank_{(abs)}} \; x \; 100$$

Quantification of phenolic compounds by HPLC analysis
Phenolic acid and flavonoid standards were quantified by preparing in methanol solution (HPLC grade, Merck, Germany), and serial dilutions were carried out by double-distilled water. Various standard concentrations were injected into HPLC system to establish standard calibration curves. Analyses were developed by HPLC system (Agilent Technologies, Waldbronn, Germany, modular model 1200 series instrument), equipped with Eclipse DB-C18 column (5 µm, 4.6 x 250 mm i.d.) according to Neo *et al.* [17]. Isocratic elution was carried out with a mobile phase consisting of acidified water and acetonitrile in the ratio of 90:10 (v/v), at a flow rate of 1 ml/min. Detection was done using a diode array detector (DAD) at 280 and 320 nm. The injection volume for all samples was 20 µL. Identification of phenolics was based on retention times in comparison with standards. Signals were acquired and processed using a Chemstation soft-ware. Quantitation in each gram of sample was carried out using external standard method. The concentration of each of the phenolics was calculated using peak area and the calibration curves obtained from the phenolics standard solutions. The amount of each phenolic compound was expressed as micrograms per gram of dry plant weight (µg/g).

HPLC analysis for the determination of flavonols was carried out using 5% acetic acid and acetonitrile in a gradient elution mode and column temperature of 30°C.

Antimicrobial Activity
Bacterial strains
Seven *S. aureus* strains were used. SA1199B is a fluoroquinolone-resistant NorAoverexpresser derived from a methicillin-susceptible *S. aureus* bloodstream isolate from a patient with endocarditis [18], RN4220 contains plasmid pU5054 (that carries the gene encoding the MsrA macrolide efflux protein), and XU212 possesses the TetK efflux pump and is also a MRSA strain. These strains were provided by S. Gibbons (University College London, UK) [19-22]. Three clinical MRSA (MJMC001, MJMC002, MJMC004) were isolated from the Hospital Centre of Trás-os-Montes and Alto Douro, EPE, Vila Real (Portugal). *S. aureus* CECT 976 was already used in other studies with phytochemical compounds [23-26]. Bacteria were grown overnight at 37 ℃ and under agitation (150 rpm) in Mueller-Hinton (MH) broth (Merck, Germany).

Antibiotics
Ampicillin (AMP), ciprofloxacin (CIP), erythromycin (ERY), oxacillin (OXA) and tetracycline (TET) were obtained from Sigma (Portugal) and prepared in dimethyl sulfoxide (DMSO), (SigmaAldrich, Portugal).

Antibacterial susceptibility testing
The Minimum Inhibitory Concentration (MIC) of each extract was determined by broth microdilution testing according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [27]. MIC was defined as the lowest concentration of the antimicrobial compound that inhibited bacterial growth. Three independent experiments were performed for each compound. The highest concentration of DMSO remaining after dilution (10%, v/v of the well) caused no growth inhibition (data not shown).

Disc Diffusion Method
Antibiotic-extracts antibacterial effects were determined according to Abreu et al. [24]. FP extract was added to MH agar (after autoclaved and cooled) yielding the final concentration desired. Samples were applied at several

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concentrations, in order to define the minimal/optimal concentration causing antibiotic potentiation. Then, the medium was poured into 90 mm Petri dishes to give a uniform depth of approximately 4 mm (~20 mL). The bacterial suspensions were adjusted to 0.5 McFarland standards and seeded over hardened MH agar Petri dishes using a sterilized cotton swab and allowed to set (for 10 to 15 min).Sterile blank discs (6 mm diameter; Oxoid, Portugal) were placed on the agar plate seeded with the respective bacteria. A volume of 15 µL of each antibiotic prepared according to the CLSI guidelines (AMP – 10 µg/disc; CIP – 5 µg/disc; ERY – 15 µg/disc; TET – 30 µg/disc; and OXA – 1 µg/disc) was added to the blank discs [26]. After incubation at 37 °C for 24 h, each inhibition zone diameter (IZD) was recorded and analyzed according to CLSI guidelines [27]. According to the scheme proposed in a previous study [24] for non-antibacterial compounds, it was characterized the combination between two bioactive agents as additive if $4 \leq$ (IZD combination – IZD most active agent) < 6 mm, as potentiation if (IZD combination – IZD most active agent) $\geq$ 6 mm, indifferent if -6 < (IZD combination – IZD most active agent) < 4 and as negative if (IZD most active agent – IZD combination) $\geq$ 6 mm.

