Research Article

Antifungal Activity of 23 Seaweeds from the Coast of El Jadida Morocco against *Fusarium culmorum* and *Alternria alternata*

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ABSTRACT

Marine macroalgae are considered to be an excellent natural biosource of a large active biocidal substance against plant-infecting pathogens. For that, the inhibitory effect of 23 marine algae (13 Rhodophyta, 6 Phaeophyta and 4 chlorophyta) collected from the coast of El Jadida (Sidi bouzid) Morroco has been evaluated against 2 fungal strain (*Fusarium culmorum* and *Alternria alternata*) using five organic extracts (Methanol, Butanol, Methanol/Dichloromethane (50/50), Dichloromethane and Hexane). Data revealed that the *Fusarium culmurum* was the most sensitive pathogen, the important inhibitions was obtained with methanol/ dichloromethanolic extract of red algae *Cystoseira humilis* (29±0.2 mm) followed by the extract with the same solvent of the green algae *Ulva rigida* with 27±0.6 mm. The highest activity against *Alternaria alternata* is obtained with the methanolic extract of *Bifurcaria bifurcata* (22±0.3 mm) followed by the methanolic extract of *Fucus spiralis* and the methanol / dichloromethanolic extract of *Cystoseira humilis* (20±0.5 mm). These marine macro-algae appear to have immense potential source of antifungal compounds, they could be used in treating diseases caused by various organisms tested.

Keywords: *Marine macroalgae, Fusaruim culmorum, Alternaria alternata*, coast of El Jadida, antifungal activity.

INTRODUCTION

On the globe, more than 150 000 macroalgae species are found in oceans, in Morocco there are 451 species present on the two marine facades (Atlantic and Mediterranean) including Green algae (Chlorophyta), red algae (Rhodophyta) and brown algae (Phaeophyta).[1,2,3]

Marine macroalgae are rich source of structurally novel and biologically active metabolites that used in biological control in the agricultural field,[4] but unfortunately, most available literatures on marine macroalgae and their derivatives mainly focused on their pharmaceutical applications but their potential utilization in sustainable agriculture development is still often regarded as a secondary goal. However, a relatively considerable dataset on marine macroalgae showed that they could play a major role in plant protection and improvement.

They also showed great potential due to their importance as a useful bioindicator for heavy metals pollution in ecosystems and its multiusage for many other purposes medicinal, antimicrobial…. [5,6] Many investigations revealed that macroalgae have a broad range and potential use in pharmacology researches as antibacterial or/and antifungal.[7-12] Inhibitory effects is related to the presence of bioactive compounds as secondary metabolites phenol and carotenoids compounds, [13] or due to the presence of saponins, flavonoids, tannins and cardiac glycosides. [9]

The current investigation focused on algae utility as antifungal agents also reported in many investigations. [14, 15] So, the most potent algae will be handled in the future research as a cheaper source for antimicrobial treatment.

The present study aims to screen the antifungal properties of five extracts from 23 algae against *Fusarium culmorum* causal agent of foot and root rot and head blight on wheat and *Alternaria alternata* causal agent of leaf blight (Alternaria blight).

MATERIALS AND METHODS

Algal materials

Seaweeds was collected by hand-picking during March to April 2016 from the coast of El Jadida (33 ° 33 °16'09''N, 8 °30' 8 °45'W) (fig. 1). The algae were cleaned, washed in distilled water, then dried at room temperature and crushed to a fine powder.

The algae investigated were identified from fresh species as ; Phaeophyceae: *Bifurcaria bifurcata, Fucus spiralis, Laminaria digitata, Cystoseira humilis, Sargassum muticum, Sargassum vulgaris* ; Rhodophyceae: *Osmundea pinnatifida, Gelidium* Sp1*, Hypnea musciformis, Plocamium cartilagineum, Gelidium pulchellum, Gracilaria multipartita, Ellisolandia elongata, Coralina officinalis, Bornetia secundiflora, Gelidium* Sp2*, Gracilaria cervicornis*, *Halopitys incurvus, Gymnocongrus norvrgicus* and Chlorophyceae: *Codium elongatum, Enteromorpha ramulosa, Ulva rigida, Ulva* sp2.,

