In vitro anticancer, antioxidant and antimicrobial potential of *Lyngbya aestuarii* (Cyanobacteria) from the Atlantic coast of Morocco

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Abstract
The aim of this study is to investigate *in vitro* anticancer, antioxidant and antimicrobial activities of the crude extract of *Lyngbya aestuarii*, a marine filamentous non-heterocystous cyanobacterium frequently found in the rocky shores of the Moroccan Atlantic Ocean, where it forms epilithic hairy mat-like biomasses in the uppermost part of intertidal zone. The species was first taxonomically identified based on a combined phenotypic attributes and molecular approach using the partial sequencing of 16S rRNA. The *in vitro* anticancer activity of *Lyngbya aestuarii* extract was performed on HepG2, HT-29, T47D and MG-63 human cell lines using MTT assay. Moderate cytotoxicity was revealed within 48h of incubation in all cells and the most pronounced responses were seen in HT29 and HepG2 cells with reduced cell viability of 61.38±3.26 and 62.78±2.13%, respectively. The significant antioxidant activity demonstrated by DPPH$^\bullet$ radical scavenging assay (EC_{50}=213.95 µg mL^{-1}) and ferrous ion chelating ability (EC_{50}=219.76 µg mL^{-1}) implies the presence of various potent antiradicals presumed as an adaptation strategy of the species to harsh environmental conditions in the upper tidal limit. The cyanobacterial extract screened for antimicrobial activity using agar disc diffusion method, exhibited moderate to good antibacterial activity against three gram positive microorganisms (*Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*) and the gram negative bacterium *Pseudomonas aeruginosa*, as well as a moderate inhibition towards two tested fungal species *Candida albicans* and *Candida parapsilosis*. This study suggests that *Lyngbya aestuarii* may be a potent source of bioactive molecules and further research will focus on the separation and identification of metabolites responsible for these anticancer, antioxidant and antimicrobial activities.

1. Introduction

Over the past few decades, marine organisms have been found to be a source of many new molecules with a broad spectrum of biological activities [1]. Generally, the synthesis of highly potent bioactive metabolites is one of the evolutionary strategies to cope with the dangers posed by marine planktivorous grazers or environmental rivals [2,3]. Among marine flora, several species of Cyanobacteria (blue-green algae) have been studied for their potential biological properties. These Gram negative oxygenic autotrophs are among the oldest organisms known, inhabiting the earth for more than three billion years [4]. They occupy almost all habitats and are highly diversified in terms of morphology, physiology and metabolism [5]. Cyanobacteria are deemed ecologically important for their contributions to global nitrogen fixation, and carbon flux [6,7] and their global biomass is a relevant component of both terrestrial and marine biomes. Biotechnologically, they possess a great potential to act as cell factories by virtue of their relatively simple structure, minimal nutritional requirements, and an ability to synthesize a wide variety of metabolites [8]. They are well-recognized producers of bioactive secondary metabolites with constantly rising interest. While only about 200 cyanobacterial metabolites have been

structurally characterized until 1996 [9], this number raised recently to about 1100 [10]. Probably owing to their high capacity to adapt to almost all kinds of habitats, the metabolic activities of Cyanobacteria also produce a wide variety of secondary metabolites with biological activities useful for therapeutic purposes, e.g. strong antiviral, antibacterial, antifungal, anti-inflammatory and antitumoral activities. They correspond to a heterogeneous group of secondary metabolites belonging to phenolics, polychlorinated aromatics, alkaloids, cyclic peptides and depsipeptides, lipopeptides, glyco-and sulfolipids, fatty acids, amides, macrolides, isonitriles, lactones and nucleosides [11].

Species of the Oscillatoriales order are mainly prolific producers of bioactive natural products. This chemical diversity is mainly demonstrated in several species of the genus *Lyngbya* which have proven to be prodigious producers of secondary metabolites, including toxins. Indeed, there are an increasing number of *Lyngbya* species from marine, brackish and fresh environments worldwide which have been found to produce an impressive array of structurally varied compounds with diverse biological activities [12]. To date, the most important species of the genus *Lyngbya* in terms of secondary metabolite production are *Lyngbya majuscula*, *Lyngbya aestuarii*, *Lyngbya martensiana* and *Lyngbya wollei* [13].

In other hand, marine blue-green algae still globally the subject of far fewer studies than freshwater forms, and as a result they are poorly described and only a small number appear in determinative manuals [14]. This is also the case of the Moroccan marine shores where no research was conducted on the diversity and natural products of marine Cyanobacteria in spite of their widespread occurrence and their known chemical richness. Particularly, diversity and toxicology of Cyanobacteria are relatively well documented in Moroccan freshwater habitats [e.g. 15-18]. In this context and as part of natural products discovery program focused on marine Cyanobacteria in Morocco, monoalgal hairy mats of *Lyngbya aestuarii*, harvested from the Moroccan Atlantic coast and identified through morphological and molecular approaches, were investigated for their anticancer, antibacterial and antioxidant potentials.