RESULTS

TPC, TFC, TC and scavenging activity
Table 1; reported the yield percentage, TPC, TFC, TC and IC_{50} for both FP and BP extracts of *P. oceanica* leaves. A higher yield (7.55%) was observed for FP extract; whereas BP extract yield was about 5 times lower (2.75%).

The TPC of both extracts, as estimated by the Folin-Ciocalteau reagent method with reference to the standard curve gave a higher value, 27.71 ± 2.4 for FP extract and 12.35 ± 1.1 mg GAE/g DW for BP extract. TFC in the extracts, was expressed with reference to a standard curve as 15.33 ± 1.4 for FP extract, and higher to 19.16 ± 1.8 mg QE/g DW for BP extract. As for TC of both, FP and BP extracts, was close and corresponded to 2.08 ± 0.2 and 3.34 ± 0.5 mg CE/g DW respectively. The scavenging activity of FP, BP extracts and standard (Trolox) on DPPH radical expressed as IC_{50} values were in the following order, 4.0 ± 0.03, 2.5 ± 0.02 and 0.7 ± 0.0 mg/ml, respectively.

Table 1. Content of total phenolics, flavonoids, tannins and antioxidant activity in FP and BP extracts obtained from *P. oceanica* leaves

Sample	Yield (%)	TPC (mg GAE/g DW)	TFC (mgQE/g DW)	TC (mg CE/gDW)	IC_{50} (mg/ml)
FP	7.55	27.71± 2.4	15.33± 1.4	2.08± 0.2	4.0± 0.03
BP	2.75	12.35± 1.1	19.16± 1.8	3.34± 0.5	2.5± 0.02
Total	10.30	40.06± 3.5	34.49± 3.2	5.42± 0.7	Trolox 0.7± 0.00

Results are expressed in mean of triplicates± standard deviation (S.D.); GAE, gallic acid equivalent; QE, quercetin equivalent; CE, catechin equivalent

HPLC analysis
HPLC analysis of FP extract was assayed by external standard calibration method at 280 and 320 nm by comparing the retention times of phenolic compounds with those of commercial standards. The results showed that the main constituents were protocatechuic acid, catechin, and ferulic acid, in the order of 192.32, 56.41, and 45.36 µg/g of the dry leaves weight, respectively. Vanillic acid, caffeic acid, cinnamic acid and syringic acid were also identified as minor constituents (Table 2, fig. 1&2).

Table 2. Results of HPLC analysis of phenolic compounds in FP extract obtained from *P. oceanica* leaves

Peak No.	Compound	Rt	Conentration(µg/g)
1	Protocatechuic acid	10.17	192.32± 2.56
2	Catechin	18.70	56.41± 0.04
3	Caffeic acid	21.73	10.36± 0.02
4	Syringic acid	23.14	6.15± 0.01
5	Vanillic acid	25.16	25.84± 0.03
6	Ferulic acid	32.77	45.36± 0.03
7	Sinapic acid	33.70	2.82
8	Cinnamic acid	42.96	6.48± 0.01

Results are expressed in mean of triplicates± standard deviation (S.D.)
R_t= *retention time in min*