Figure 1: Localization of the collection site of Sidi Bouzid

Preparation of extracts

Each powder from dried algae was extracted in different solvents, namely Methanol, Butanol, Methanol/ Dichloromethane (50/50), Dichloromethane and Hexane, as described by Caccamese and Azolina.[16] The resulting extracts were concentrated by drying in a rotary evaporator under reduced pressure at 45°C. A crude extract obtained was stored at 4°C until utilization

Plant material

The plant material used in this study consists of the leaves of common wheat showing the typical symptoms of *Alternaria alternata*, collected in 2016 and 2017 in the cereal region of Casablanca-Settat (Morocco) during the active development period of the disease (April and May).

Antifungal strain

Strains used to evaluate the antifungal activity were *Fusarium culmorum* and *Alternaria alternata*. The first one was obtained from laboratory of phytopathology of National Institute of Agronomic Research (INRA), Settat-Morocco and the second strain was isolated, purified and identified in our laboratory (Laboratory of Marine Biotechnology and environment-Faculty of Sciences- El Jadida).

Isolation and purification of *Alternaria alternata*

The naturally infected plant tissues are immersed 2 min in 50 ml of NaClO 2% to remove the superficial saprophyte flora and then rinsed three times with sterile distilled water. The plant tissues are then dried on sterile filter paper (Whatman N°1) and placed in Petri dishes supplemented with the potato carrot agar (PCA) medium (15 ml per dish), the dishes are sealed with cellophane and then incubated at 25 ± 1 ° C. After 4 to 5 days of incubation, the dishes are examined under the binocular loupe (magnification × 40), the identification of the colonies developed from the infected fragments is based on the morphology of the spores. Implants of 5 mm^2 are cut with the loop from the edge of colonies then transferred individually to a new box of PCA medium or potato dextrose agar (PDA). The cultures representing bacterial contaminations are transferred to an amended medium with streptomycin 50µg ml^{-1}.

Identification of *Alternaria alternata*

The *Alternaria alternata* strain was identified by a macroscopic Petri dish observation and a microscopic observation with reference to other works that have worked on the identification of different altenaria species.[17]

Antifungal bioassays

Antifungal assays were carried out using the agar disc-diffusion assay. Three colonies of each fungus were removed with a wire loop from the original culture plate and were introduced into a test tube containing 5 ml of Nutrient broth .An overnight culture yielded a suspension of 10^6 spores per ml (evaluated by the absorbance value of 0.5 at 620 nm .(This solution was diluted 100-fold and the fungal density was then adjusted to 0.2×10^4 spores per mL with sterile water to inoculate petri dishes containing Mueller-Hinton agar culture media .Plates were dried for about 30 min before inoculation and were used within 4 days of preparation .The organic extracts were tested using cellulose disks (6 mm diameter) satured with the solution .After the temperature was equalized at 4 °C ,the microorganisms were incubated overnight at 24 °C during 36 hours .Diameters of inhibitory zones were then measured .Discs impregnated with standard antifungal such ,amphotericine B (50µg/ml) were used as reference and discs impregnated with each solvent are used like control .All tests were performed in triplicate) .

Antifungal efficiency of extracts was evaluated according to the following scale:

- Ø ≤ 10 mm: No-significant antifungal activity
- 10 < Ø < 15 mm: Moderate antifungal activity
- 15 ≤ Ø < 20 mm: Significant antifungal activity
- Ø ≥ 20 mm: Very significant antifungal activity

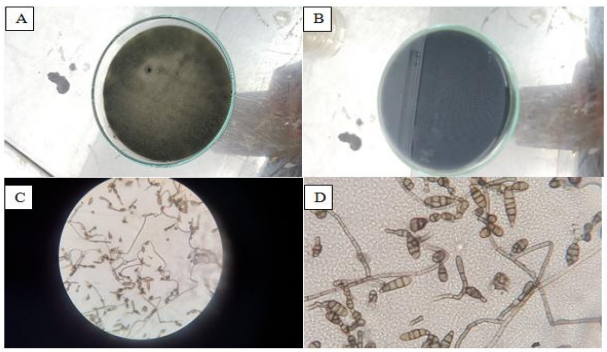
Statistical analysis

The data were statistically analyzed by applying a one-way ANOVA for comparison of mean values. All tests were considered to be statistically significant at *P<0.05.

