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Comparative Antibacterial Activity of Some Selected Seaweed Extracts from Agadir Coastal Regions in Morocco

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A B S T R A C T

Seaweeds are rich sources of natural bioactive products. Their interesting biological activities contributed to the investigation of natural therapeutic compounds used in biomedical and pharmaceutical applications. In this study, methanolic extracts of four seaweeds collected from the Moroccan Atlantic coastline (Agadir) *Bifurcaria bifurcata*, *Corallina elongata*, *Corallina officinalis* and *Ulva fasciata* were investigated for antimicrobial activities using the disc diffusion and broth microdilution methods. The extract bioactivity was expressed as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against four human pathogenic bacteria *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. The results indicated that among the algal samples screened, the methanolic extract of *B. bifurcata* and *U. fasciata* exhibited the highest potency against all pathogens tested, with MIC ranged from 0.11 to 3.75 mg/ml depending on the bacterial species. The lowest MIC and MBC values were recorded for methanolic extract of *B. bifurcata* against *Bacillus subtilis* (0.11 mg/ml, 7.50 mg/ml) and *E. coli* (0.23 mg/ml, 7.50 mg/ml). Therefore, the present findings suggest that local seaweeds, chiefly *B. bifurcata* may be considered as an interesting biological source of bioactive compounds and should be investigated for natural antibiotics to control some pathogenic bacteria strains such as *E. coli* and *B. subtilis*.

Introduction

Nowadays, intensive use of several forms of synthetic antibiotics to treat bacterial infections has resulted dramatically in emergence of resistant pathogenic microorganisms, often due to indiscriminate use of these substances. Hence, giving treatment against these infections becomes more difficult and generates constraints for clinicians about therapeutic options (Kandhasamy and Arunachalam, 2008). This serious worldwide problem has led researchers to explore new sources of natural products to control bacterial pathogens, with low toxicity and side effects. These

alternatives are also expected to be biodegradable and environmentally friendly. In this context, several studies have indicated that seaweeds are an interesting source of many biologically active compounds with a broad range of antibacterial activity (Ravikumar *et al.*, 2011). Like terrestrial plants, the marine macroalgae contain various kinds of organic and inorganic substances beneficial to human health (Farasat *et al.*, 2014). Compared with agricultural crops they have a high biomass productivity and do not rely on arable land and freshwater. Aside from their ecological impacts in coastal ecosystems, their socio-economic value and their natural role, seaweeds are a promising source of bioactive molecules that can be used in pharmaceutical field, biomedicine, food and agriculture (Abowei and Ezekiel, 2013; Rebours *et al.*, 2014). Bioactivity of marine seaweed extracts and fractions have recently been the research interest in various fields, with relatively little regard to their potential antimicrobial applications. These organisms are subjected to diverse environmental conditions, and thus have developed special physiological adaptations that improve endogenous defense mechanisms, which induce synthesis of different secondary metabolites (Duffy and Hay, 1990; Águila-Ramírez *et al.*, 2017).

Many substances isolated from red, brown and green marine macroalgae have been associated with a wide variety of pharmacological properties, such as antibacterial, antiviral, antifungal, insecticidal, antitumor and antioxidant activities (Shelar *et al.*, 2012; Alves *et al.*, 2016; Abirami and Kowsalya, 2017). Numerous chemical compounds were identified as antimicrobial agents including phenolic compounds, phlorotannins, terpenoids, fatty acids, chlorellin and steroids (Kavita *et al.*, 2014). However, the quantitative ranges of these bioactive molecules can fluctuate widely according to season, environmental conditions, geographical location and reproductive stage (Marinho-Soriano *et al.*, 2006). It has been reported that among the algae groups, Phaeophyceae exhibited the highest antibacterial activity and 75% of the most secondary metabolites were derived from brown algae (Alves *et al.*, 2016). Currently, a large number of structurally unique bioactive agents derived from seaweeds have been identified and some of them are under experimentation or are being developed as new pharmaceutical drugs (Manikandan *et al.*, 2011).

