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Research of the antifungal activity of the extracts of certain species of Macroalgae from the Atlantic coast of Morocco

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Keywords

Antifungal activity, seaweed extracts, Disk diffusion method, fungus, MIC.

Currently, marine organisms are of paramount importance source of new bioactive molecules. This study was carried out with an objective to investigate the antifungal activity of seaweed extract from 5 marine algae species, *Cystoseira* spp., *Fucus spiralis*, *Bifurcaria bifurcata*, *Ulva rigida* and *Corallina elongata*. Extraction, by soxhlet, was carried out using methanol, ethanol, ethyl acetate, dichloromethane or hexane. The extracts obtained were tested against three fungal species: *Botrytis cinerea*, *Altenaria alternata* and *Rhizoctonia solani*.

Evaluation of this antifungal activity was performed by using the disk diffusion method, followed by determining the minimum inhibitory concentration (MIC) and its effect on spore germination. This study revealed that methanol and ethanol gave the highest yield extraction with 14.31% and 13.9% percentages in *Fucus spiralis* and *Cystoseira* spp. respectively. With regards to the antifungal activity, the hexane extract is the most active on *B. cinerea* with a zone of inhibition of 24.3 mm and a spore germination which did not exceed 10%. This effect is observed at a MIC of 250µg/ml. However, inhibition of 70-80% spores and a most inhibition of mycelial growth of the species tested were observed for the methanolic and ethyl acetate extracts in the case of Pheophyceae species. We have concluded that the methanolic and ethyl acetate extracts of certain

species Cystos*eira* spp., *Fucus spiralis* and *Bifurcaria bifurcata* do represent a considerably important antifungal activity.

1. Introduction

Morocco has two maritime coastlines on both the Mediterranean Sea and the Atlantic Ocean. These seaboards extend over about 3500 km in length, which offers the existence of many marine species, particularly algae, which have a substantial biomass [1].

Algae are organisms poorly evolved, lacking defense weapons, living in harsh environmental and ecological conditions (pressure, salinity, inter and intra-specific competition, etc.). These factors lead those organisms to produce defense molecules that are secondary metabolites biologically active and with a great structural originality.

Nevertheless, out of nearly 220 000 listed natural substances, only 10% are belonging to marine origin. It could be explained by the fact that marine organisms have been much less studied than their terrestrial analogues, mainly because of the lack of knowledge of the marine environment and the difficulties involved in collecting samples.

In fact, the first research on marine natural products dates back in the end of the 1960s, whereas terrestrial substances have been intensively studied for more than a century [2].

Since marine algae are huge reservoir of potentially active natural molecules [3]; [4], [5], [6], studies have isolated and identified a very large number of new molecules that most of them have an interesting biological activities as antimicrobial, antiviral, antifungal, antiallergic, anticoagulant, anticancer, and antioxidant effect.

In recent years, the agricultural world is moving towards a sustainable and reasoned agriculture in order to preserve the environment and improve food safety by developing integrated biological protection.

In addition, although pesticide treatments generally provide effective protection against fungal diseases of crops, their efficacy is relatively unsustainable because of the rapid adaptation of pathogenic populations [7].

In order to remedy the problem of pesticide resistance, and also to enhance the maritime algal resources, we studied the antifungal activity of the crude extracts of five species of brown, red and green algae from the Atlantic coast of Morocco.

2. Material and methods

2.1. Plant material

2.1.1. Harvesting algae species

Algae (*Cystoseira* spp., *Bifurcaria bifurcata* and *Corallina elongata*) were collected from the Moroccan Atlantic coast in the Mirleft beach, southern Agadir region, while *Ulva rigida* and *Fucus spiralis* were collected at Sidi Brahim beach, El Jadida region.

After the harvest, the samples were rinsed with fresh seawater to remove associated debris and epiphytes. In the Laboratory they were washed with tap water to remove salt, thereafter with distillated water. They were after, dried at room temperature.

2.1.2. Preparation of algae extracts

For each species, the algae powder was extracted separately with five different solvents: Methanol, Ethanol, Ethyl acetate, Dichloromethane and Hexane according to the following protocol: 20 g of algae powder was extracted in a Soxhlet, with 200 ml of each solvent for 6 hours. The liquid extracts were evaporated under reduced pressure with a rotary evaporator at 45 °C. The dry extracts were recovered in 2 ml of DMSO 5% and stored at 4 °C until used.