2. Material and Methods

2.1. Sampling site and biomass harvesting

Cyanobacterial biomass was sampled from high intertidal shallow rock pools of El Jadida located on the northwestern Atlantic coast of Morocco (33°15'48.5"N 8°30'45.9"W) (Figure 1) where extensive *Lyngbya aestuarii* monospecific epilithic tufted mats commonly occurred (Figure 2). Selected specimens were previously preserved with 5% formalin, microscopically examined and putatively identified based on morphology according to Komárek and Anagnostidis [19].

2.2. Molecular identification

Total genomic DNA was isolated from 100 mg of lyophilized *Lyngbya aestuarii* sample using the MO-BIO UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol for maximum yields and was performed in triplicate. DNA integrity was checked by agarose gel electrophoresis with ethidium bromide staining.

PCR was performed to amplify cyanobacteria-specific fragment of 422 bp from the 16S rRNA gene (16S–CYA) using the primer pair 359F–GC (GGG GAA TYT TCC GCA ATG GG) and 781R (GAC TAC WGG GGT ATC TAA TCC CWT T) [20]. The 40-nucleotide GC-rich sequence, referred to as a GC clamp, attached to the 5´-end of primer 359F was used to improve the detection of sequence variation in amplified DNA fragments by subsequent denaturing gradient gel electrophoresis (DGGE). PCR reactions were performed in triplicate with a final volume of 20 µL containing 1X GoTaq buffer, 2.5 mM $MgCl_2$, 125.0 mM of each deoxynucleotide triphosphate, 1.0 µM of each primer, 0.5 U of GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), 10 mg.L^{-1} of bovine serum albumin (BSA) and 10 ng of template DNA. Thermal cycling was carried out using T-Professional Standard (Biometra, Goettingen, Germany) and the PCR conditions were as follows: initial denaturation at 94ºC for 2 min, 12 touchdown cycles of denaturing (at 94ºC for 1 min), annealing (at 65-55ºC for 1 min, decreasing 0.5ºC each cycle) and extension (at 72ºC for 1 min) followed by 32 standard cycles of denaturing (at 94ºC for 1 min), annealing (at 55ºC for 1 min) and extension (at 72ºC for 4 min) and a final extension step at 72ºC for 4 min. PCR products were verified by agarose gel electrophoresis with ethidium bromide staining. PCR products were pooled and separated by agarose gel electrophoresis and purified from gel using Cut&Spin Gel Extraction Spin Columns (GRiSP, Porto, Portugal), according to the manufacturer's instructions. The integrity of purified DNA was checked by agarose gel electrophoresis with ethidium bromide staining.

Samples corresponding (16 µL of purified PCR product plus 4 µL of loading buffer) were loaded onto 6% polyacrylamide 1 mm gels, using a 35–65% denaturing gradient (100% denaturing conditions correspond to 7 M

urea and 35% (v/v) formamide). The electrophoresis was performed using 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), at 60 V for 16 h, in a DCode system (Bio-Rad, CA, USA). The gel was stained with 1×SYBR Gold nucleic acid stain (Invitrogen, San Diego, CA). The central part of each DGGE band was excised from the gel with a scalpel carefully cleaned after each incision and the DNA was resuspended in 25 µL of sterile water and incubated at 37°C for 30 min to allow diffusion of the DNA. Two microliters of the eluted DNA were used for the amplification of the16S rRNA gene fragment with the same set of primers without the GC clamp as described by Nübel et al. [20]. PCR products were separated by agarose gel electrophoresis and purified from gel using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany) and sequencing was performed by GATC Biotech Company (Germany). Obtained sequences were queried against GenBank (http://www.ncbi.nlm.nih.gov/blast) by BLASTN algorithm to identify the closest matching sequences available in GenBank database. The sequences generated in this study were deposited in the GenBank database under the accession numbers MF962581-MF962582.
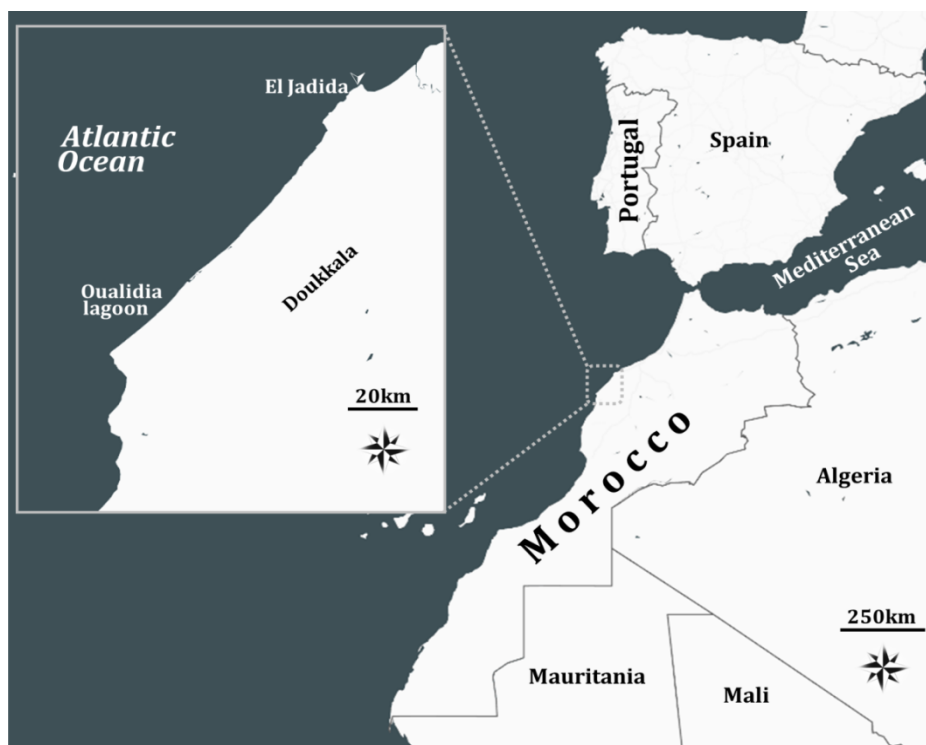