In Morocco, seaweeds are abundant and widely distributed along its two vast coastlines of more than 3500 km. This algal richness represents an important biodiversity reserve of various species, with more than 500 taxa (Bengueddour *et al.*, 2014). However, these resources having high economic value, are poorly exploited, except the agarophyte *Gelidium sesquipedale*, which is overharvested along the Atlantic littoral and used for the industrial production of agar-agar (Givernaud *et al.*, 2005).

Investment in this area requires the inventory of Moroccan algal resources on both its Atlantic and Mediterranean coasts, as well as exploration of new approaches to manage and valorize these marine natural resources, which represent a source of useful products. Therefore, the present study was aimed to investigate antibacterial activity of methanolic extracts, prepared from four local abundant seaweeds *Bifurcaria bifurcata*, *Corallina elongata*, *Corallina officinalis* and *Ulva fasciata* collected from the Moroccan Atlantic coastline (North of Agadir city) against four human pathogenic bacteria *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*.

Materials and Methods

Sample collection

In this work, four intertidal marine macroalgae *Corallina officinalis* and *Corallina elongata* (Rhodophyceae), *Bifurcaria bifurcata* (Phaeophyceae) and *Ulva fasciata* (Chlorophyceae) were harvested during low tide on May 2017 from the Moroccan Atlantic coast at Tamri National Park area, northern Agadir coastline. Immediately after their arrival at the laboratory, the algae were cleaned, washed thoroughly with distilled water to eliminate salt, epiphytes and sand particles, and then were air-dried in the shade at room temperature. The dried algae were grounded to fine powder, packed in an airtight container and stored at 4°C until analysis.

Preparation of algal extracts

The extraction with methanol, which has the ability to dissolve both non-polar and polar compounds, was conducted by cold maceration method as described by Sridharan and Dhamotharan (2012) with modifications. This technique was chosen since it is less destructive to most chemical compounds with comparison to hot extraction. A weighed amount of 20 g of each seaweed powder was soaked in 200 ml of methanol (90%) by maceration with constant agitation for 48h at room temperature.

The extracts were sonicated in an ultrasonic bath for 30 min then filtered. The filtrates were concentrated in a rotary evaporator under reduced pressure with controlled temperature (45°C) to remove the solvent. The dried extracts were then dissolved in a small volume of methanol and sonicated for twenty minutes to increase solubility. After that, the resulting extracts were stored at -4°C in airtight brown glass bottles. The methanolic extracts were further tested for their antibacterial activity against the investigated pathogens.

In-vitro antibacterial tests

Disc diffusion method

Antibacterial activity was carried out using the disc diffusion method (Seenivasan *et al.*, 2012) against four human pathogenic bacteria *Staphylococcus aureus* (CECT 976), *Escherichia coli* (ATCC 8739), *Bacillus subtilis* (DSM 6633) and *Pseudomonas aeruginosa* (CECT 118) obtained from the bacterial collection of the Microbial Biotechnology and Vegetable Protection Laboratory, Faculty of Sciences, Agadir, Morocco. Twenty microliters of each seaweed extract was applied to a sterile Whatman No. 1 filter paper disc of 6 mm diameter. Negative control was done using paper disc loaded with 10 µl of the methanol solvent. After evaporation, the discs were placed on sterile Muller Hinton agar (MHA) plates previously inoculated with a fresh cell suspension adjusted at 10^6 CFU/ml of the tested bacteria.

