ANTIMICROBIAL PROPERTIES OF SEVEN BROWN ALGAE HARVESTED FROM THE COAST OF SIDI BOUZID (EL JADIDA-MOROCCO)

NAZHA SAMRI, LAILA HSAINE, SOUKAINA ELKAFHI, SAMIRA KHLIFI, SAMIRA ETAHIRI

Marine Biotechnology and the Environment Laboratory (BIOMARE) Faculty of Science, University Chouaib Doukkali-El Jadida-Morocco

Email: samri.nazha@gmail.com

ABSTRACT

Objective: This work aims at the screening of the antimicrobial activity of the seven brown marine algae of the Coast of Sidi Bouzid (El Jadida-Morocco).

Methods: The aim of this study was to evaluate the antimicrobial activity of seven brown marine algae against three Gram-positive bacteria (Staphylococcus epidermidis, Staphylococcus aureus and Streptococcus pyogenes). Three Gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumonia) and two fungi (Aspergillus Niger and Candida tropicalis). Thus, 35 algal extracts were prepared with five organic solvents methanol/water, methanol, dichloromethane/methanol, dichloromethane and ethyl acetate. The antibacterial activity was evaluated through the disk diffusion method.

Results: Data revealed that the Staphylococcus aureus bacteria was the most sensitive pathogen by showing the highest zone of inhibitions of 20 mm with the lowest Minimum Inhibitory Concentration (MIC) of 2 μgmL⁻¹ methanol/water extract of Cystoseira tamariscifolia. Whereas, antifungal activity, the highest zone of inhibitions of 21 mm and 22 mm with the lowest Minimum Inhibitory Concentration (MIC) of 5 μgmL⁻¹ was respectively shown in the methanol/water extract of Laminaria ochroleuca against Candida tropicalis and in the dichloromethanol extract of Sargassum vulgare against Aspergillus niger.

Conclusion: The results indicate that these algal extracts can further be analyzed and purified for relevant antibacterial and antifungal compounds which can be used in therapeutics and other applications.

Keywords: Brown algae, Antibacterial activity, Antifungal activity, Solvent extracts

INTRODUCTION

Marine species, comprising approximately a half of the total global biodiversity, are a rich source of structurally diverse bioactive compounds with various biological activities. Thus, their importance as a source of novel bioactive substances is growing rapidly. Among marine organisms, algae are rich sources of bioactive compounds with various biological activities. Recently, their value as a source of novel bioactive substances has become important. Moreover, researchers have revealed that marine algal originated compounds exhibit various biological activity [1-3].

Most of the secondary metabolites biosynthesized by the marine algae are well-known for their cytotoxic [4], anti-inflammatory [5-8] property, their numerous studies have revealed the anti-bacterial [9-13], antifungal activity [14, 2, 3] and antioxidant [15, 16] properties in different macro-algae.

The growing resistance of bacteria to present antibiotics is a major problem worldwide. One way to prevent this resistance is the development of new compounds different from the existing synthetic antimicrobials. Thus, the search for new natural sources of marine ecosystems has led to the isolation of new algal antibiotics such as acrylic acid, halogenated aliphatic compounds, terpenes, sulfur-containing heterocyclic compounds and phenolic inhibitors [17-19].

This work aims to evaluate the antimicrobial activity of seven seaweeds collected from the coast of Sidi Bouzid, El Jadida against clinical multidrug resistant bacteria and fungi in order to discover new compounds with important antimicrobial activity.

MATERIALS AND METHODS

Sampling

The brown algae were carefully removed manually along the coast of Sidi Bouzid, El Jadida (33°3’36”N, 8°30’05”W), in April 2015. The collected algae were: Sargassum muticum (Yendo) Fensholt 1955, Fucus spiralis Linnaeus 1753, Cystoseira tamariscifolia (Hudson) Papenfuss 1950, Sargassum vulgare C. Agardh 1820, Cystoseira humilis var. myriophylloides (Sauvageau) J. H. Price and D. M. John 1978, Bifurcaria bifurcata R. Ross 1958 and Laminaria ochroleuca Bachelor Pylae 1824. Algae were initially washed in seawater to remove the macroscopic epiphytes, particles and other extraneous matters and then rinsed in distilled water. Later, algae were air-dried at room temperature and ground to a fine powder for further analysis.