2.2. Fungal material

The fungus was provided by the Botany and Plant Protection Laboratory, University Ibn Tofail, Faculty of Sciences Kenitra. They kept on a PDA medium. The species studied were *Botrytis cinerea*, *Alternaria alternata* and *Rhizoctonia solan*i.

2.2.1. Preparation of the spore suspension

A Culture of ten days of the phytopathogens *Botrytis cinerea* and *Alternaria alternata* were used for preparing of the spore suspension.

The culture's surface was scraped with a sterile metal spatula and diluted in sterile distilled water. The suspension obtained was centrifuged at 1000 rpm for 5 min. Spore density was determined using a Malassez's hemocytometer. The concentration was adjusted to 10^8 spores / ml [8].

2.2.2. Assessment of antifungal activity by the disk diffusion method

Mycelial discs of 6 mm diameter were cut from the periphery of fungal cultures and placed in the center of steril Petri dishes with the PDA medium. Disks of 6 mm diameter were impregnated with 10 µl of each extract (1mg / disc) and placed around of the fungal central disk. After incubation at 25 ± 2

° C for 7 days, the results were read by measuring the diameter of the inhibition zone (mm). DMSO (5%) was used as a negative control [8].

2.2.3. Determination of the minimum concentration of inhibition (MIC)

The MIC was determined following the broth dilution method. Potato Dextrose Broth medium (PDB) was supplemented with 10 µl of the spore suspension and 10 µl of each extract concentrations (1000, 500, 250, 125 and 62.5 µg / ml). The broth culture prepared was incubated at 25 ± 2 °C for 7 days. The results were done visually. The negative control contained the PDB with the spore suspension [8].

2.2.4. Effect on spore germination

The test was carried on slides. 10 µl of spore suspension (10^8 spores / ml) was mixed with 10 µl of three concentrations of each extracts (250, 500 and 1000 µg / ml). The slides were incubated at 25 ± 2 ° C for 24 hours, and then each slide was stained with lactophenol cotton blue. This test was carried out only on the extracts presenting a important MIC [8].

2.3. Statistical analysis

All tests were done in triplicate and the data were processed using analysis of variance (ANOVA). The statistical comparison was performed by Tukey's post-hoc tests. Variations were considered significant when the probability was greater than 95% ($p < 0.05$).

3. Results and discussion

3.1. Results

3.1.1. Yield of extracts

The results presented in figure 1 show the important fluctuations in the yield of the crude extracts obtained due to the different polarities according of the solvent used and also depend on the species. Indeed methanol gave the highest yield with a maximum value of 14.31% in *Fucus spiralis* and a minimum value of 3.01% in *Corallina elongata*, followed by ethanol with a maximum yield of 13.9% in *Cystoseira.* spp. and a minimum yield of 1.95% in *Corallina elongata*, while hexane gave the lowest yield.

Thus, the yield was proportional to the polarity of the solvent and according to the groups of algae, of which Rhodophyceae give the highest yield followed by Chlorophyceae, then Phaeophyceae.