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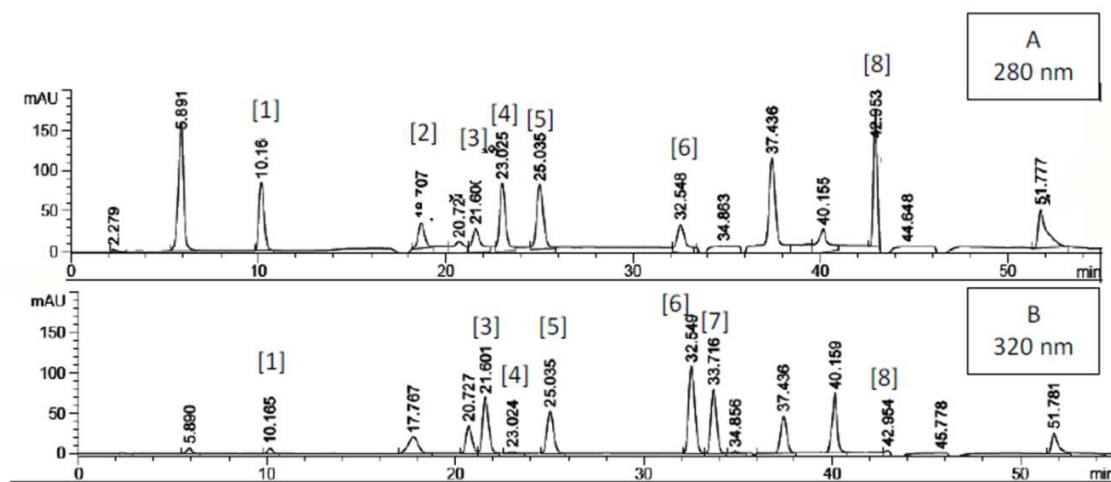
(Fig: 1) HPLC–DAD chromatograms at 280 and 320 nm of Standards; 1- Protocatechuic acid, 2-Catachin, 3- Caffeic acid, 4- Syringic acid, 5-Vanillic acid, 6- Ferulic acid, 7- Sinapic acid and 8- Cinnamic acid
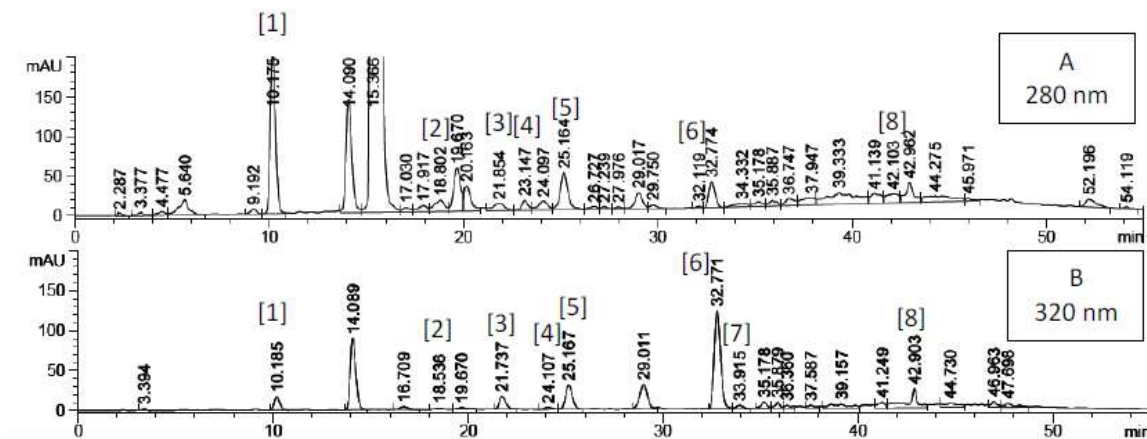
(Fig: 2) HPLC–DAD chromatograms at 280 and 320 nm of FP extract of *P. oceanica* leaves; Peak numbers refer to Table 2

Results of HPLC analysis of BP extract (obtained after hydrolysis) showed that the main flavone aglycone was quercetin (6.20± 0.10 µg/g DW), while myricetin and kaempferol were present in minor amounts (0.21± 0.00 and 0.42± 0.00 µg/g DW, respectively). Identification of flavonols was performed by comparing the chromatograms obtained from the BP extract to the one obtained for a mixture of authentic samples of myricetin, quercetin and kaempferol (Table 3, fig.3) using retention time, together with UV-spectra of the studied flavonols. Consequently, their retention pattern depends on the number of OH-groups in the B ring, therefore, the order of elution from the reverse phase column follows the rule: more polar (OH groups, myricetin with three, quercetin with two and kaempferol with one OH group)→less retention→shorter retention time.