Each plate contained six paper discs placed about equidistance to each other: Four treated discs impregnated with different seaweed methanolic extracts (5 mg/ml), one positive control, which is a standard commercial antibiotic disc Penicillin (10 units/disc) and one negative control disc. The plates were kept for 2h in the refrigerator at 4°C to allow diffusion of the extracts into the media, and then incubated overnight at 37°C. Antibacterial activity was evaluated by measuring the growth inhibition zone diameter around the discs in millimeters. It was calculated as an average of tree determinations measured in three different directions. Each experiment was tested in triplicate, to minimize the error, and results were expressed as mean ± standard deviation.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

To quantify the antibacterial activity, MIC and MBC were determined using a broth micro dilution method and tetrazolium salt, 2,3,5-Triphenyltetrazolium Chloride (TTC) (Favarin *et al.,* 2019). Mother solutions obtained for each seaweed extract tested were dissolved in methanol and then serially diluted in 96-well microtiter plates to obtain concentrations ranging from 0.05 to 30 mg/ml. Under laminar flow, 20 µl of an actively growing inoculum of the tested bacterial species, adjusted at 10^6 CFU/ml, was added to each well. The microplates were covered with sterile plastic cover and cultures were incubated for 24h at 37°C with shaking on an orbital shaker to fully mix the contents. In each plate, some wells were reserved to test the medium sterility control (MHB alone) and inoculum viability control (MHB with inoculum only). Microbial growth was revealed by adding to each well 20 µl of solution containing 2% of triphenyl tetrazolium chloride (TTC) and the microplates were again incubated at 37°C for 2 hours (Klhar *et al.,* 2019). The TTC indicator solution changes from colourless to purple-red in the presence of bacterial activity. The lowest sample concentration showing no visible antimicrobial growth was considered as the MIC value expressed in mg/ml.

To determine the minimum bactericidal concentration, 10 µl of suspension from each well that did not exhibit any visible growth during MIC assays, were transferred into MHA plates and incubated at 37°C for 24h. MBC was determined as the lowest concentration of extract that completely inhibited bacterial growth. The tests were performed in triplicate and the experiment is repeated twice.

Statistical analyses

All assays were carried out in triplicate and results were expressed as mean ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Newman-Keuls multiple comparison test, when the F value was significant ($p<0.05$). Experimental data were statistically processed by Xlstat software for windows version 2016.

Results and Discussion

Antibacterial activity

The antimicrobial activity of the methanolic extract was evaluated against two Gram-negative strains of *Escherichia coli* and *Pseudomonas aeruginosa* and two Gram-positive strains of *Staphylococcus aureus* and *Bacillus subtilis.* In vitro antibacterial potential of extracts was detected by the presence of inhibition zone observed around the filter paper discs (figure 1).

The methanolic extracts prepared from the four different macroalgae belonging to three classes (Chlorophyceae, Phaeophyceae and Rhodophyceae) showed various degrees of activity against the pathogenic bacteria at 5 mg/ml (Table 1).

Among the algal samples screened for antibacterial activity, the methanolic extract of *B. bifurcata* (Phaeophyceae) showed the highest inhibition zone diameter against most tested organisms, especially *Bacillus subtilis* followed by *Escherichia coli* and *Staphylococcus aureus* with 14.33 mm, 13.00 mm and 12.00 mm respectively. It is important to highlight that *B. subtilis* value was closely near that of the positive antibacterial standard Penicillin (10 units/disc). Moderate zone of inhibition (8.33 to 10.66 mm) was recorded in methanol

extract of *U. fasciata* (Chlorophyceae) toward all tested microorganisms, except *P. aeruginosa*, which was also resistant to all the other algal extracts examined in this study. On the other hand, little or no antibacterial activity was recorded for the rest of seaweed extracts, except for *C. officinalis*, which revealed an inhibition zone diameter of 9.66 mm toward *B. subtilis* strain.

Determination of MIC and MBC

The antibacterial activity was confirmed by determining the minimum inhibitory concentration (MIC). As presented in Table 2, the extracts exhibited varying degrees of antimicrobial effect against the microorganisms tested (MIC were ranged between 0.11 to 15 mg/ml). *Bifurcaria bifurcata* methanolic extract were found to be the most effective in inhibiting the four bacteria (MIC was between 0.11 to 1.87 mg/ml) and the lowest MICs values was recorded for *Bacillus subtilis*, followed by *Escherichia coli* (0.23 mg/ml), *Staphylococcus aureus* (0.46 mg/ml) and *Pseudomonas aeruginosa* (1.87 mg/ml) (Figure 2). Except for this last strain, *B. bifurcata* extract showed the strongest bactericidal activity, especially on *E. coli* and *B. subtilis* at 7.50 mg/ml. The same trend was observed for the other seaweed extracts but with significantly lower levels (MBC values greater than 7.50 mg/ml). The most sensitive pathogen to all the extracts was *Bacillus subtilis*, whereas all the extracts showed high MBC value (>30 mg/ml) for *P. aeruginosa*, which was the most resistant.