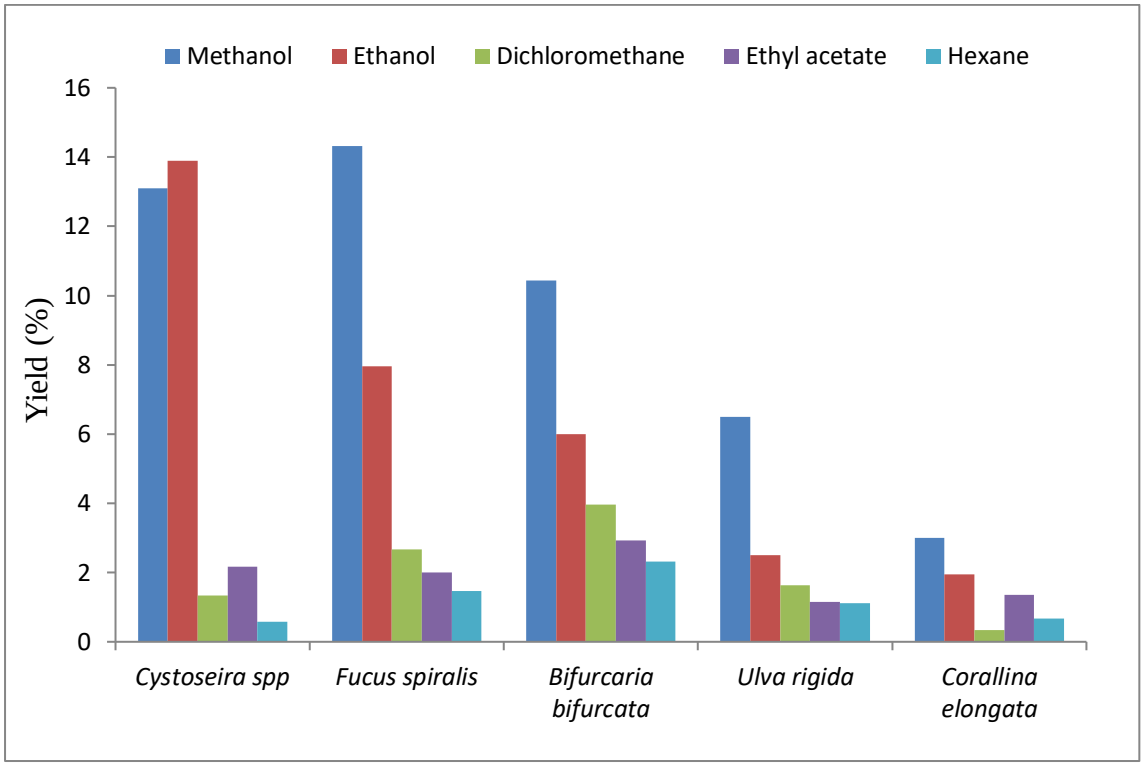

Figure 1: Yield (expressed as a percentage) of the crude extracts of different species of algae by different solvents.

3.1.2. Study of antifungal activity by disk diffusion method

The results of the antifungal activity of the extracts are shown in Table 1. The negative results obtained by the DMSO 5% prove that it had no inhibitory effect.

The two extracts, hexanic and ethyl acetate of *Cystoseira* spp. had a significant activity with respect to *B. cinerea*, expressed by an inhibition diameter of 24.3 and 21 mm respectively, whereas its hexanic extract was average towards *A. alternata* and *R. solani* with an inhibition diameter of 17.6 and 10.3 mm respectively. However, the hexanic extracts have no inhibition effect when applied to other algae species, except the effect of *Corallina elongata* on A. alternate (7.3 mm).

The ethyl acetate extract of *Bifurcaria bifurcata* inhibited the mycelial growth of *B. cinerea* (21 mm), *A. alternata* (23 mm) and *R. solani* (23 mm).

The test with *Fucus spiralis* shows that its dichloromethanolic extract exhibited remarkable anti-fungal activity against *B. cinerea* and *R. solani* with an inhibition diameter of 23 and 22.3 mm respectively. The methanolic extract proved an inhibitory effect only against *R. solani* (21 mm).

The ethanolic extract of *Corallina elongata* was actively repressor on *R. solani*, with an inhibition diameter of 23 mm, similarly its methanolic extract had an inhibitory effect on *B. cinerea*, with an inhibition diameter of 21 mm.

Table 1: Evaluation of the antifungal activity of the different extracts of algae, expressed in diameter of inhibition (mm).

