



Cytotoxicity test on *Artemia Salina*, antibacterial and antifungal activities of *Cystoseira Stricta* extracts from the coast of Mostaganem, western Algeria

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Abstract

Cystoseira stricta sp, with its abundance and its dimensions, is undoubtedly the most important brown seaweed of the Mediterranean Sea. Within the objective to promote this alga by researching natural products for possible pharmaceutical use, four organic extracts of *Cystoseira stricta* sp, harvested from the Mostaganem coast, west of Algeria, were evaluated for their cytotoxicity on *Artemia salina* and their antibacterial activities against three gram-positive bacteria (*Bacillus cereus* ATCC 1087, *Bacillus subtilis* ATCC 66336 and *Staphylococcus aureus* ATCC 33862) and two other gram-negative (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) as well as antifungal activity against *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 106404 using the diffusion test on wells. We found that acetone extract exhibited significant antibacterial and antifungal activity against all strains used in contrast to the other extracts. The toxicity test of the acetone extract of our seaweed on the *Artemia salina* has shown that the DL₅₀ is more than 1000 µg/mL which proves the non-toxicity of our extract

1. Introduction

In recent decades, macroalgae have always been at the forefront of chemical and biological research and have been widely recognized as producers of a wide range of bioactive substances such as heterocyclic compounds, acrylic acid, cyclic polysulphides, sterols, polysaccharides, terpenoids, peptides, proteins, vitamins, terpenes, chlorophyllides, phenols and halogenated ketones and alkanes [1-5], which exhibit a wide variety of biological activities including antibacterial, antiinflammatories, antifungal, antitumorals, antiviral, and antioxidant [7-12]. On the other hand the large-scale screening for antimicrobial activity was only done in the 1970s [13-14]. However, several studies have shown that the production of these antimicrobial compounds by the same species varied and that this intraspecific variability could be due to the ecology of active growth stage[15]. Macroalgae can be classified in three broad categories, green algae (chlorophytes), red algae (rhodophytes) and brown algae (phaeophytes), the latter two are mainly used as a source of human food because of their richness in proteins, vitamins and minerals [16], the nutritional composition of these algae varies and is affected by geographical areas, seasons of the year and water temperature. Among the brown algae, the *Cystoseira stricta* sp. is a large photophyle, consisting of one or more trunks carrying many ramifications ; it colonizes the beaten and enlightened rocks of the infralittoral floor of the Mediterranean[17]. They are known to play a role in the formation and maintenance of habitats for a large number of species. *Cystoseira stricta* sp. is very sensitive to water pollution and when its populations are affected, it is replaced by *C. compressa*, which tolerates better the environment fluctuations [18].

Many studies have reported the presence of *Cystoseira stricta* sp. especially on the western shores of the Mediterranean. It has also been reported in El Marsa, El Kala, Kristel, Cherchel and Tamenfoust in Algeria [19]. In this study, we exploited a brown seaweed, *Cystoseira stricta* sp. harvested in spring on the Mediterranean coast around Khadra, east of Mostaganem in Algeria, as a source of pharmaceutical molecules by studying the antibacterial and antifungal potential of their extracts, as well as the cytotoxicity test carried out on the *Artemia salina* collected at the salt lake of Bethioua in Oran, Algeria. This micro-crustacean has been used in ecotoxicological tests and, more recently, it has demonstrated high sensitivity, precision and reliability in detecting toxic and bioactive compounds in plant extracts [20, 21].

2. Material and Methods

2.1. Algae collection

8 kg of *Cystoseira stricta* sp. were harvested at various sites on khadra coast, approximately 70 km east of Mostaganem, at 36° 15' 29" N and 0° 31' 19" E. To avoid contamination, the fresh material was hand selected and packed in plastic bags. The botanical identification of this algae was carried out by Benarous Ahlam at the Aquaculture and Fishiers laboratory of ENSSMAL, Algeria. The macroalgae were washed several times with fresh water to remove salt and epiphytes, then with distilled water, then dried in the shade at 30 ° C to dryness. The dried samples were finely powdered and wrapped in plastic bags and kept away from the light at 4 °C until they are used