Figure 1. Sampling site of *Lyngbya aestuarii* (⊻) from Sidi Daoui, El Jadida
on the Atlantic coast of Morocco.

Figure 2. *Lyngbya aestuarii* macroscopic hairy mat-like colonies in shallow rock-pools
of the uppermost part of intertidal zone in Sidi Daoui, El Jadida, Morocco.

2.3. Cyanobacterial extract preparation
The freeze-dried (32.7 g) grounded biomass of *Lyngbya aestuarii* was repeatedly extracted by immersion on a 2:1 mixture of CH_2Cl_2/MeOH (< 40ºC). The resulting slurry was evaporated under reduced pressure yielding 2.4 g of crude extract.

2.4. Anticancer activity by MTT assay
All cell lines included in the study are of human origin. Hepatocellular carcinoma cell line HEPG2, colon adenocarcinoma cell line HT-29, breast carcinoma cell line T47D were purchased from Sigma-Aldrich and MG-

63 osteosarcoma was obtained from the ATCC. Tumor cells were cultured in Dulbecco's modified Eagle medium (DMEM Glutamax), supplemented with 10% fetal bovine serum (FBS), 2.5 µg mL^{-1} fungizone, penicillin-streptomycin (100 IU mL^{-1} and 100 µg mL^{-1}, respectively). Cells were incubated in a humidified atmosphere with 5% of CO_2, at 37°C. The cellular viability was evaluated by the reduction of the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [21]. Cells were seeded in 96-well culture plates at a concentration of 10^4 cells cm^{-2}. After 24 h of adhesion, cells were exposed to 100 µL fresh medium supplemented to a final concentration of the *Lyngbya aestuarii* extract of 300 µg mL^{-1}, for a period of both 24 and 48 hours. After incubation, cells were exposed to 10 µL of 0.5 mg mL^{-1} MTT. Solvent control cells (DMSO 1%) and DMSO 20%; were used respectively as negative and positive control. Following exposure, purple-coloured formazan salts were dissolved in 100 µL DMSO and the absorbance measured at 550 nm in a microplate reader (Synergy HT, Biotek, USA). All tests were run in triplicate and averaged. Viability of cells is expressed relative to the solvent control.

2.5. Antimicrobial assay by disc diffusion method

The antimicrobial activity of *Lyngbya aestuarii* crude extract was tested against three Gram positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Micrococcus luteus* ATCC 9344), two Gram negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two yeast species (*Candida parapsilosis* and *C. albicans*) using the disc diffusion method. Briefly, 38 g of Mueller-Hinton agar (MH) (BioKar Diagnostics, Beauvais, France) were suspended in distilled water, mixed well and distributed homogenously. The medium was sterilized by autoclaving at 121°C for 15 min. In parallel, overnight fresh grown microbial colonies were used to prepare the bacterial inocula in Mueller-Hinton broth (MHB) (BioKar Diagnostics) equal to 0.5 McFarland. Tenfold serial dilutions were performed and the third obtained dilution was incorporated (100 µL/100 mL) in Mueller-Hinton agar previously cooled to 45 °C, which was then poured into a Petri dish. After this, 6 mm filter discs (Oxoid, Basingstoke, England) were laid flat on growth medium containing 100 µL of *Lyngbya aestuarii* extract (3 mg mL^{-1}). The DMSO and ciprofloxacin (CIP, 5 µg) were used as solvent control and antibiotic standard, respectively. The Petri plates were then incubated at 37°C for 24 h and the zone of growth inhibition (IZ) was measured.

2.6. DPPH$^{\bullet}$ scavenging activity

The scavenging effect of *Lyngbya aestuarii* crude extract on DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical was performed according to Brand-Williams et al. [22] with some modifications. The reaction mixture consisted of adding 0.5 mL of the tested sample (*Lyngbya* crude extract or pure ascorbic acid solution) at different concentrations, 0.3 mL of 0.5 mM DPPH solution and 3 mL of absolute methanol. Absorbance of stable DPPH free radical at 515 nm (Metashe 5200 HPC UV–VIS spectrophotometer) will be reduced when it encounters proton-donating compounds like antioxidants. The ability to scavenge the DPPH radical was calculated according to the following equation:

$$DPPH^{\bullet} \text{ scavenging activity (\%)} = [(A_c - A_s)/A_c] \times 100$$

where A_c is the absorbance of the negative control and A_s is the absorbance of the sample.