Preparation of extracts

The prepared powder for each species was extracted in different solvent: methanol/water (40/60), methanol, dichloromethane/methanol (50/50), dichloromethane and ethyl acetate at a rate of 1g of alga powder/5 ml of solvent during 72 h at ambient temperature according to the extraction protocol described by caccamese [20], then the extracts are filtered on Whatman paper N°1 and evaporated in a rotary evaporator. The dry extracts obtained are stored at 4 °C. For methanol water extract the evaporated extract was also lyophilised until later use for the biological tests.

Bacterial and fungal pathogens

The strains used for these test were obtained from the Collection of Institute Pasteur of Paris (CIP), from American Type Culture Collection (ATCC) and from Mohamed V Hospital (El Jadida Morocco). The Gram-positive bacteria included: Staphylococcus epidermidis, Staphylococcus aureus (ATCC25925) and Streptococcus pyogenes. Gram-negative bacteria were Pseudomonas aeruginosa (ATCC9027), Escherichia coli (ATCC10536) and Klebsiella pneumonia. Fungi used were Candida tropicalis (ATCC127581) and Aspergillus niger (CIP 1275).
Antimicrobial bioassays

Antibacterial assays were carried out using the agar disk-diffusion assay Bauer [21]. Three colonies of each bacterium were removed with a wire loop from the original culture plate, and were introduced into a test tube containing 5 ml broth. An overnight culture yielded a suspension of 10^8 bacteria/ml (evaluated by the absorbance value of 0.5 at 620 nm) with sterile water to inoculate Petri dishes containing culture media (12 ml Mueller-Hinton agar, 3 mm thick). Plates were dried for 30 min before inoculation. The organic extracts were tested using paper disks (6 mm diameter) impregnated with the 150 µg of each extract, after the temperature was equalized at 4 °C; the plates were incubated overnight at 37 °C. Diameters of inhibitory zones were then measured. The Streptomyces (100 µg) and Ofloxacin (50 µg) susceptibility test discs were used as the positive control.

For fungidical activity, zones of inhibition were determined after 24 h of incubation at 27°C. Discs impregnated with standard antibiotics such as Amphotericin B were used at 100 µg as reference in the test of antifungal activity. In addition, control disks were prepared with each solvent. All tests were at least triplicate.

The antimicrobial activity was classified from low active (+: diameter of inhibition<10 mm), moderately active (++: 10 mm ≤ diameter of inhibition<15 mm) to highly active (+++: 15 mm ≤ diameter of inhibition) and inactive (-: no/very hazy inhibition zone) [22].

Minimum inhibitory concentration (MIC)
The minimum inhibitory concentration (MIC), using microdilution plate method with resazurin Sarker [23], was determined. Briefly, the 96-well microplate was prepared by dispensing 100 µl of Mueller-Hinton broth (bacteria strain) or PDA (fungi strains) into each well. 100 µl from the stock solution of tested extract (concentration of 40 mg/ml) were added into the first row of the plate. Then, two-fold, serial dilutions were performed by transferring 100 µl of solution from one row to another, using a multichannel pipette. The obtained concentration range was from 20 mg/ml to 3.10^-3 mg/ml. 10 µl of each 106 CFU/ml bacterial suspension were added to wells. Finally, 10 µl of resazurin solution were added. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37 °C for 24 h for bacteria strains but for fungi strains the plates were incubated at 22 °C for 48 h. MIC was defined as the lowest concentration of the tested algae extracts that prevented resazurin color change from blue to pink. Antibiotic streptomycin and ofloxacin, dissolved in Mueller-Hinton broth, were used as positive controls for bacteria strains, for fungi strains amphotericin B was used as antibiotic. Solvent control test was performed to study an effect of 10% DMSO on the growth of bacteria. It was observed that 10% DMSO did not inhibit the growth of bacteria. Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

RESULTS AND DISCUSSION

The paper described the antimicrobial activity of seven brown algae collected from the coast of Sidi Bouaïd El Jâdida against clinical multidrug resistant bacteria and fungi. The results of the screening of antibacterial and antifungal activities against bacteria and yeast are summarized in tables 1 and 2.