The majority of the methanolic algae extract in this study exhibited relatively good to moderate activity against the investigated human pathogens *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*, with the MIC values ranging from 0.11 mg/ml to 7.5 mg/ml. This may be related to the ability of methanol solvent to extract both lipophilic and lipophobic phytoactive components with strong antimicrobial properties (Jepkoech and Gakunga, 2016). Indeed, while different solvents have been used in screening seaweed properties, many researchers reported that methanol is the greatest and appropriate solvent for extraction of bioactive compounds from marine algae, with higher antioxidant and antibacterial activity (Kolsi *et al.,* 2015; Arulkumar *et al.,* 2018).

From the present results, it was found that the antibacterial effects of the methanolic extracts were more pronounced with the brown algae *B. bifurcata*. The best activities were detected against *B. subtilis*, *E. coli* and *S. aureus*. Similarly, the results of Oumaskour *et al.,* (2012) coincided with this study, they found also that methanolic extract of *B. bifurcata* inhibited the growth of most pathogens studied, but in contrast to our findings, it did not affect *E. coli*. As well, several other seaweed species extracts tested towards this enteric pathogen, showed low or no antibacterial activity (Arunkumar *et al.,* 2010). This difference in results may be due to the extraction method, season in which samples were collected and intraspecific diversity in bioactive compounds production (Karabay-Yavasoglu *et al.,* 2007; Kandhasamy and Arunachalam, 2008). Both MIC and MBC values confirmed the disc test results, and showed that the different extracts had more significant bacteriostatic than bactericidal activities. The lowest values (0.11 and 7.50 mg/ml respectively) was recorded for *B. bifurcata*, which indicated that the latter might more easily reach therapeutic concentration in further biological studies. These values were below that reported earlier by Pais and al. (2019), where extraction was performed from the same seaweed using dichloromethane solvent.

Table.1 Antibacterial activity of methanolic seaweed extracts tested based on disc diffusion method, inhibition zones in millimeters

Seaweeds	*E. coli*	*S. aureus*	*B. subtilis*	*P. aeruginosa*
B. bifurcata	13.00±1.73[b]	12.00±1.12[b]	14.33±0.57[b]	-
C. elongata	-	-	-	-
C. officinalis	-	-	9.66±1.52[d]	-
U. fasciata	9.00±1.20[c]	8.33±1.15[c]	10.66±0.57[c]	-
Positive control**	16.83±1.41[a]	15.50±0.57[a]	15.20±0.34[a]	13.66±0.76
Negative control*	-	-	-	-

The values represent the average of three measurements ± SD. For each column, means having the same ending letter do not differ significantly ($p<0.05$) by Newman-Keuls test. **: Penicillin (10 unit/disc), *: Methanol (10 µl/disc), -: No effect at tested concentration

Table.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values, expressed in mg/ml, of seaweed methanolic extracts against the four pathogenic bacteria

Seaweeds	*E. coli*		*S. aureus*		*B. subtilis*		*P. aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. bifurcata	0.23	7.50	0.46	15	0.11	7.50	1.87	>30
C. elongata	7.50	>30	7.50	>30	0.46	>30	15	>30
C. officinalis	3.75	15	7.50	>30	0.23	15	7.50	>30
U. fasciata	0.46	15	0.93	15	0.11	15	3.75	>30

>30 indicated no bactericidal activity or the bactericidal concentration was beyond the tested concentration