Algal species	Solvents	Fungal species		
		Botrytis cinerea	*Alternaria alternata*	*Rhizoctonia solani*
***Cystoseira* spp.**	Me	5,3±2,5^{e}	8,3±1 ,5^{f}	7,6±1,5^{fg}
	Et	14,6±2,5^{c}	0	20,3±1,5^{abc}
	DCM	4,6±2,1^{ef}	7,6±1,5^{f}	7,3±1,5^{g}
	Eac	21±3^{ab}	15,6±3,5^{cd}	0
	Hex	24,3±2,5^{a}	17,6±2,1^{bc}	10,3±1,5^{ef}
Bifurcaria bifurcata	Me	0	14,3±1,5^{d}	16,3±1,5^{d}
	Et	0	0	0
	DCM	10,6±1,5^{d}	0	0
	Eac	21,6±3,1^{ab}	23,3±2,3^{a}	23,3±1,5^{a}
	Hex	0	0	0
Fucus spiralis	Me	0	0	21±1,7^{abc}
	Et	0	0	16,3±2,3^{d}
	DCM	23±4^{a}	0	22,3±2,3^{ab}
	Eac	15,3±2,5^{c}	7,3±2,5^{f}	6,3±1,5^{g}
	Hex	0	0	0
Ulva rigida	Me	0	0	19,6±0,5^{bc}
	Et	0	0	0
	DCM	18,3±2,5^{b}	0	12±2,1^{e}
	Eac	0	0	0
	Hex	0	0	0
Corallina elongata	Me	21±2^{ab}	0	11,6±1,5^{e}
	Et	0	19,6±2,5^{b}	23±2,6^{a}
	DCM	10,3±1,5^{d}	17,3±2,5^{bc}	7,6±1,5^{fg}
	Eac	10,6±2,3^{d}	11,6±2,1^{e}	18,3±1,5^{cd}
	Hex	0	7,3±2,5^{f}	0
	DMSO (5%)	0	0	0

Me: Methanol ; Et: Ethanol ; DCM: Dichloromethane ; Eac: Ethyl acetate ; Hex: Hexane

For each extract, the averages of the same column with the same letter don't differ significantly at the 5% threshold (tukey post hoc test).

3.1.3. Determination of the MIC

The minimum inhibitory concentration (Table 2) was determined for the various extracts and different species.

The hexanic extract of *Cystoseira* spp. inhibited the growth of *B. cinerea* and *A. alternata* at a concentration of 250 µg/ml and 500µg/ml respectively. The methanolic extract of the Phaeophyceae exhibits antifungal activity against *B. cinerea* and *A. alternata* at a MIC of 250-500 µg / ml. The ethyl acetate extract of *Cystoseira* spp., *Fucus spiralis* and *Bifurcaria bifurcata* had a MIC of 250-1000 µg / ml with respect to *B. cinerea* and *A. alternata*. Dichloromethane extract of all seaweed showed an inhibitory effect against *B. cinerea.*

This test is not applicable on *Rhizoctonia solani* because it is a sterile fungus.

Table 2: Minimum inhibitory concentration of each extracts of algae.

		MIC (µg/ml)		
		Botrytis cinerea	***Alternaria alternata***	***Rhizoctonia solani***
***Cystoseira* spp.**	Me	500	250	na
	Et	1000	nd	na
	DCM	1000	250	na
	Eac	250	500	na
	Hex	250	500	na
Fucus Spiralis	Me	250	500	na
	Et	nd	nd	na
	DCM	1000	nd	na
	Eac	250	250	na
	Hex	nd	nd	na
Bifurcaria Bifurcata	Me	250	250	na
	Et	nd	nd	na
	DCM	1000	nd	na
	Eac	250	1000	na
	Hex	nd	nd	na
Ulva Rigida	Me	nd	nd	na
	Et	nd	nd	na
	DCM	1000	nd	na
	Eac	nd	nd	na
	Hex	nd	nd	na
Corallina elongata	Me	1000	nd	na
	Et	nd	1000	na
	DCM	1000	1000	na
	Eac	1000	500	na

	Hex	nd	250	na

nd: no activity was detected ; na: not applied

3.1.4. Effect of algae extracts on spore germination

The results obtained for the organic extracts of the spore germination assay of the tested fungi were shown in Figures 2 and 3. DMSO (5%), as a negative control, did not inhibit spore germination. There was significant inhibition of fungal spore germination by different concentrations of algal extracts.

100% of *B. cinerea* and *A. alternata* spores were germinated in the absence of algae extracts.

90% of *B. cinerea* spores were inhibited by the hexanic extract of *Cystoseira* spp. at a concentration of 1000 µg/ml. At a concentration of 250 µg/ml, the ethyl acetate extract of *Cystoseira* spp. and *Fucus spiralis* and methanolic extract of *Bifurcaria bifurcata* inhibited germination of *B. cinerea* spores of 50%. These extracts at a concentration of 1000 µg/ml permitted germination of only 30% of the spores of both fungal species tested. The ethyl acetate extract of *Bifurcaria bifurcata* and the methanolic extract of *Fucus spiralis* inhibited germination of *B. cinerea* spores with a percentage inhibition between 70-80% at concentrations ranging from 500-1000µg / ml.