2.7. Ferrous ion-chelating ability

The Ferrous ion-chelating (FIC) ability by *Lyngbya aestuarii* crude extract was estimated by the original method of Decker and Welch [23] with minor modifications. This assay is based upon the formation of blue colored ferrous ion-ferrozine complex which has a maximum absorbance at 562 nm. Briefly, 1ml of varying concentrations of extract and standard were mixed with2.75 ml distilled water, $FeCl_2$ (0.05 ml, 2 mM) and ferrozine (0.2 mL, 5 mM). After 10 min in dark and at room temperature, the absorbance of the mixture was measured at 562 nm. Distilled water was used instead of sample as a control, and instead of ferrozine solution as a blank. Ethylene diamine tetra-acetic acid (EDTA) was tested as positive control at different concentrations. The FIC ability was calculated using the equation given next:

$$FIC \text{ Ability (\%)} = [A_c - (A_s - A_b)/A_c] \times 100$$

where A_c is the absorbance of the control, A_s the absorbance of the sample or EDTA, and A_b is the absorbance of the blank.

3. Results and discussion

3.1. Morphological and molecular identification

Phenotypic attributes

The studied cyanobacterial mats are monospecific formed exclusively by *Lyngbya* (Figure 3A-B), exceptionally in co-occurrence with thalli of *Enteromorpha* (Chlorophyta) (Figure 3C). The cyanobacterium forms extensive epilithic hairy mat-like biomasses in the uppermost part of intertidal zone particularly in shallow tide pools and puddles left in the rocks as water recedes when the tide goes out. The filaments are very long reached 10-15 cm, flexibles, densely crowded and arranged in fixed tufts forming extensive mats which can range in color from black to green black or dark blue-green when viewed on mass. Cells 15-20 µm wide, 1.5-2 µm long, slightly narrowed towards apex. Sheaths thick, lamellate, colorless at first, later becoming yellowish or brownish (Figure 3D-G). Based on these phenotypic features and on traditional morphological criteria [19], the studied blue-green alga can be identified as *Lyngbya aestuarii*. It is well known that this species forms worldwide extensive mats in many marshes and intertidal mud flats [24-27] under environments that are extreme in many respects, with repeated cycles of desiccation and wetting, intense exposure to ultraviolet radiation, and changing regimes of salinity [8].
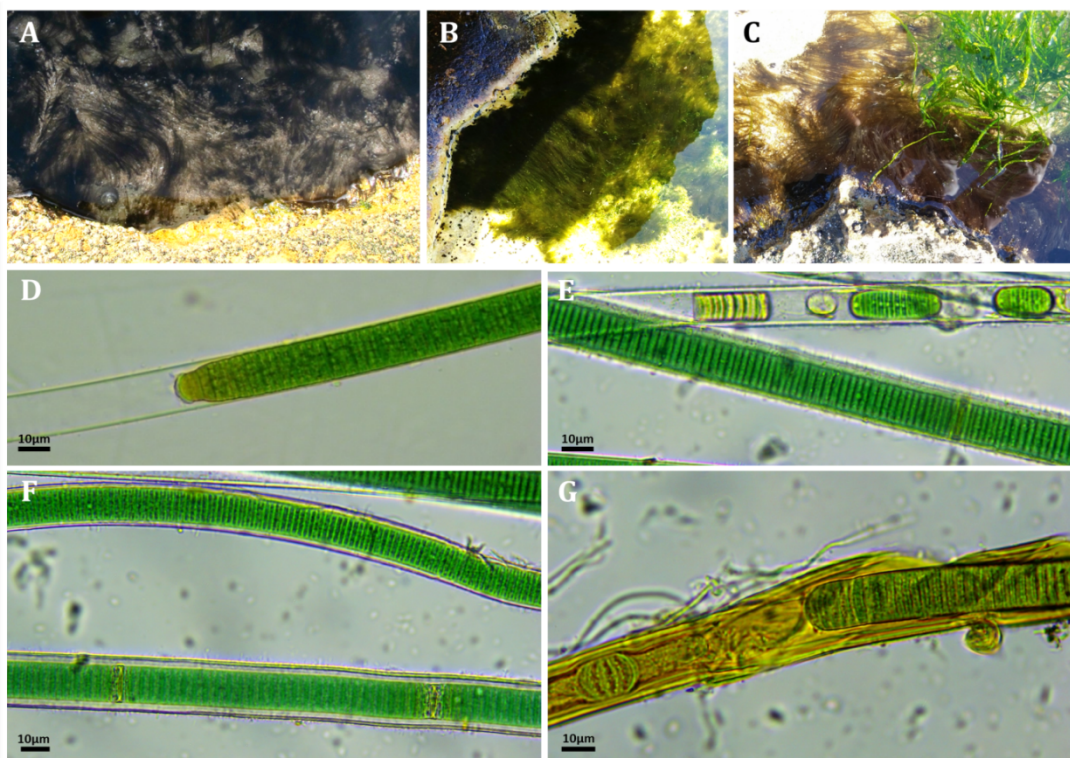

Figure 3. Illustrations of field cyanobacterial biomass (A-C) and photomicrographs showing trichomes of the mat-forming *Lyngbya aestuarii* (D-G).