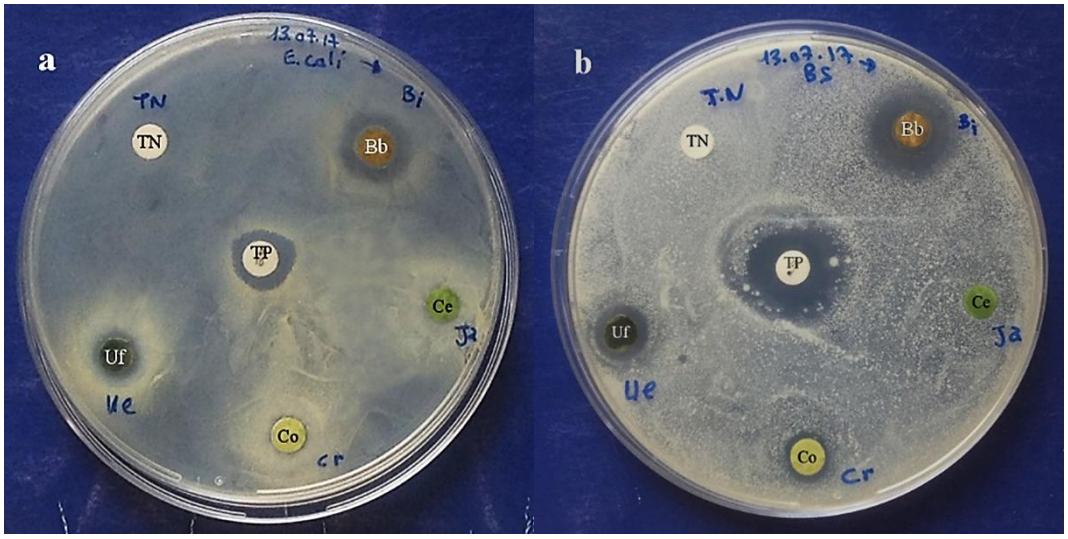

Figure.1 Antibacterial activity against *E. coli* (a) and *B. subtilis* (b). Bb: *B. bifurcata*, Ce*: C. elongata*, Co: *C. officinalis*, Uf: *U. fasciata*,TP*:* Standard commercial antibiotic disc (Penicillin), TN: Negative control disc (methanol)