At a concentration of 1000 µg/ml, the ethyl acetate extract of *Bifurcaria bifurcata* inhibited germination of *A. alternata* spores of 87%. Also 63-73% of *A. alternata* spores were inhibited by the ethyl acetate and methanolic extract of *Cystoseira* spp. and *Fucus spiralis* at a concentration of 1000 µg/ml. The same solvents in *Fucus spiralis* and *Bifurcaria bifurcata* inhibited germination of *A. alternata* spores of 50-60% at a concentration of 250 µg/ml.

The dichloromethane extract show de least inhibitory effect on spore germination compared to the other extract. The same *Corallina elongata* and *Ulva rigida* shows the least important effect of the species studied.

Overall, the inhibition of spore germination increases with concentration of algae extracts.

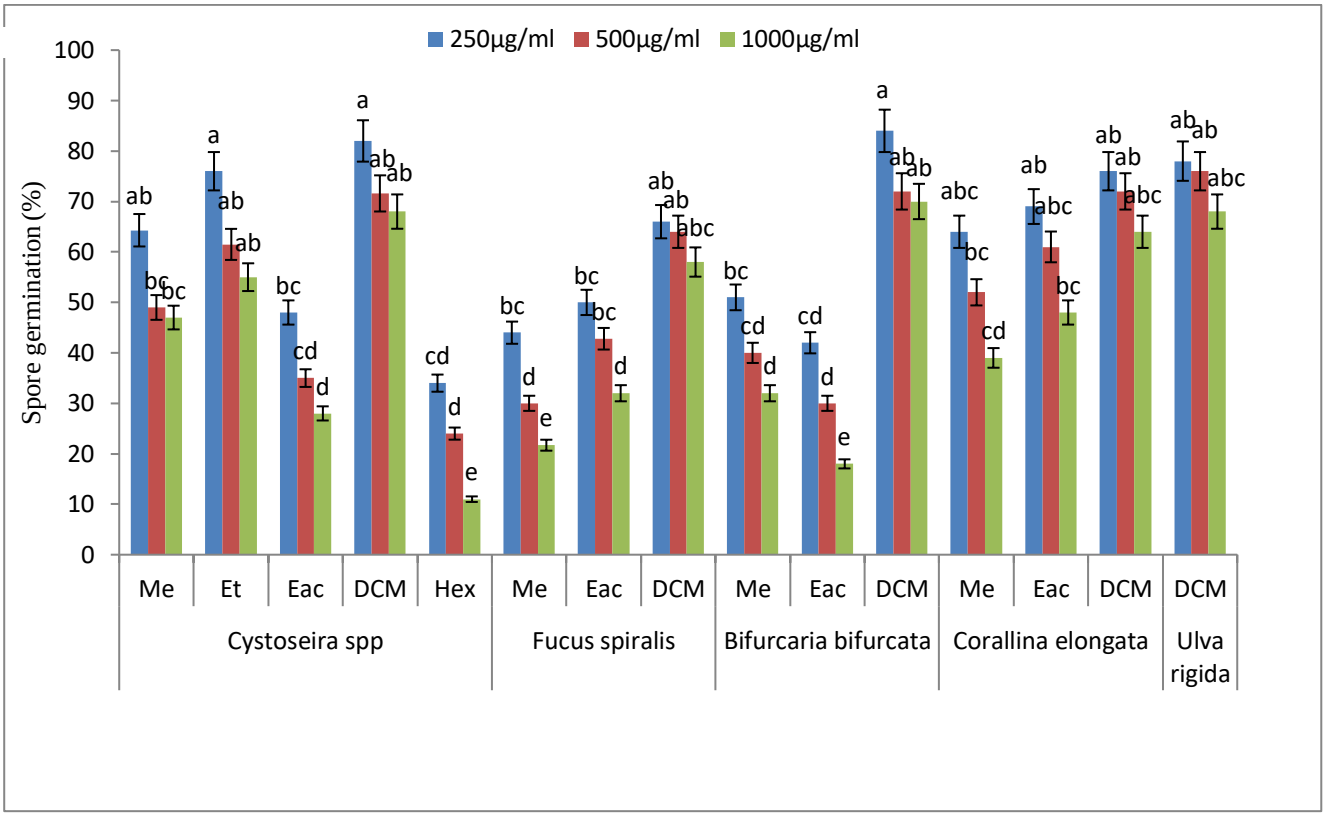

Figure 2: Effect of different concentrations of algae extracts on spore germination of *Botrytis cinerea*.