Molecular identification

The most closely related sequences to the studied cyanobacterium were identified using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). As expected from the morphological identification, the blasted sequences (DGGE bands 1 and 2) correspond to several cyanobacteria closely related to *Lyngbya aestuarii* (Table 1). The sequence from band 1 showed 98% pairwise sequence identity with both an environmental sample of *Lyngbya aestuarii* from the Arabian Gulf [28] and the strain *Lyngbya cf. aestuarii* LEGE 07165 isolated from the Portuguese coast [29]. This tool also confirms the very close genomic similarity of the sequence from band 2 sharing 97-100% 16S rRNA gene similarity with several strains such as *Lyngbya aestuarii* PCC 7419 [20], *Lyngbya aestuarii* kopara-LY [30], *Lyngbya aestuarii* CNP 1005 [31] and *Lyngbya aestuarii var. tenuis* CNP3007 (Unpublished, GenBank Accession Number KT347317.1).

3.2. Cytotoxicity against HEP G2, HT-29, T47D and MG-63 cancer cell lines

The *in vitro* anticancer activity of *Lyngbya aestuarii* crude extract was performed on HepG2, HT-29, T47D and MG-63 human cell lines using MTT assay as it is one of the most popular tests used for assessment of cell viability and proliferation studies [32].

Table 1. BLAST sequence information for two DGGE bands obtained from16S-CYA PCR product and similarity relationships with other strains of *Lyngbya* available in NCBI database.

Accession Number	DGGE Band N°	Closest Isolate Relative (Accession Number)	Similarity (*)	References
MF962581	1	*Lyngbya aestuarii* (KP276706.1)	98%	[28]
		Lyngbya cf. *aestuarii* LEGE 07165 (HQ832912.1)	98%	[29]
		Lyngbya aestuarii PCC 7419 (NR_114680.1)	98%	[20]
		Lyngbya aestuarii kopara-LY (AJ621838.1)	99%	[30]
MF962582	2	*Lyngbya aestuarii* var. *tenuis* CNP3007 (KT347317.1)	99%	Unpublished
		Lyngbya aestuarii CNP 1005 (JX519572.1)	99%	[31]
		Lyngbya aestuarii kopara-LY (AJ621838.1)	100%	[30]
		Lyngbya aestuarii PCC 7419 (NR_114680.1)	97%	[20]

*% of identical nucleotides in the sequences obtained from the DGGE bands and the closest matching sequences available in GenBank database.

Exposure to the algal extract led to distinct responses depending on the cell line and the exposure time (Table 2). After 24h of exposure, no cell stimulatory above control levels was observed except for MG-63 cells where a slight increase in cell viability (112.26±0.42%) was showed. Moderate cytotoxicity was revealed with a 48h of incubation in all cells and the most pronounced responses were seen in HT29 and HepG2 cells with a reduction of cell viability of 61.38±3.26 and 62.78±2.13%, respectively. Since the extraction solvent was a mixture of dichloromethane/methanol, the compounds responsible for the observed antitumoral activity are polar like phycobilins, phenolic compounds and polysaccharides reported to induce apoptosis of cancer cells [33,34].

Table 2. Anticancer activity of *Lyngbya aestuarii* crude extract against HEP G2, HT-29, T47D and MG-63 human cancer cell lines. Values represent means and standard deviations.

	% of cell viability					
	Solvent control (DMSO 1%)		Positive control (DMSO 20%)		*Lyngbya aestuarii* crude extract	
Cell lines	24h	48h	24h	48h	24h	48h
T47D	100±22.84	100±27.02	38.10±3.63	34.57±4.54	66.74±5.79	76.93±12.98
HT-29	100±5.88	100±7.16	44.45±12.31	5.08±1.02	90.19±9.34	61.38±3.26
HEPG2	100±12.98	100±18.34	42.97±7.48	25.94±1.78	106.66±5.23	62.78±2.13
MG-63	100±24.85	100±5.11	22.47±2.70	17.13±1.39	112.26±0.42	69.68±4.89

Cyanobacterial species are prodigious producers of interesting anticancer secondary metabolites such as the anti-mitotic curacin A, the protein kinase C activators lyngbyatoxin, the debromoaplysiatoxin, and the V-ATPase inhibitor Iejimalide A [35]. Many compounds were obtained from *Lyngbya* species, namely *Lyngbya majuscula*, *Lyngbya martensiana*, *Lyngbya polychroa* and *Lyngbya wollei*. In a recent review, Swain et al. [36] described 144 *Lyngbya*-compounds as the source of antineoplastic agents, which have been screened primarily with cancer cell lines. For example, the Dragonamides C–D extracted from *Lyngbya majuscula* and *Lyngbya polychroa* showed anticancer activity against HT29 colon adenocarcinoma cells [37,38]. Furthermore, Lyngbyaloside , Lyngbyabellin A; Lyngbyastatin 4; Lyngbyastatin 5-7, Lyngbyastatin 8-10 and Lagunamide C has been reported to be remarkably cytotoxic against lung cancer, KB nasopharyngeal carcinoma, LoVo colon adenocarcinoma, cancer prostate PC3 and ileocecal colorectal cancer HCT8 [39-42].