RESULTS AND DISCUSSION

Identification and isolation of *Alternaria alternata*

The strain of *Alternaria alternata* was identified by a macroscopic observation on petri dish and by a microscopic observation with reference to the identification keys.



A) Frontal observation of the petri dish; B) Dorsal observation of the petri dish; C) Microscopic observation X 400 and D) Microscopic observation X 1000

Antifungal activity

The results of the antifungal test of each extracts (Methanol, Butanol, Methanol/Dichloromethane (50/50), Dichloromethane and Hexane) against *Fusarium culmorum* and *Alternaria alternata* are summarized in table 1 to 3.

Table 1: Antifungal activity of brown seaweeds extracts against *Alternaria alternata* and *Fusarium culmorum*

		Diameter of inhibition (mm) against *Alternaria alternata* and *Fusarium culmorum*									
		MeOH		But		DC/MeOH		Dc		Hex	
		A.a	F.c	A.a	F.c	A.a	F.c	A.a	F.c	A.a	F.c
Bifurcaria bifurcata		22±0.3	12±0.2	16±0.4	16±0.3	15±0.2	17±0.1	13±0.2	18±0.5	13±0.1	7±0.0
Cystoseira humilis		8±0.0	25±0.3	12±0.3	15±0.6	20±0.3	29±0.2	13±0.3	23±0.4	8±0.0	7±0.1
Fucus spiralis		20±0.5	9±0.0	11±0.2	20±0.8	12±0.4	10±0.3	14±0.2	7±0.3	12±0.1	11±0.0
Laminaria digitata		8±0.1	15±0.4	9±0.3	12±0.3	12±0.0	7±0.1	8±0.1	7±0.1	7±0.0	11±0.3
Sargassum muticum		12±0.2	7±0.1	12±0.0	17±0.0	14±0.1	10±0.1	7±0.0	7±0.0	7±0.0	11±0.3
Sargassum vulgaris		9±0.1	10±0.3	11±0.2	11±0.1	11±0.1	11±0.2	13±0.1	7±0.1	7±0.0	11±0.2
Amphotericine B (50 µg/mL)	F.c	13±0.3									
	A.a	17,3±0.6									

A.a., *Alternaria alternate, F.c Fusarium culmorum*, MeOH: Methanol, But: Butanol, DC/MeOH: Dichloromethane/Methanol, DC: Dichloromethane, Hex: Hexane.

Of the sex brown algae tested, the majority of species showed a positive activity against *Fusarium culmorum* but just three species showed a positive activity against *Alternaria alternata*.

An important activity against *Fusarium culmorum* has been observed in the dichloromethane/methanolic, methanolic and dichloromethane extract of *Cystoseira humilis*, (successively 29, 25 and 23 mm) followed by butanolic extract of *Fucus spiralis* (20 mm). Significant inhibition (diameter of inhibition higher than 15 mm) was observed in the dichloromethane, dichloromethane/methanol and butanol extract of *Bifurcaria bifurcata*, Butanol extract of *Sargassum muticum,* methanol extract of *Laminaria digitata* and butanol extract of *Cystoseira humilis* wish is successively 18, 17, 16, 17, 15 and 15mm compared to the antifungal control (**table 1**). Concerning *Alternaria alternata* an important activity was observed in the methanolic extract of *Bifurcaria bifurcata* (22 mm), followed by the methanolic extract of *Fucus spiralis* and the dichloromethane / methanolic extract of *Cystoseira humilis* (20 mm) (**table 1**).

According to these results, it can be noted that F*usarium culmorum* is more sensitive than *Alternaria alternata* against brown algae tested (**table 1**).