Antibacterial activity

The antibacterial activity of thirty five organic extracts (methanol/water (40/60), methanol, dichloromethane/methanol (50/50), dichloromethane and ethyl acetate) obtained from seven seaweeds species against six pathogenic bacteria was studied in comparison to the reference drugs Streptomycin (100 µg) and Ofloxacin (50 µg). Results obtained were reassembled in table 1.

### Table 1: Antibacterial activity of seven brown seaweeds extracts against pathogenic bacteria

| Algae            | Diameter of inhibition (mm) | Gram positive bacteria | Gram negative bacteria |
|------------------|----------------------------|------------------------|------------------------|
|                  | Solvent extraction         | Streptococcus pyogenes | Staphylococcus aureus  | Staphylococcus epidermidis | Klebsiella pneumonia | Pseudomonas aeruginosa | Escherichia coli |
| S. muticum       | MeOH                       | 11±0.768               | 12±0.577               | 7±0.000                  | 14±0.408             |
|                  | MeOH/DC                    | 9±1.424                | 7±0.000                | 8±0.000                  | 12±0.734             |
|                  | DC                         | 10±1.577               | 12±1.154               |
| F. spiralis      | MeOH/DC                    | 8±0.416                | 10±0.000               | 8±0.000                  | 7±0.000               |
| C. tamariscifolia| MeOH/DC                    | 8±0.000                | 7±0.000                | 8±0.000                  | 7±0.000               |
| S. vulgare       | MeOH/DC                    | 7±0.000                | 9±1.000                | 10±1.154                 | 9±0.577               |
| C. humidis       | MeOH/DC                    | 8±0.000                | 9±1.154                | 7±0.333                  | 13±0.433              |
| L. digitata      | MeOH/DC                    | 8±0.000                | 8±1.000                | 7±0.000                  | 12±0.734              |
| B. bifurcata     | MeOH/DC                    | 7±0.000                | 7±0.000                | 8±0.000                  | 11±0.000              |

MW: methanol water (40/60), M: Methanol, M/DC: Methanol/Dichloromethane, DC: Dichloromethane (50/50), EA: Ethyl acetate. Resistant values are mean ± standard deviation of three replicates.
The presence of a positive activity on Gram-positive and Gram-negative bacteria was observed in all seven brown algae tested (table 1). This result is similar to the one described by Ara [24] who revealed that extracts of brown algae were active against a number of Gram-positive and Gram-negative bacteria.

For Gram-positive bacteria, higher activity (was obtained against Staphylococcus aureus with methanol/water extracts of F. spiralis, S. muticum, C. tamariscifolia, S. vulgare and with dichloromethane extract of S. muticum. Against Staphylococcus epidermidis, the best inhibition was obtained with methanol/water extract of F. spiralis, C. tamariscifolia, L. ochroleuca, ethyl acetate extract of C. tamariscifolia, C. humilis and methanol extract of F. spiralis. For these species, other extracts prepared exhibited a moderate activity against Gram-positive bacteria; their diameter of the inhibition was ranged from 10 mm to 15 mm.

Against Pseudomonas aeruginosa (Gram-negative bacteria), higher activity was obtained with methanol/water extracts of F. spiralis, C. tamariscifolia and S. vulgare. While, for Escherichia coli, the moderate activity was obtained by methanolic extract of S. muticum, dichloromethane/methanol extract of F. spiralis and with methanol/water extract of B. bifurcata who exhibited an important antibacterial activity against other pathogenic bacterial strains [25, 26, 7]. In earlier work, metabolite with antibacterial activity against Escherichia coli has been isolated from C. tamariscifolia and was characterized as methoxy bifurcarenone [27].