The mechanisms implicated in the cytotoxicity of marine cyanobacteria compounds in tumor cell lines are still largely overlooked but several studies point to an implication in several apoptotic aspects such as cell cycle arrest, mitochondrial dysfunctions and oxidative damage, alterations in caspase cascade, alterations in specific proteins levels and alterations in the membrane sodium dynamics [43]. Also, an increasing number of marine cyanobacterial compounds are found to target tubulin or actin filaments in eukaryotic cells, making them an attractive source of natural products as anticancer agents [44]. Prominent molecules such as the anti-microtubule agents, curacin A and dolastatin 10, have been reported in preclinical and/or clinical trials as potential anticancer drugs or served as drug leads for the development of synthetic analogues, e.g. compound 4, TZT-1027, ILX-651, and LU-103793, usually with improved pharmacological and pharmacokinetic properties [12, 45-48].

3.3. In vitro antiradical activity
The antioxidant activity of *Lyngbya aestuarii* crude extract was investigated by using two *in vitro* antioxidant assay systems: DPPH$^{•}$ radical scavenging activity and ferrous ion chelating ability (FIC) assay.

DPPH˙ radical scavenging assay

The model of scavenging the DPPH˙ radical is a widely used method to evaluate the free radical scavenging ability of various natural samples [49]. Absorbance of DPPH˙ radical at 515 nm reduces when it encounters hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [22]. Figure 4 shows DPPH˙-scavenging profile of the tested extract and of ascorbic acid with respect to their concentrations. As depicted in this figure, antioxidant activities of *Lyngbya aestuarii* are low as compared to the positive control but at higher doses of 0.5-1mg mL^{-1}, almost 85-94% DPPH-radicals inhibition was achieved by the cyanobacterium extract which is comparable to that of ascorbic acid (88-96%).
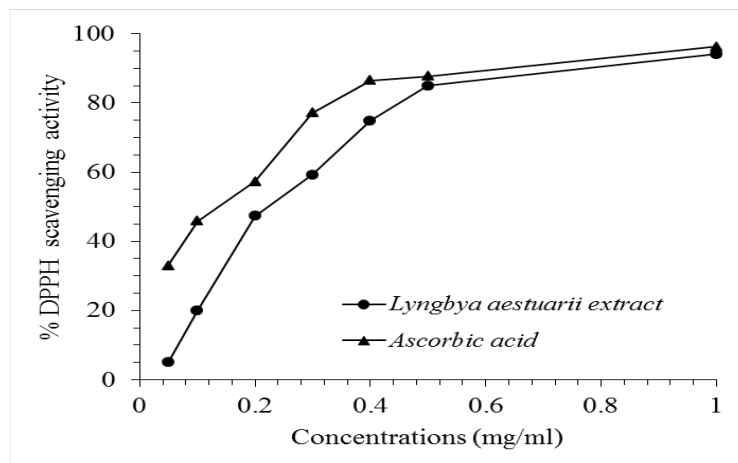

Figure 4. DPPH radical scavenging activity of ascorbic acid and crude extract of *Lyngbya aestuarii*.

Based on DPPH˙ assay, the antioxidant activities was also expressed as the effective concentration (EC$_{50}$) defined as the dose of the sample leading to 50% reduction in the initial DPPH˙ concentration. The lower the EC$_{50}$ value the higher the antioxidant activity of a sample. As showed in table 3, the EC$_{50}$ of *Lyngbya* extract (213.95 µg mL^{-1}) was approximately 50% of pure ascorbic acid (109.36 µg mL^{-1}). These results are an indicator of a significant antioxidant scavenging activity of the studied cyanobacterium and highlight the potential bearing antiradical metabolites. The involvement of free radicals, especially their increased production, appears to be a feature of several human diseases, including cardiovascular disease and cancer [50]. Singh et al. [51] reported that the methanol extracts of twenty cyanobacterial species contained high quantity of total phenol and total flavonoid that were supposed to impart prominent antiradical properties in terms of DPPH free-radical scavenging. Similarly, Abd El-Aty et al. [52] reported that the highest antioxidant activities as well as the highest phenolic contents were showed in methanol extracts from the filamentous cyanobacteria *Oscillatoria agardhii* and *Anabaena sphaerica*. Furthermore, the hydrosoluble phycobiliproteins – phycocyanin from cyanobacteria has been reported as a strong antioxidant and protects the cells against apoptosis by attenuating the free radicals and reactive oxygen species (ROS) formation [53-55]. These implications are important as radical scavengers from natural sources like cyanobacteria may protect cell tissues from free radicals and ROS such as hydroxyl radical (HO˙), superoxide radical (O2˙⁻), peroxyl radical (ROO˙), nitric oxide radical (NO˙) and hydrogen peroxide (H$_2$O$_2$) which are highly reactive molecules produced from aerobic metabolism [56]. Such oxidants are associated with important pathological processes including inflammation, neurodegenerative diseases, artherosclerosis and cancer [57].