Table 2: Antifungal activity of red seaweeds extracts against *Alternaria alternata* and *Fusarium culmorum*

		Diameter of inhibition (mm) against *Alternaria alternata* and *Fusarium culmorum*									
		MeOH		But		DC/MeOH		DC		Hex	
		A.a	***F.c***	***A.a***	***F.c***	***A.a***	***F.c***	***A.a***	***F.c***	***A.a***	***F.c***
Bornetia secundiflora		15±0.1	12±0.0	7±0.0	10±0.6	11±0.3	7±0.0	8±0.0	7±0.0	7±0.1	7±0.3
Coralina officinalis		7±0.1	10±0.0	7±0.1	7±0.5	9±0.0	7±0.0	10±0.0	7±0.1	7±0.0	7±0.1
Ellisolandia elongata		10±0.3	7±0.1	10±0.3	7±0.0	7±0.0	7±0.0	11±0.1	7±0.0	7±0.0	7±0.0
Gelidium pulchellum		7±0.3	15±0.5	8±0.6	15±0.0	7±0.1	7±0.1	7±0.3	7±0.3	7±0.1	7±0.0
***Gelidium* sp1.,**		7±0.1	19±0.5	9±0.3	7±0.3	11±0.3	12±0.3	14±0.0	12±0.6	10±0.0	7±0.3
***Gelidium* sp2.,**		16±0.5	11±0.1	8±0.3	7±0.1	11±0.1	12±0.0	14±0.3	10±0.0	13±0.3	7±0.0
Gracilaria cervicornis		7±0.0	10±0.3	10±0.1	16±0.0	7±0.2	10±0.0	7±0.1	9±0.3	7±0.6	8±0.3
Gracilaria multipartita		13±0.1	24±0.4	14±0.8	13±0.3	8±0.3	7±0.0	13±0.5	7±0.0	7±0.3	7±0.3
Gymnocongrus norvegicus		13±0.5	11±0.1	10±0.0	8±0.1	11±0.0	12±0.1	9±0.0	13±0.3	9±0.3	10±0.1
Halopitys incurvus		7±0.3	9±0.0	10±0.0	10±0.3	7±0.1	7±0.0	7±0.0	10±0.1	7±0.5	8±0.5
Hypnea musciformis		7±0.1	22±0.3	10±0.5	20±0.5	9±0.0	7±0.1	7±0.1	12±0.3	8±0.0	15±0.6
Osmundea pinnatifida		7±0.0	7±0.0	8±0.0	7±0.1	11±0.1	7±0.1	11±0.3	7±0.3	11±0.3	7±0.1
Plocamium cartilagineum		7±0.0	17±0.3	10±0.1	14±0.1	12±0.1	7±0.0	13±0.3	25±0.5	14±0.0	23±1.3
Amphotericine B (50 µg/mL)	F.c	13±0.3									
	A.a	17,3±0.6									

A.a.,: *Alternaria alternata, F.c.: Fusarium culmorum*, MeOH: Methanol, But : Butanol, DC/MeOH: Dichloromethane/Methanol, DC: Dichloromethane, Hex : Hexane.

Concerning the red algae, six algae showed a significant activity against *Fusarium culmorum* in at least one of the solvents used, while only two algae show significant activity against *Alternaria alternata*.

A very important activity against *Fusarium culmorum* was observed in the dichloromethanolic extract of *Plocamium cartilaginum* (25 mm) followed by the methanolic extract of *Gracilaria multipartita* (24 mm), the hexanolic extract of *Plocamium cartilaginum* (23 mm), methanolic and butanolic extract of *Hypnea musciformis* (22 and 20 mm). The significant inhibition with diameter of inhibition higher than 15 mm was observed in the methanolic extract of *Gelidium* sp1., and *Plocamium cartilagineum*, butanolic extract of *Gracilaria cervicornis*, methanolic and butanolic extract of *Gelidium pulchellum* and hexanolic extract of *Hypnea musciformis* which represent a diameter of 19, 17, 16, 15, 15 and 15 mm respectively. While the two species *Gelidium sp2.,* and *Bornitia secundiflora* show significant activity against *Alternaria alternata* in the methanolic extract which is successively 16 and 15 mm (table 2).