2.2. Algal Extracts

For the preparation of the algae extracts, 10 g of the sample was added to 150 mL of organic solvent. The resulting mixture is then macerated continuously in the dark for 24 hours, three times at room temperature. The extraction was carried out separately with different organic solvents: acetone, methanol, methanol-water (6 :1 v/v) and ethanol. After filtration on paper Wattman N° 1, the organic extracts were concentrated by evaporation under reduced pressure using a rotary evaporator at 40 °C. The crude extracts were lyophilized and weighed. The weight yield of the powdered samples obtained was 2.22 % for the methanolic extract, 8.75 % for the acetone solvent, 6.18 % for the ethanolic extract and 3.69 % for the methanol / water mixture. These four extracts were then tested for cytotoxicity on *Artemia salina* and their antibacterial and antifungal activities against certain human pathogens

2.3. Pathogens used for the assay

The strains used for the anti-bacterial and anti-fungal tests were obtained from Pasteur institute of Algeria, are clinical isolates of fungal strains (*Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 106404), gram-positive bacterial strains (*Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 33862) and gram negative bacterial strains (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Schigella ssp*).

2.4. Anti-bacterial and anti-fungal tests

The anti-bacterial and anti-fungal activities of the extracts is demonstrated by the well diffusion method on Mueller-Hinton agar and Sabouraud agar consecutively. Petri dishes containing the two media were seeded aseptically with 100 µL of microbial suspension whose turbidity was adjusted to 10⁸ CFU / mL for the bacteria, 10⁶ CFU / mL for the yeasts [22]. and 10⁶ spores / mL for fungi [23]. In wells 4.5 mm in diameter, we introduced 50 µL of crude extract. In order to allow the radial diffusion of the inhibitory agent, the Petri dishes thus prepared were preincubated for 2 to 4 hours at 4 °C. and then incubated at 37 °C. for 24 hours for the bacteria and at 25 °C. for 48 hours for yeasts and 7 days for fungi. The antimicrobial activity of the crude extract was determined by measuring the diameter of the inhibition zone formed around each well.

2.4.1. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by the broth microdilution method, by serially diluting the test sample in broth, which is then inoculated with a bacterium into a microtiter with a 96-well plate.

For each line of the microplate, we deposited 100 µL of Sabouraud broth for yeast and fungus and Mueller Hinton broth for bacteria in the 12 wells except the first one in which we added 200 µL of pure extract to be tested. The last two wells of the line that serve to control the growth and sterility of the medium. Then, we took 100 µL of the first well by passing them to the second and from the second to third, and so on to the well number 10 so as to obtain the following successive dilutions: 1/2, 1/4, 1 / 8, 1/16, 1/32, 1/64 and 1/128. Afterwards, we introduced 100 µL of the microbial suspension with turbidity adjusted in all the wells except the last of each line. The plates are then sealed and placed in the oven and the reading of the results is made with the naked eye by observing the change in turbidity in the tubes after incubation and with respect to the controls.

2.6. Cytotoxicity test

The toxicological study was carried out on the *Artemia salina* collected from the salt lake of Bethioua in Oran, Algeria, according to the Bine Shrimp 'BS' test developed by P.i Vanhaecke [24].

2.6.1. Incubation and hatching of *Artemia salina* cysts

After collection, the cysts undergo a treatment according to the standard method of Sorgeloos and al [25], which requires the following steps, separation by diameter, separation by difference of density in seawater, washing with fresh water, separation by density in fresh water and drying for hatching, 250 mg of treated cysts were incubated in a cylindrical conical glass vessel containing 100 mL of filtered natural seawater with a salinity of 3.3%, maintained at room temperature (26-28 °C). After 48 hours of aeration of the container, the cysts hatch and the nauplii were collected under intense light and were used for the test.