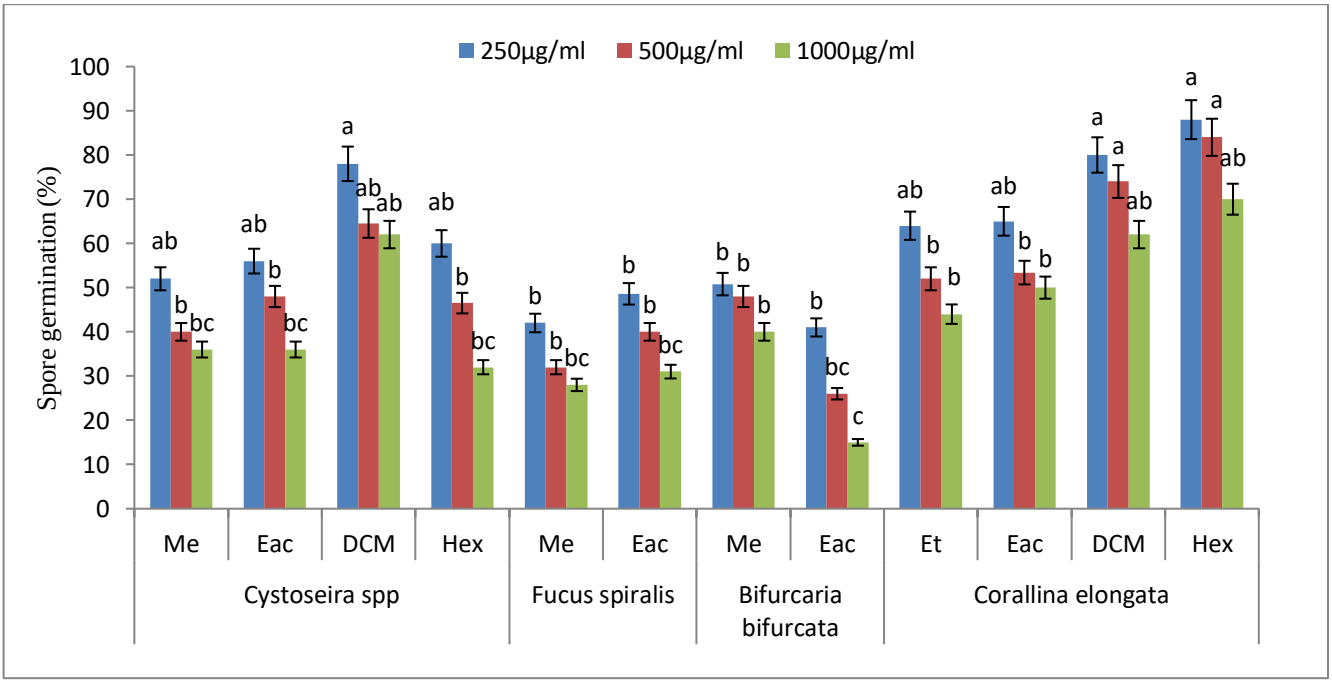

Figure 3: Effect of different concentrations of algae extracts on spore germination of *Alternaria alternata*.

3.2. Discussion

Organic natural products isolated from algae recommended as biocidal potential and pharmaceutical agents. Numerous data are now available concerning the antifungal activity of different families of algae.

This study shows that the methanolic and ethyl acetate extracts, obtained from the species *Cystoseira* spp., *Fucus spiralis* and *Bifurcaria bifurcata* inhibited the mycelial growth of *B. cinerea* and *A. alternata*. These results were comparable with those obtained by other researchers demonstrating an inhibitory action of ethyl acetate extracts of the Cystoseiraceae family on the growth of *Alternaria alternata, A. brassicicola* and *Fusarium oxysporum* [9]. As well the dichloromethanolic extract of *Fucus spiralis* induced a marked inhibition of the growth of *Aspergillus niger*, with an extent of inhibition of approximately 1 cm [10]. Similarly, the study of the antifungal activity of the various extracts of *C. tamariscifolia* showed that the ethanolic extract inhibited all the species tested: *Aspergillus flavus*, *Penicillium* sp. [11]. In addition, its ether-ethyl and hexanic extract showed an inhibitory effect on *Botrytis cinerea* and *Fusarium oxysporum* [12].