Table 3: Antifungal activity of green seaweeds extracts against *Alternaria alternata* and *Fusarium culmorum.*

		Diameter of inhibition (mm) against *Alternaria alternata* and *Fusarium culmorum*									
		MeOH		But		DC/MeOH		DC		Hex	
		A.a	F.c	A.a	F.c	A.a	F.c	A.a	F.c	A.a	F.c
Codium elongatum		7±0.1	17±0.0	8±0.0	7±0.0	14±0.3	7±0.5	15±0.8	10±0.1	12±0.3	7±0.0
Enteromorpha ramulosa		8±0.0	10±0.1	7±0.0	7±0.0	14±0.1	9±0.0	11±0.5	7±0.3	7±0.0	7±0.3
Ulva rigida		7±0.0	20±0.3	8±0.1	20±0.5	10±0.5	27±0.6	12±0.3	10±0.0	8±0.0	11±0.3
***Ulva* sp2.,**		7±0.1	12±0.0	7±0.3	12±0.3	7±0.6	11±0.3	12±0.7	11±0.0	12±0.0	13±0.1
Amphotericine B (50 µg/mL)	F.c	13±0.3									
	A.a	17,3±0.6									

A.a; *Alternaria alternata, F.c*: *Fusarium culmorum*, MeOH: Methanol, But: Butanol, DC/MeOH: Dichloromethane/Methanol, DC: Dichloromethane, Hex: Hexane.

About the green algae, two of the four species tested showed significant activity against the fungal species *Fusarium culmorum* while only one specie showed activity against *Alternaria alternata*.

The important activity against *Fusarium culmorum* was found in methanol/dichloromethane extract of *Ulva rigida* (27 mm) followed by the methanolic extract and butanolic extract of the same algae (20 mm) and methanol extract of *Codium elongatum* (17 mm). The only activity observed against *Alternaria alternata* is in the dichloromethane extract of *Codium elongatum* with a 15 mm inhibition diameter (table 3).

For the 23 species studied that belongs to pheophyceae and rhodophyceae are the most active against *Fusarium culmurum* and *Alernaria alternata.*

Regarding the solvents used, Methanol is the one that gave the most active extract.

Our results are in affirmation with the results of Lakhdar et al. [18, 19] which shows that in the different species studied, those belonging to the Phaeophyceae and Rhodophyceae were the most active, while Chlorophyceae have a low inhibition. The most inhibition diameter was 25 mm and 21 mm in the methanolic extract of *Halopitys incurvus* and *cystoseira humilis* respectively against *Pectobacterium brasiliensis* and *Dickeya dadantii* (*Erwinia chrysanthemi*), that causes soft rot in potato (*Solanum tuberosum* L). The same result was showed by Bouhraoua et al.[20] which shows that algae belonging to phyophyceae and rhodophyceae are the most active against *Bipolaris sorokiniana*. Similar finding was reported by Khallil et al., [15] In vitro antifungal screening of six extracts of five seaweeds belong to Phaeophyta (*Sargassum vulgare, Cystoseira barbata, Dictyopteris membranacea, Dictyota dichotoma, and Colpomenia sinuosa*) against eight fungal species (*Alternaria alternata, Cladosporium cladosporioides, Fusarium oxysporum, Epicoccum nigrum, Aspergillus niger, Aspergillus ochraceus, Aspergillus flavus*, and *Penicillium citrinum*) showed an important antifungal activity whith a broad spectrum inhibitory action. Cyclohexanic extracts were almost the most active, whereas both acetone and ethyl acetate extracts exhibited the lowest antifungal activity.