Among the seven algae tested, F. spiralis exhibited higher antibacterial activity against four bacteria, followed by C. tamariscifolia which exhibited in important antibacterial activity against three bacteria. The present study reveals that methanol/water mixture is better for extraction of anticancer fraction compared with other solvents.

Some studies concerning the effectiveness of solvent used for extraction reported that methanol extraction yielded higher antibacterial activity than other organic solvents [28-30].

The difference between different results may be due to the strain sensitivity, seasonal variation [31-34], ecological parameters such as: temperature, salinity, light, dissolved oxygen and nutrients, or related to the biology and physiology of the seaweed itself [35] and efficiency of extraction methods to recover active metabolites. Different solvents used show different antimicrobial activity depending upon their solubility and polarity [9, 36, 37]. Therefore, chemical compounds should be extracted from different seaweeds in order to optimize their antibacterial activity by selecting the best solvent system [2].

In addition, the seven brown algaes tested strong activity against Gram-positive bacteria than Gram-negative bacteria which was observed. This result is in agreement with earlier reports [30, 38, 39] who proved that Gram-positive bacteria was more sensitive than Gram negative bacteria. Among six bacterial strains tested, Staphylococcus epidermidis is the most sensitive. While, Streptococcus pyogenes and Escherichia Coli are the most resistant.

The present screening revealed that the highest antibacterial activity in some brown algae indicates the presence of active compounds which can be exploited for the production of innovation drugs. In other species such as C. humilis, the inhibitory activity was only observed in the extract obtained with one kind of solvent but not in extracts obtained with other solvents. Which may suggest that a particular solvent is required to extract some antibacterial substances within the algal plant. Therefore, the percentage of inhibitory activity will go up when several solvents are used in the screening [2].

### Antifungal activity

The results of the antifungal test of each extracts (methanol/water [60/40], methanol, dichloromethane/methanol [50/50], dichloromethane and ethyl acetate) against Candida tropicalis and Aspergillus niger are summarized in table 2.

| Seaweed species | MW | MeOH | MeOH/DC | DC | EA | Amphotericin B (100µg) |
|----------------|----|------|---------|----|----|----------------------|
|                | C. t | A. n | C. t | A. n | C. t | A. n | C. t | A. n | C. t | A. n | C. t | A. n |
| S. muticum     | 9±0.60 | - | 17±0.76 | - | - | - | 10±0.00 | - | 7±0.577 | - | 12±0.50 | - |
| F. spiralis    | 8±1.00 | 3 | 12±1.00 | - | - | - | 10±0.00 | 17±0.88 | - | 10±1.04 | - |
| C. tamariscifolia | 8±1.33 | - | 10±1.52 | - | - | - | 14±0.50 | 7±0.577 | - | 23±1.000 | 15±0.577 |
| S. vulgare     | - | 9±0.731 | - | - | - | 22±0.76 | - | - |
| C. humilis     | 8±0.57 | - | - | - | - | 7±0.000 | - | - | - |
| L. digitata    | - | 18±0.86 | 16±0.76 | 21±0.98 | - | - | 12±1.73 | - |
| B. bifurcata   | - | 18±0.57 | 9±1.201 | 12±0.33 | - | 7±1.00 | - | - |

C. t: Candidas tropicalis, A. n: Aspergillus Niger, MW: methanol water [50/50], M: Methanol, M/DC: Methanol/Dichloromethane [50/50], DC: Dichloromethane, EA: Ethyl acetate; -: No activity. Values are mean±standard deviation of three replicates.

Of the seven brown algae tested, six species showed a positive activity against Candida tropicalis and Aspergillus niger. Concerning Candidas tropicalis, very important activity was observed in the dichloromethane/methanol extract and in methanolic extract of L. ochroleuca, methanolic extract of B. Bifurcata and methanolic extract of S. muticum (table 2). These results are in agreement with those obtained by Rizzo [40] which shows that B. bifurcata, F. spiralis and C. humilis possess an important antifungal activity.