2.6.2. The lethal dose (DL50)

The lethal dose LD₅₀ is the concentration of the tested extract that kills 50% of artemia nauplii in 24 hours under standard conditions, for the determination of this concentration the samples were dissolved in 2% of dimethylsulfoxide (DMSO) and diluted to different concentrations (1000, 800, 600, 400, 200, 100, 50 and 10 ppm). Determined volume of the prepared solutions was added to the test tubes each containing 10 nauplii . After 24 hours, the results were read by counting under a dissecting microscope. The percentage of mortality was calculated by the following formula:

$$\% \text{ Mortality} = ((\text{DNE} - \text{DNW}) / \text{NT}) \times 100$$

DNE : Average number of dead nauplii in the Presence of the extract in three assay

DNW: Average number of dead nauplii in the Presence of Witness in three assay

NT: the numbers of the nauplii tested

3. Results and discussion

3.1. Antibacterial activity of algal extract

Four organic extracts (acetone, methanol, methanol / water and ethanol) of the brown alga *Cystoseira stricta* sp. from the Algerian coast were studied to evaluate their antibacterial activity against three Gram-positive bacterial strains (*B.Cereus*, *B.Subtilis* and *S .aureus*) and three other Gram-negative (*P.aeruginosa*, *E. coli* and *Schigela ssp*) compared to the reference drug Gentamycin (10mg) using the well diffusion on Mueller-Hinton agar method. As shown in Table 1, unlike the other three seaweed extracts, only the acetone extract showed significant activity against the bacteria tested (figure 1) and the zones of inhibition ranged from 12 to 20 mm.

3.2. Antifungal activity

Table 2 shows that the acetone extract also has antifungal activity against *Candida albicans* ATCC 10231 with a zone of inhibition of 21 ± 1.2 mm (Figure 2) , greater than that of the drug Amphotericin B (20 mg / well) and has no effect on *Aspergillus niger* ATCC 106404. This study showed that the other extracts had no positive activity on the fungal strains tested.

Table 1: Antimicrobial activity of *Cystoceira stricta* sp. extract

Marine algae	Organic extract	Gram-positive bacterial strain		
		<i>B.Cereus</i>	<i>B.Subtilis</i>	<i>S .aureus</i>
<i>Cystoceira stricta</i> sp.	Acetone	12 ± 1.2	17 ± 0.5	18 ± 0.9
	Methanol	-	-	-
	Ethanol	-	-	-
	Methanol /water	-	-	-
Gentamycine (Reference drug)		15 ± 0.7	18 ± 0.9	19 ± 0.6
Marine algae	Organic extract	Gram-negative bacterial strain		
		<i>P.aeruginosa</i>	<i>E. coli</i>	<i>Schigela ssp</i>
<i>Cystoceira stricta</i> sp.	Acetone	15 ± 0.8	19 ± 0.3	20 ± 0.8
	Methanol	-	-	-
	Ethanol	-	-	-
	Methanol /water	-	-	-
Gentamycine (Reference drug 10 mg/well)		16 ± 0.7	20 ± 0.9	19 ± 1.2

The data are expressed as the average of three repetitions ± a standard deviation (SD)