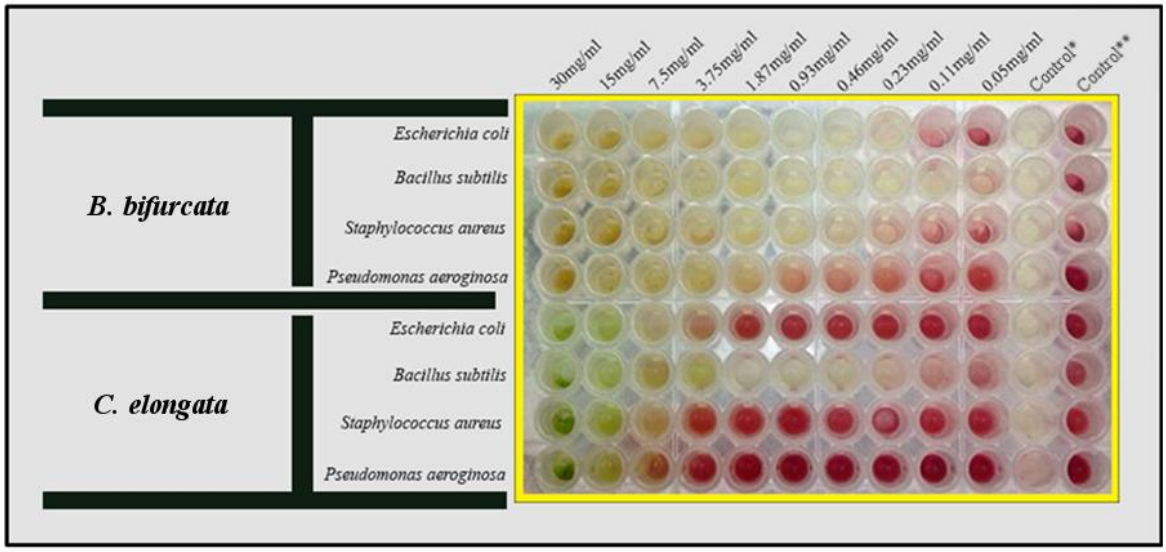

Figure.2 Microtiter plate prepared for MIC determination of *B. bifurcata* and *C. elongata* methanolic extracts against tested pathogenic bacteria. Red wells indicate the absence of bacterial inhibitory, *: Sterility control, **: Viability control

Previous reports noted that the high antibacterial activity observed in brown seaweeds was probably due to the presence of various bioactive compounds such as sulphated polysaccharides, peptides, amino acids, lipids and polyphenols (Vallinayagam *et al.*, 2009; Gupta and Abu-Ghannam, 2011). Nagayama and al.(2002) reported in more detail the chemical composition of *B. bifurcata*, and based on their observations, they suggested that the antibacterial activity of this genus would be related directly to the interactions of phlorotannins with bacterial proteins. These molecules are the exceptional group of tannins in Phaeophyceae class, which are polar, very reactive, and exhibit prominent antioxidant activity. They are highly hydrophilic and constitute polyphenolic secondary metabolites derivatives obtained by polymerization of phloroglucinol-based phenolic compounds (Meillisa *et al.*, 2013).

The green marine macroalga *U. fasciata* also displayed good antibacterial activity, although higher concentrations were needed to achieve better control of the bacteria growth. This result agreed with previous studies suggesting the richness of its methanolic extract in bioactive compounds that can act as antibacterial agent (Christabell *et al.*, 2011; Chandrasekaran *et al.*, 2014). Phytochemical screening of this seaweed indicated the presence of carbohydrates, phenol, terpenoids, flavonoids and steroids (Lake, 2016). In general, the complexity of antimicrobial properties in seaweeds may be attributed to their multiple inhibitory mechanisms. Hence, several research indicated that these beneficial bioactivities are attributed to the synergetic effect of different active compounds mainly present in the same macroalgal extract (Kotnala *et al.*, 2009).

The lowest antimicrobial activity was observed for the two red algae, mainly *C. elongata*, for which the MIC values obtained against the four tested bacteria were found to be comparatively very high. This result was in good accordance with other studies reporting that this species of seaweed did not inhibit the growth of the above microorganisms (Salvador *et al.*, 2007; Farid *et al.*, 2012). These results, however, were in contrast to

those of Osman and al. (2012) who found that methanolic extract of *C. elongata* collected from Egyptian Mediterranean coast exhibited significant antibacterial effect. However, these differences in activity might be due to geographical variations, developmental stages or extraction methods.

On the other hand, it should be noted that *P. aeruginosa* was the only bacteria that showed very low or no activity with all the tested extracts (MBC>30), since it is known to have high level of intrinsic resistance to a wide variety of antimicrobial and antibiotic agents. Resistance to this clinical pathogen, causing severe nosocomial infections, was also reported against twenty macroalgal extracts tested (Selvi *et al.*, 2001; Christabell *et al.*, 2011). It has been suggested that the primary mechanism of this natural multidrug resistance may be due to the extremely restrictive permeability of the *P. aeruginosa* outer membrane barrier (Solórzano-Santos and Miranda-Novales, 2012).

The conclusion of this study clearly showed that the Moroccan seaweed extracts collected from northern Agadir coastline had antimicrobial activity against some pathogenic bacteria strains including *E. coli*, *B. subtilis* and *S. aureus*. Consequently, these marine algae, chiefly *B. bifurcata*, may be considered as a biological source of bioactive compounds and should be investigated for natural antibiotics in treating diseases such as enteric infections caused by these microorganisms. Nevertheless, further phytochemical investigations are needed to identify and characterize the chemical components responsible for the antimicrobial effect of each seaweed extract.

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