Ferrous ion chelating ability

Among various species of transition metals, ferrous ions are the most powerful pro-oxidants which can directly interact with hydrogen peroxide via the Fenton reaction and generate the ROS and hydrogen free radicals [58]. Fe^{2+} not only catalyzes formation of hydroxyl radicals but also accelerate autoxidation reactions and lipid peroxidation, thus minimizing its concentration affords protection against oxidative damage [59]. Antioxidants having metal chelating ability may act as preventive or secondary antioxidants as they forms-bonds with metal ions and reduce the redox potential thereby stabilizing the oxidized form of the metal ions [60]. For that reason, the FIC ability of the studied cyanobacterium, estimated by the disruption of Fe^{2+}–ferrozine complex, may have an important contribution toward their antioxidant virtue. The results of this assay are depicted in Figure 5 and table 3. In all concentrations, *Lyngbya aestuarii* extract interfered with the formation of Fe^{2+}–ferrozine complex, suggesting that it has chelating activity. Overall, FIC activity was found to be concentration-dependent and increased with the increasing concentration of the extract.

Table 3. EC$_{50}$ values of *Lyngbya aestuarii* crude extract and positive controls derived from DPPH$^\bullet$ and ferrous chelating *in vitro* antioxidant assays. Values represent the mean ± standard deviation.

| | EC$_{50}$ values (µg mL^{-1}) | | |
| | Positive controls | | *Lyngbya aestuarii* |
Assays	Ascorbic acid	EDTA	crude extract
DPPH$^\bullet$ scavenging activity	109.36±1.25	-	213.95±2.38
Ferrous ion chelating activity	-	26.31±0.71	219.76±1.46

However, the crude extract of *Lyngbya aestuarii* showed a moderate chelating ability when compared to EDTA particularly under low-range doses (0.1-0.3 mg mL^{-1}). Indeed, the FIC ability of *Lyngbya* extract (20-59.22%) at these concentrations was 1.65 to 4.26 times lower than EDTA iron chelating activity (85.19-97.97%). Accordingly, the EC$_{50}$ value of the extract was 219.76µg/ml and that of the EDTA was 26.31 µg mL^{-1} (Table 3). Nonetheless, significant activities were exhibited at highest tested concentrations by the cyanobacterial extract as compared with the activities of the pure synthetic standard. The extract of *Lyngbya aestuarii* recorded a prominent Fe^{2+} chelating activity of 94.16% while in case of EDTA, 100% activity obtained at the same concentration of 1 mg mL^{-1}.
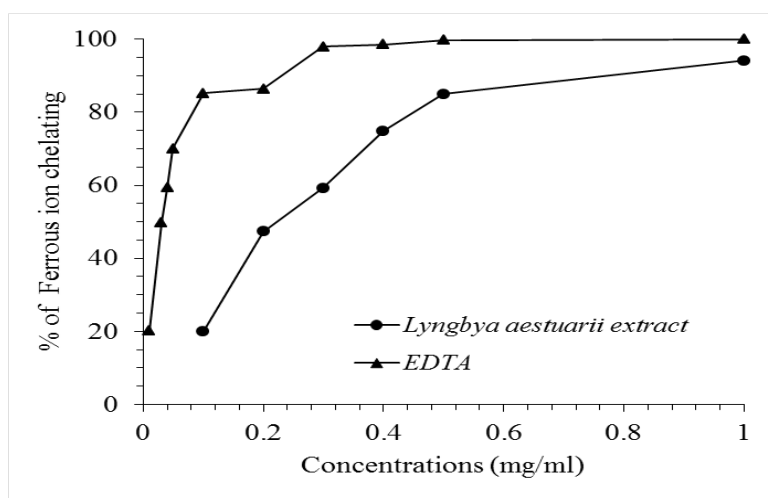

Figure 5. Ferrous chelating ability of the crude extract of *Lyngbya aestuarii* compared to EDTA.

The antioxidant activity from both DPPH (EC$_{50}$=213.95 µg mL^{-1}) and FIC (EC$_{50}$=219.76 µg mL^{-1}) assays implies the presence of various groups of antiradical compounds in the extract of *Lyngbya aestuarii* and may be presumed as an intrinsic adaptation strategy of the species to overcome abiotic stresses under its specific extreme habitat in the upper limit of the intertidal zone. According to Singh et al. [51], the presence of phenolic acids like phenylpropanoids gallic, chlorogenic, caffeic, vanillic and ferulic acids and flavonoids rutin, quercetin and kaempferol in cyanobacterial extracts and their correlation with antioxidant properties add functional values such as free-radical quenching, metal chelation and ROS-scavenging activity and make these organisms potentially viable source of biomolecules. However, when using crude cyanobacterial extracts as a source of natural antioxidants, not only polyphenolic molecules but also other compounds should be considered. Likewise, the well described cyanobacterial carotenoids (α- and β-carotene, lycopene, zeaxanthin, lutein, echinenone, astaxanthin, and canthaxanthin) show important antioxidant activity against radicals [61]. Furthermore, other compounds such as polyunsaturated fatty acids and polysaccharides may also play an important role in radical scavenging activity [62,63]. To sum up, since cyanobacteria are complex matrices of various compounds, antioxidant activity would not be closely connected to a specific compound but multi-component antioxidant systems, which are generally more effective due to additive or synergistic interactions between the different antioxidant components [64,65].