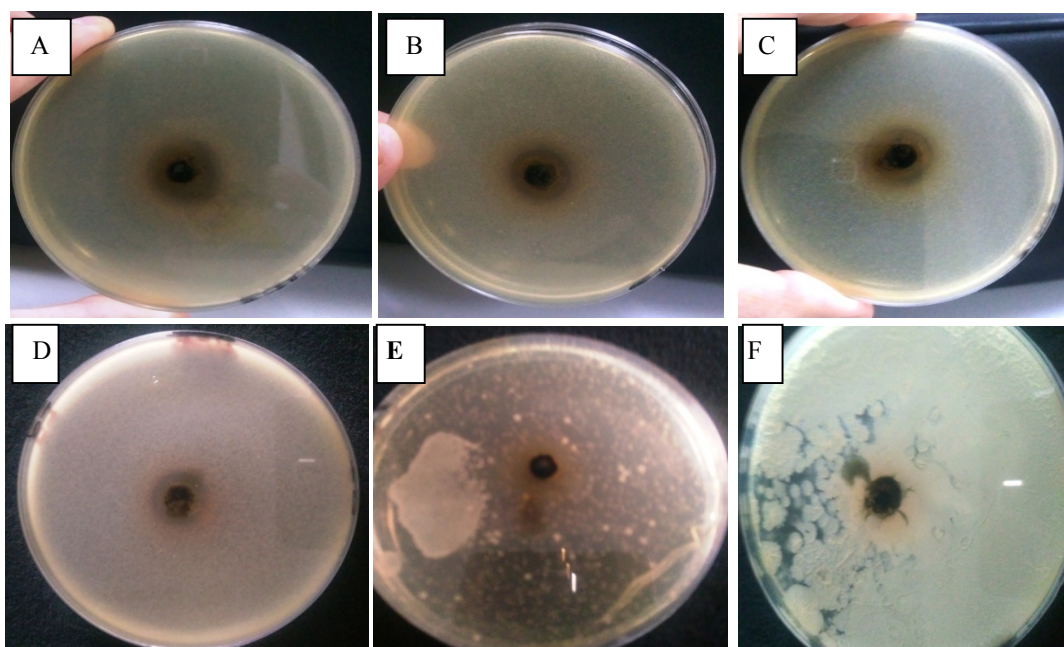


Figure1: Zones of inhibition of the acetonetic extract of *Cystoceira stricta* sp. on bacterial strains
A : *Escherichia coli* ATCC 25922 ; B : *Bacillus cereus* ATCC 10876 ; C : *Staphylococcus aureus* ATCC 33862 ; D :
Pseudomonas aeruginosa ATCC 27853; E : *Bacillus subtilis* ATCC 6633 ; F : *Schigela ssp*

Table 2: Antifungal activity of *Cystoceira stricta* sp. extracts

Marine algae	Organic extract	fungal strain	
		<i>Candida albicans</i>	<i>Aspergillus niger</i>
<i>Cystoceira stricta</i> sp.	Acetone	21 ± 1.2	-
	Methanol	-	-
	Ethanol	-	-
	Methanol /water	-	-
Amphotericin B (20 mg / well)		13 ± 1.2	15 ± 0,8

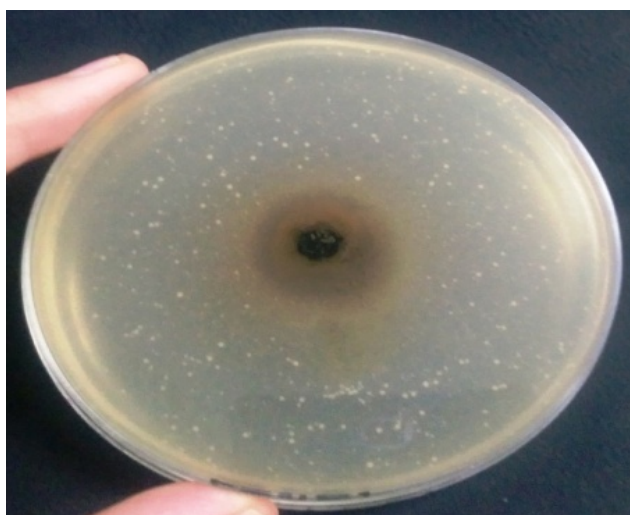


Figure 2: Zones of inhibition of the acetonetic extract of *Cystoseira stricta* sp. on fungal strains *Candida albicans* ATCC 10231

3.3. Minimal inhibitory concentration

The lowest minimum concentration of acetone extract of the brown alga studied was 12.5 mg / mL against *Candida albicans*, *Escherichia coli* and *Schigella ssp* and the highest concentration of 100 mg / mL was recorded against *Pseudomonas aeruginosa*. all MIC's values are shown in Table 3.