Concerning Aspergillus niger, a very important activity has been observed in the dichloromethane extract of S. Vulgare; this activity was better compared to that obtained with the control (Amphotericin B at100µg): diameter of inhibition of S. vulgare and the control was respectively 22 mm and 15 mm. Very important activity has been also observed in the dichloromethane extract of F. spiralis and in methanolic extract of L. ochroleuca. Wagh [41] and Saleh [3] showed that extracts of some brown algae are the most active against Aspergillus niger.

Estimated minimum inhibitory concentration (MIC)

In the current investigation MIC values, as useful parameters, have been estimated in order to screen algal inhibitory effects (table 3). MIC results for the algal species tested against the different microorganisms are presented in the tables 3 and 4.
The extracts prepared by methanol/water solvent from C. tamariscifolia were more active against Gram-positive bacteria Staphylococcus aureus (MIC = 2 µg/ml). Followed by methanol/water extract of S. muticum, F. spiralis and S. vulgare against the same strain (MIC = 5 µg/ml). The methanol/water and methanolic extracts of F. spiralis were more active against respectively Gram-negative bacteria Pseudomonas aeruginosa and Klebsiella pneumoniae (MIC = 5 µg/ml). Followed by methanol/water extract of B. bifurcata against Escherichia Coli (MIC = 10 µg/ml). Grozdanic [43] reported that the dichloromethane/Methanolic extract of C. humilis was more effective against following bacterial species: Staphylococcus aureus, Escherichia coli (MIC = 6250 µg/ml) and Klebsiella pneumoniae (MIC = 12500 µg/ml).

Sónia and al. [44] studied brown seaweed B. bifurcata. They found that activity against Staphylococcus aureus (MIC = 1024 µg ml⁻¹) and Escherichia Coli (MIC = 2048 µg ml⁻¹) was observed. While no growth inhibition of Staphylococcus epidermidis and Pseudomonas aeruginosa was verified in the range of concentrations tested (MIC=2048 µg.ml⁻¹).

Our results show that B. bifurcata extract exhibited inhibition against both Gram-negative and Gram-positive bacteria. In opposition to that observed by Alves [45], which only verified activity of B. bifurcata dichloromethanolic extract against Gram-negative bacteria.

### Table 3: Algal minimum inhibitory concentration (MIC) values using different solvents against pathogenic bacteria

| Algae            | Solvent extraction | Streptococcus pyogenes | Staphylococcus aureus | Staphylococcus epidermidis | Klebsiella pneumoniae | Pseudomonas aeruginosa | Escherichia Coli |
|------------------|--------------------|------------------------|-----------------------|---------------------------|-----------------------|------------------------|------------------|
| S. muticum       | M/Dc               | 312                    | 625                   | 78                        | 1250                  | 156                    | 2500             |
| F. spiralis      | M/Dc               | 78                     | 312                   | 39                        | 625                   | 78                     | 20               |
| C. tamariscifolia| M/Dc               | 156                    | 2500                  | 156                       | 625                   | 312                    | 2500             |
| L. ochroleuca    | M/Dc               | 5000                   | 78                    | 5000                      | 156                   | 78                     | 2500             |
| B. bifurcata     | M/Dc               | 156                    | 78                    | 156                       | 78                    | 312                    | 2500             |
| F. spiralis      | EA                 | 20                     | 19                    | 312                       | 312                   | 312                    | 2500             |
| C. humilis       | MW                 | 156                    | 78                    | 78                        | 39                    | 2500                   | 78               |
| L. ochroleuca    | M/Dc               | 312                    | 312                   | 312                       | 78                    | 78                     | 78               |
| B. bifurcata     | EA                 | 1250                   | 1250                  | 1250                      | 1250                  | 1250                   | 1250             |
| S. muticum       | EA                 | 78                     | 78                    | 78                        | 78                    | 78                     | 78               |
| F. spiralis      | EA                 | 5000                   | 5000                  | 5000                      | 5000                  | 5000                   | 5000             |

MW: methanol/water (40/60), M: Methanol, MdC: Methanol/Dichloromethane, DC: Dichloromethane (50/50), EA: Ethyl acetate.