The ethanolic extract of brown algae had antifungal effect against *R. solani*. This inhibitory effect was probably due to phenolic compounds, whose brown algae contain high levels [13].This had also been confirmed by [14] in 2010 who found that phenolic compounds were responsible for the antifungal activities of algae. Other substances like terpene had been isolated by [13] from the genera *Cystoseira* and *Bifurcaria*. Indeed, a chromatographic analysis had shown that these extracts contain substances of terpene nature responsible for the inhibitory activity obtained (Moreau *et al.*, 1984).

Moreover, in *Bifurcaria bifurcata*, [15] isolated the first linear diterpenes such as: Epoxyeleganolone and Eleganediol. Then, interesting works of extraction and purification of the linear diterpenes were realized by several researchers [16]; [17]; [18]. Also, [19] in (1991) for isolating two linear diterpenes and for the first time cyclic diterpenes of *Bifurcaria bifurcata*, derived from Eleganolone. [20] identified different compounds of algae studied following geographical variations, at the Rabat-Casa site, they identified Eleganediol, in Oualidia: Bifurcadiol and Eléganolone in Casa-Oualidia. On the north-western coast of France, between Cherbourg and Nantes, the same compound was observed "Eleganolone". [17] also identified five other diterpenes compounds in the etheric extract in *Bifurcaria bifurcata*, harvested on the French Atlantic coast. Antifungal tests carried out with hexane extracts of brown algae belonging to the order Dictyotales show a marked inhibition of certain fungi such as *Aspergillus fumigatus* [15].

The methanolic extract of *Corallina elongata* showed positive activity against *Aspergillus niger* and *Candida albicans* [21]. Sulphated galactans isolated from *Corallina elongata* showed inhibitory activity and bactericidal activity against all Gram-positive strains tested. But, carrageenan was only able to inhibit the growth of *Staphylococcus epidermidis*. However, the two polysaccharides formed isolated from *Corallina elongata* exhibit inhibitory or bactericidal activity against Gram-negative bacterial strains [22]. The green alga *Ulva rigida* didn't give very significant results. These results were consistent with those obtained by [23] who found that aqueous extracts of ethyl acetate, chloroform and hexane from *Ulva rigida* showed no inhibitory effect against *A. alternata*.

It should be noted that the yield of natural products extracted from marine macroalgae is higher with methanol. [24] tested several solvents and found that methanol was the best solvent for the isolation of bioactive secondary metabolites from marine algae followed by ethanol, ethyl acetate and dichloromethane. These results also indicate that the polarity of the solvents had effects on the isolation of the active ingredients and therefore on the antifungal activity. Extraction methods also affect this activity, which was why there were contradictions reported by several studies, hence the importance of the choice of the appropriate solvent and the appropriate extraction method to ensure the presence of specific bioactive compounds for certain objectives [9].

Conclusion

In this study, we were interested in the antifungal power of certain algal species of our Moroccan Atlantic coast belonging to the three groups: Phaeophyceae (*Cystoseira* spp., *Bifurcaria bifurcata* and *Fucus spiralis*), Rhodophyceae (*Corallina elongata*) and Chlorophyceae (*Ulva rigida*).

The phytopathogenic species tested represent a substantial economic interest, case of *B. cinerea, A. alternata* and *R. solani*. Our results showed that the methanolic and ethyl acetate extracts showed the strongest inhibitory effect of the other extracts. Extracts obtained from Phaeophyceae were the most endowed with antifungal activity. In addition, *B. cinerea* was more sensitive to the hexane extract of *Cystoseira* spp., where we recorded an inhibition zone of 24.3 mm and 90% inhibition of spore germination. We can deduce that there was an existence of secondary metabolites responsible for this antifungal activity.

However, this work is just the first step in the search for these bioactive molecules in these marine algae. So, in order to validate these results, it is important to test other extraction solvents, to

extrapolate this study to other pathogens, to consider *in vivo*, greenhouse and field trials and to carry out a complete screening of the major potentially active chemical groups (HPLC, CCM ... etc).

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