Table 3: Minimal inhibitory concentrations of the acetone extract against the different strains tested

Bacterial strains	MIC
<i>Bacillus cereus</i> ATCC 10876	50 mg/mL
<i>Bacillus subtilis</i> ATCC 6633	50 mg/mL
<i>Staphylococcus aureus</i> ATCC 33862	25 mg/mL
<i>Pseudomonas aeruginosa</i> ATCC 27853	100 mg/mL
<i>Escherichia coli</i> ATCC 25922	12.5 mg/mL
<i>Schigella ssp</i>	12.5 mg/mL
Fungi strains	MIC
<i>Candida albicans</i> ATCC 10231	12.5 mg/mL

3.4. Cytotoxicity test

Different concentrations of acetone extract from our algae have been tested on *Artemia salina* nauplis and the percentage of mortality is shown in Table 4.

Table 4. Results of Cytotoxicity test of *Cystoseira Stricta* sp. acetone extract

Algal extract	Extract concentrations (ppm)	NNT	NDNE	NDNW	M%
Acetone extract of <i>Cystoseira stricta</i> sp	50	10	0.33	0	3.33
	100	10	1.66	0	16.66
	400	10	3	0	30
	800	10	4.33	0	43.33
	1000	10	4.66	0	46.66

From Table 4, we can see that the percentage of *Artemia salina* nauplii mortality increases with the increase in concentration of the acetone extract of our seaweed. This is clearly shown in the graph giving the variation of

M% as a function of the logarithm of the concentration, in figure 3. From the linear expression of the graph of figure 3, we obtained the LD₅₀ value of this extract, ie 1359 ppm, which proves that the acetone extract of this alga is not toxic.

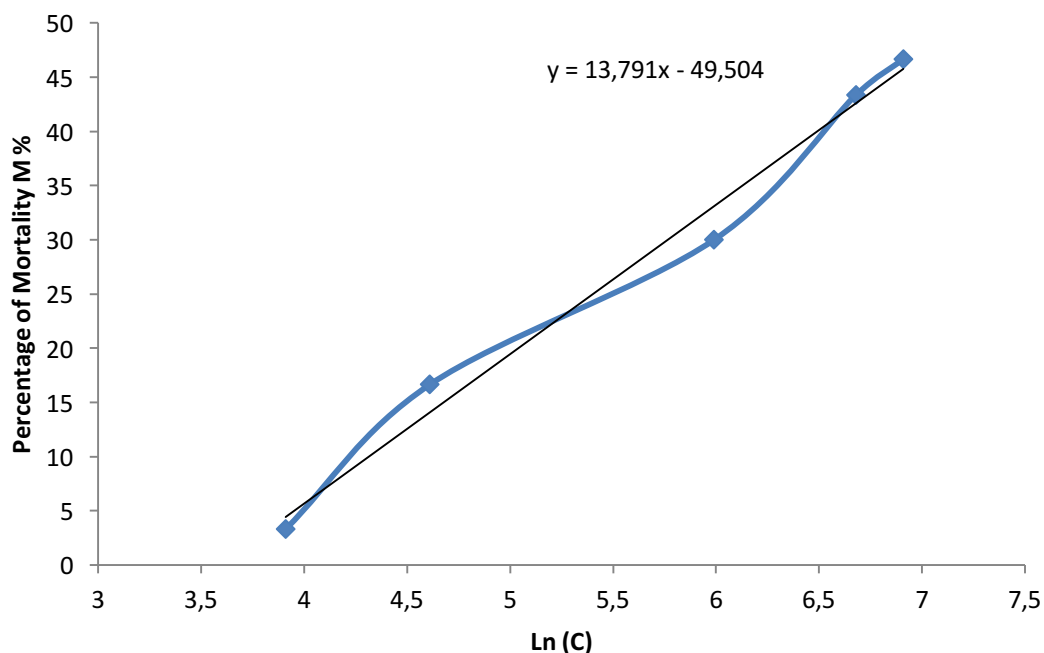


Figure.3: Curve of percentages of *Artemia salina* nauplii mortality as a function of the logarithmic concentrations of algae extract

Conclusion

In summary, this study indicated that the acetone extract of *Cystoseira stricta* sp. collected from the west coast of Algeria, was not toxic and had a significant capacity for antibacterial and antifungal activities. Therefore, the screening of the chemical composition of this brown algae would be of great interest and further studies should be conducted to characterize their active compounds and evaluate the effects of each substance on the microorganisms.

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