Table 3. Results of HPLC analysis of free flavonoids in BP extract obtained from *P. oceanica* leaves

Peak No.	Compound	Rt	Concentration (µg/g)
1	Myricetin	10.07	0.21± 0.00
2	Quercetin	11.06	6.20± 0.10
3	Kaempferol	16.57	0.42± 0.00

Results are expressed in mean of triplicates± standard deviation (S.D.)
R_t= retention time in min

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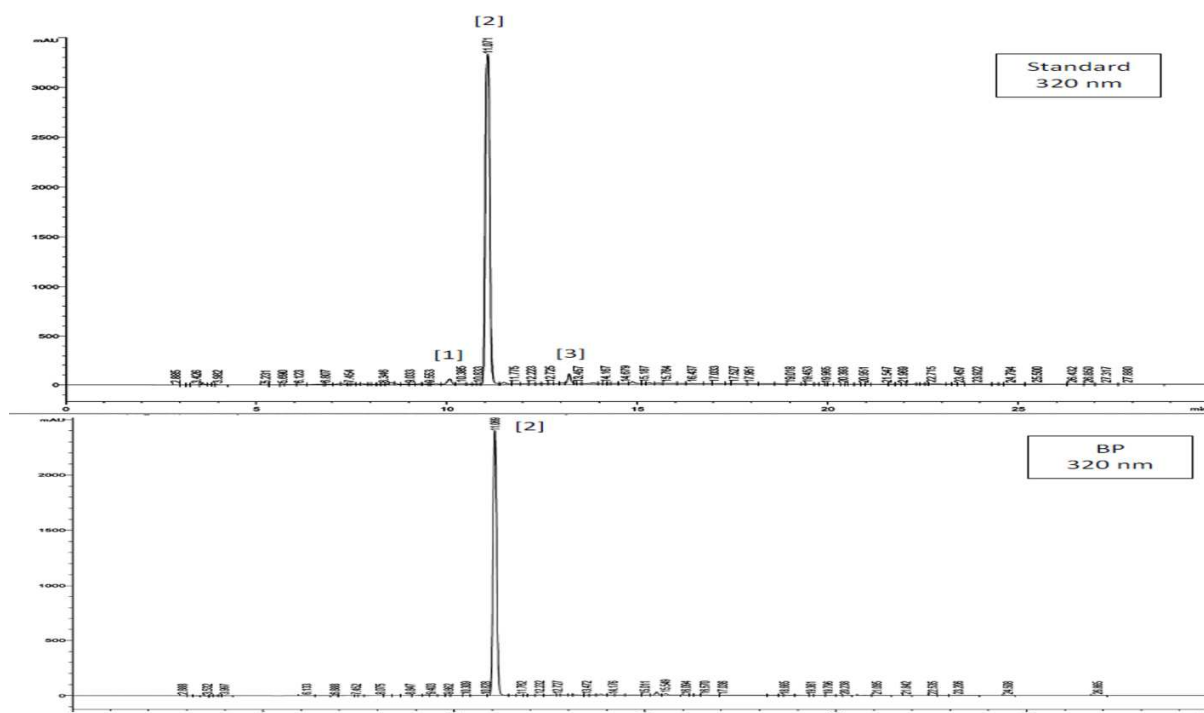
(Fig: 2) HPLC–DAD chromatograms at 320 nm of Standards and BP extract of *P. oceanica* leaves; Peak numbers refer to Table 3