### Table 4: Minimum inhibitory concentration (µg/ml) seaweed extracts against species of fungi

| Seaweed species | Minimum inhibitory concentration (MIC) (µg/ml) | Amphotericin B (100µg) |
|-----------------|-----------------------------------------------|-----------------------|
|                 | MW | M | M/Dc | DC | EA | A. n | C. t | A. n | C. t | A. n | C. t | A. n | C. t | A. n | C. t | A. n | C. t | A. n |
| S. muticum      | 78 | 1250 | 78 | 312 | 3750 | 78 | 312 | 156 | 78 |
| F. spiralis     | 156 | 625 | 59 | 468 | 156 | 938 | 156 | 15 | 625 | 78 |
| C. tamariscifolia| 156 | 2500 | 117 | 234 | 2500 | 1250 | 625 | 78 | 156 | 2500 |
| S. vulgare      | 2500 | 78 | 5000 | 938 | 1875 | 1250 | 2500 | 156 | 78 | 2500 | 156 | 2500 | 156 | 2500 | 156 | 2500 |
| C. humilis      | 78 | 5000 | 938 | 1875 | 1250 | 234 | 156 | 2500 | 156 | 2500 | 156 | 2500 | 156 | 2500 | 156 | 2500 |
| L. ochroleuca   | 625 | 5000 | 938 | 1875 | 1250 | 234 | 156 | 2500 | 156 | 2500 | 156 | 2500 | 156 | 2500 | 156 | 2500 |
| B. bifurcata    | 625 | 2500 | 10 | 234 | 156 | 78 | 312 | 78 | 1250 | 2500 |

C. t: Candidas tropicalis, A. n: Aspergillus Niger, MW: methanol water (50/50), M: Methanol, M/Dc: Methanol/Dichloromethane (50/50), DC: Dichloromethane, EA: Ethyl acetate; No activity.
This difference in results may be due to the fact that the extraction conditions in both studies were not the same. The macroalgae origins are distinct, which may influence the metabolite composition and therefore their bioactivities. The methanol/water extract of the same seaweed exhibited the maximum growth inhibition against *S. muticum* (MIC = 7 µg/ml) and *B. bifurcata* (MIC = 10µg/ml) as the most active extracts against *Candida tropicalis*. Against *Aspergillus niger*, the minimum inhibitory concentration was determined for *S. vulgare* (MIC = 5 µg/ml), *L. ochroleuca* (MIC = 14 µg/ml) and *F. spiralis* (MIC = 15 µg/ml) as the most active extracts (Table 4).

Saleh [46] found that the methanolic extracts of *S. vulgare* recorded a MIC of 150µg/ml against *Candida albicans* and a MIC of 110µg/ml against *Aspergillus niger*. However, our results show that the MIC of the methanolic extract of the same algae against *Aspergillus niger* is better (MIC = 9.7µg/ml).

**CONCLUSION**

The seaweed extracts of seven species studied possessed noticeable activity antibacterial and antifungal against bacteria and fungi strains compared with standards solution (Ofloxacine, Streptomycin and Amphotericin B). The methanol water extracts of the red alga *Fucus spiralis* (MIC = 10µg /ml) and *B. bifurcata* (MIC = 15 µg /ml) as the most active extracts against *Aspergillus niger* (MIC = 7 µg /ml) and *Candida albicans* (MIC = 5µg/ml) as the most active extracts against *Candida albicans*. Against *S. vulgare* (MIC = 15 µg /ml) as the most active extracts against *Candida albicans*. Therefore, further works may be performed on the isolation and identification of the antimicrobial components.

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**AUTHORS CONTRIBUTIONS**

Both authors have contributed equally.

**CONFLICT OF INTERESTS**

Declared none

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