3.4. Antimicrobial activity
The agar disc diffusion method, known to produce predominantly qualitative results, was quite useful to obtain preliminary information on the antibacterial and antifungal potential of the crude extract of *Lyngbya aestuarii*. The data expressed as the diameter of the inhibition zone are summarized in table 3. In plates where blank disks were impregnated only with DMSO no inhibitory growth was registered, while ciprofloxacin disks (used as a positive control) showed excellent growth inhibition (IZ ≥12mm). The extract of *Lyngbya aestuarii* exhibited moderate to good antibacterial activity (7< IZ <10mm) against all selected Gram positive reference strains (*S. aureus* ATCC 25923, *B. subtilis* ATCC 6633 and *M. luteus* ATCC 9344) and the Gram-negative bacterium

P. aeruginosa ATCC 27853, while no inhibitory effect was observed against *E. coli* ATCC 25922. Similarly to the observed antibacterial activity, the extract exhibited inhibition towards the two tested fungal species with moderate values (8 mm IZ) in case of *C. albicans* and *C. parapsilosis*. These different activities, although corresponding to preliminary results, suggest that the crude extract of *Lyngbya aestuarii* contained different antimicrobial substances and reflected the chemical variety of cyanobacterial metabolites often discussed in the literature [e.g. 66-69].

Cyanobacteria represent an untapped bioresource for a diverse range of bioactive compounds produced during primary and secondary metabolism, including 40% lipopeptides, 5.6% amino acids, 4.2% fatty acids, 4.2% macrolides, and 9% amides [70]. In spite of the studies carried out so far, many cyanobacteria are still largely unexplored and the antimicrobial chemicals involved are mostly unidentified, thus giving a great opportunity to discovery of new bioactive compounds [71]. Particularly, very few antibacterial compounds from cyanobacteria have been structurally characterized to date [72]. In general, it has been found that the antibacterial activity of cyanobacteria is mainly directed against Gram positive bacteria since, most Gram negative bacteria are resistant to toxic agents in the environment due to the barrier of lipopolysaccharides on their outer membrane [73]. Furthermore, several extracts of cyanobacteria have shown antifungal activity in *in vitro* test systems. They include fisherellin A, hapalindole, carazostatin, phytoalexin, tolytoxin, scytophycin, toyocamycin, tjipanazole, nostocyclamide and nostodione produced by cyanobacteria belonging to Stigonematales, Nostocales and Oscillatoriales orders [74].

Table 3. Antimicrobial activity screening of *Lyngbya aestuarii* crude extract against some bacterial and fungal strains tested by disc diffusion assay.

	Inhibition diameter (IZ, mm)*		
	Lyngbya aestuarii	DMSO	Ciprofloxacin
Bacterial strains			
Staphylococcus aureus	8	–	16
Bacillus subtilis	10	–	16
Micrococcus luteus	8	–	ND
Pseudomonas aeruginosa	8	–	14
Escherichia coli	–	–	12
Yeast strains			
Candida albicans	8	–	ND
Candida parapsilosis	8	–	ND

*: IZ is the inhibition zone including disc diameter (6 mm); ND: not determined;
−: no activity; DMSO (15 μL impregnated in a 6 mm in diameter filter paper disc was used as the negative control. Ciprofloxacin (5 μg/disk) was used as positive control for bacteria; Antimicrobial effect: inhibition zone including disc considered excellent (>10 mm), very good (>9 mm), good (>8 mm), moderate (>7 mm) and no activity (<7 mm).

Conclusion

Studies on screening of cyanobacteria with regard to their taxonomy and bioactive potential are very scarce in Morocco and those that exist have been limited to freshwater ecosystems. Thus, this research is the first one done for the assessment of antioxidant, antibacterial and anticancer activities of marine cyanobacteria from the Atlantic coast of Morocco. Based on the morphotaxonomic features and the 16S rRNA genomic similarity, the studied species was first identified as *Lyngbya aestuarii*. This species contains potent antiradicals and exhibits significant antioxidant activities by scavenging and chelating of free radical ions. The antimicrobial evaluation demonstrated that the crude extract was moderately active against the most tested pathogenic bacteria and yeasts. The antitumoral activity against HepG2, HT-29, T47D and MG-63 human cell lines using MTT assay revealed moderate cytotoxicity levels and showed that the crude extract could inhibit the growth of all of these cancer cells. Overall, these results suggest that *Lyngbya aestuarii* may be a potent source of antioxidant, antimicrobial and anticancer metabolites and further support the use of marine filamentous cyanobacteria as prominent bioresource in drug discovery efforts. However, further investigations are necessary to perform successful separation, purification and identification of the responsible metabolites having these different biological activities.

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