Table 4. IZDs obtained by disc-diffusion method for the antibiotics alone and in combination with the FP extract of *P. oceanica* leaves (at 1000 mg/l) against the *S. aureus* strains tested. Classifications of the combinations as potentiation (P), additive (A) or indifferent (I) are given in parentheses

Isolates	Antib.	Control	FP
CECT 976	**CIP**	38.9 ± 2.1	36.5 ± 1.7 (I)
	TET	29.7 ± 2.1	30.8 ± 2.6 (I)
	ERY	32.0 ± 1.4	35.4 ± 3.2 (I)
	AMP	22.2 ± 2.0	19.3 ± 1.2 (I)
	OXA	27.2 ± 2.9	22.3 ± 2.3 (I)
SA1199B	**CIP**	18.1 ± 1.5	**27.2 ± 1.8 (P)**
XU212	**TET**	0.0 ± 0.0	0.0 ± 0.0 (I)
RN4220	**ERY**	9.9 ± 1.9	10.3 ± 0.8 (I)
MRSA MJMC001	**CIP**	NI	NI(I)
	TET	24.5 ± 0.0	29.5 ± 0.7 (A)
	ERY	13.0 ± 0.0	16 ± 1.1 (I)
	AMP	NI	NI(I)
	OXA	NI	NI(I)
MRSA MJMC002	**CIP**	NI	NI(I)
	TET	20.0 ± 0.0	25.0 ± 0.0 (A)
	ERY	12.5 ± 0.7	14.0 ± 0.0 (I)
	AMP	NI	NI(I)
	OXA	NI	**26.0 ± 1.4 (P)**
MRSA MJMC004	**CIP**	NI	NI(I)
	TET	27.0 ± 1.0	22.0 ± 1.8 (I)
	ERY	12.0 ± 0.0	10.0 ± 0.0 (I)
	AMP	NI	NI(I)
	OXA	NI	**30.5 ± 0.5 (P)**

NI: no inhibition. Data are means and SD from at least three independent experiments.

Antimicrobial activity

Table 4 presents the disc diffusion method results evaluated for the antibiotics alone and combined with the FP extract (at 1000 mg/L). For *S. aureus* SA1199B, RN4220 and XU212, only CIP, ERY and TET were tested, respectively. According to the analysis of IZDs by the susceptibility breakpoints of the NCCLS, 2003 [27], *S. aureus*

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CECT976 was considered susceptible to all antibiotics, *S. aureus* SA1199B, RN4220 and XU212 were resistant to CIP, ERY and TET, respectively, the MRSA strains were classified as resistant to ampicillin, oxacillin, erythromycin and ciprofloxacin and only susceptible to tetracycline; MSSA strains were classified as susceptible to all antibiotics, with exception of ampicillin. The extract had no antibacterial activity at the highest concentration tested, 1000 mg/l (data not shown).

The tested extract exhibited potentiating activity of some antibiotics. It had no potentiation effect with TET against XU212 (contrarily to the others) but it only had an additive interaction with this antibiotic.

Analyzing the results, FP extract seems to potentiate CIP against the NorA overexpresser strain SA1199B, which could be related to a efflux pump inhibition. Also, potentiation of OXA was verified against MRSA MJMC002 and 4. This could be related to a positive interference/inhibition with the resistance mechanisms of these MRSA strains to OXA. However, no effect was observed with the other β-lactam AMP. Additive effects were also obtained with TET only against MRSA MJMC 001 and 002.

DISCUSSION

P.oceanica (L.) Delile is a marine key species in littoral Mediterranean ecosystems. It is capable of producing polyphenolic compounds, due to the presence of tannin cells, which are found in large numbers in the leaves of most of the marine phanerogams, particularly in *P. oceanica* [28]. Previous studies on *P. oceanica* have revealed great differences in the relative number and abundance of different phenolic compounds, which are much higher than that observed in other marine phanerogams of the Mediterranean [29], only rosmarinic acid is not found, despite the fact, that it is present in other marines. These phenolic compounds are often cited in allelopathic regime, playing an important role in the protection of these plants against competitors (algae dense meadows) and pathogens [30], it thus appears that when the seagrass is in interaction, it accelerates its production of secondary metabolites (phenolic compounds), so as to limit invasion of the beds [31-32]. Also deterioration of environmental conditions, pollution, as well as, resistance to colonization (indirect exploitation competition for light and nutrients) lead to stress, which is hypothesized to be the reason behind an increase in the production of tannin cells and consequently phenolic compounds in marine environments [33]. However, several studies have shown that the concentration of phenolic compounds in *P. oceanica* decreases with the age of leaves, thus the intermediate leaves show significantly greater concentrations than in the adult leaves [34-35].

The extraction procedure with aqueous ethanol allowed the identification of phenolic compounds, while acidic reflux generated anthocyanidins from homologous proanthocyanidins and flavonol aglycones from corresponding flavonol glycosides [10]. Thus, high phenolic content (TPC) is observed for the alcoholic extract (FP), on the contrary, flavonoid content (TFC) is lower. In the hydrolyzed extract (BP), TFC and TC increased, and this point, demonstrates that these compounds are present in the plant tissues mostly as esters and glycosides. The results of the HPLC quantitative analysis confirm these spectrophotometric studies. The phenolic compounds determined after extraction with ethanol, are far the most part characteristic of the monocotyledonous angiosperms, as protocatechuic acid (dihydroxy-3,4-benzoic) and ferulic acid (hydroxyl-4 methoxy-3-cinnamic), which are quantitatively by far the most abundant, accounting an average for more than 80% of the relative phenolic acids identified, being characteristic not only of the marine phanerogam, but also of land phanerogams [29-36]. Vanillic acid (hydroxyl-4 methoxy-3-benzoic), and caffeic acid (3,4-dihydroxycinnamic acid) are also among the analyzed phenolic acids, exhibiting much lower average concentrations. The remaining compounds represent less than 2% of the total phenolic compounds, including, cinnamic, syringic and sinapic acids, which are present merely as traces.

The DPPH free radical scavenging activity of FP and BP extracts of *P. oceanica* was evaluated. Both extracts show ability to quench DPPH radicals. FP extract shows lower activity, this may be due to, hydroxycinnamic and hydroxybenzoic acids are known equally as primary antioxidants acting slightly as free radical acceptors. While BP extract exhibits a higher activity, as indicated by its lower IC_{50} (2.5 mg/ml), as many flavonoids and their related compounds have been known to possess strong antioxidative characteristics linked to the presence of phenolic hydroxyl groups attached to ring structures, being able to scavenge free radicals (superoxide, peroxyl) to form stable species which can stop an oxidation chain reaction. The spatial configuration, number and location of hydroxyl groups on aromatic B ring, appeared to enhance the antioxidant activity in radical scavenging assays, notice that quercetin has an additional orthodihydroxy group in the B-ring of the flavonoid skeleton [37-38]. The results of antimicrobial effect of the FP extract is correlated positively with the previous studies [39], as it is found to exhibit

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potentiating activity of some antibiotics against *S. aureus* resistant strains. More studies would be necessary in order to isolate the specific compounds promoting this activity. Additionally, it would be necessary to identify the specific targets of the phytochemicals in order to explain the potentiation of the antibiotics.

CONCLUSION

This work could partially explain the medicinal effect of this plant by investigating its phenolic profile, however, an important extension in the future would be an *in vivo* evaluation of its biological activity on animal models, which is expected to provide important medicinal basis for its local consumption for obesity and diabetes.

We suggest that this sea wheat could afford an interesting incorporating material for the production of natural health benefit products, mostly in developing countries, and providing an indication, as well as, a solution to many environmental problems. Consequently, the management of marine resources should be achieved within comprehensive governance.

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