Tuney et al. [14] recorded positive antifungal activities using methanol, acetone, diethyl ether and ethanol extracts of *Cystoseira mediterranea* and *Ulva rigida*. Methanol extract of *Codium fragile* exhibited strong activities against *Alternaria alternata*, *Fusarium oxysporium*, *Alternaria brassicicola*, *Ulocladium botrytis* and *Botryotricum piluliferum*.[21]

The methanolic extracts of three marine phaeophytea species; *Sargassum latifolium*, *Hydroclathrus clathratus* and *Padina gymnospora* collected from Red sea, Hurghada, Safaga coastline, Egypt, have a high antifungal potential against two fungus plant disease *Fusarium solani*, *Rhizoctonia solani*. The methanolic extract of *Padina gymnospora* recorded the highest inhibition zones 24 and 21,7 mm against *Fusarium solani* and *Rhizoctonia solani* respectively.[4]

Treatment of plants with various agents, including algae extracts, can induce resistance to subsequent pathogen attack both locally and systemically.[22] Algal extracts are nowadays more applicable in controlling of plant-infecting fungi instead of synthetic fungicides, due to their higher safety and relatively negligible impacts on the environment. [21, 23, 24] The crude and purified algal preparations are able to protect the plants against several pathogenic fungi.[25, 26]

Seaweeds are known for their high original polysaccharides some of which have various degrees of sulfating. Brown algae, in particular, are already used in fertilization for a long time successfully. Some have been work specific to the eliciting properties of their constituents that are now entering products commercial. In the literature most macroalgal polysaccharides and derived polysaccharides activate defense responses of plants and protection against a range of pathogens by activating salicylic acid, jasmonic acid and/or ethylene signaling pathways at a systemic level.[27] The increased resistance of the treated plants to stress conditions has been reported by several authors. Blunden et al. [28] suggested the possibility that betaines present in the extracts of some seaweeds could be responsible for some of the reported effects. Our results are in agreement with these results, which show that brown algae (the richest in polysaccharides) have the strongest activity against the studied fungal species followed by red algae and finally the least active green algae.

Inducible reactions of plants defending against pathogens or in response to external treatment essentially require the perception of signal molecules. This kind of recognition leads to triggering of a plethora of reactions, which result in augmentation of resistance to the invading pathogens. This enhanced state of resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes, parasitic plants, and even insect herbivores. [29, 30] These reactions induced production of defense-specific signal molecules such as salicylic acid, jasmonates, and accumulation of antimicrobial compounds/proteins such as phytoalexins and pathogenesis-related proteins.[31] The chemical stimuli or elicitors, which bring about these induced reactions, are diverse and include oligosaccharides, polysaccharides, lipids, glycoproteins, peptides, and proteins.[32, 33]

Jayaraj et al.[34] found that treatment of carrot plants with an *Ascophyllum nodosum* extract resulted in them showing significantly reduced disease severity caused by pathogenic *Alternaria radicina* and *Botrytis cinerea*. The activity of certain defense-related enzymes was significantly increased in the treated plants in comparison with the controls. Treatment of the plants with the seaweed extract enhanced their disease resistance by induction of defense genes and proteins.

Kraska and Schönbeck, [35] reported that application of very low amounts of betaines extracted from algae, resulted in significant enhancement in the ability of treated plants to resist fungal attack. Of the same, Tyihák et al.[36, 37] have also shown that application to plants of very low amounts of betaines and other methylated compounds extracted from sea algae induces an immune response to fungal attack.

According to Ibraheem et al.[4] application of the powder of *Padina gymnospora, Sargassum latifolium* and *Hydroclathrus clathratus in* vivo eggplant pot experiment significantly decreased the percent of root rotting in *Fusarium solani* infected soil by 83, 56 and 27 %, respectively. Moreover, *Padina gymnospora* enhanced growth performance of eggplant in term of shoot length and plant fresh weights in the infected soil. Interestingly, the soil-free pathogen treated with *Padina gymnospora* and *Sargassum latifolium* powder alone showed significant increase in root length compared to the control treatment beside increasing of fruit fresh weights in case of *Padina gymnospora* amendment.

CONCLUSION

The present study confirms the potential use of seaweed extracts harvested from the El Jadida Atlantic coast (Morocco) as a source of antifungal compound and may constitute a basis for promising future applied research that could investigate the use of seaweeds which are renewable prosperity against deteriorating fungi.

Additional studies need to be performed to define and characterize the chemical and biochemical level, the preferential effect of algae extracts on fungal species that have adverse effects on plant and subsequently on human health. Finally we conclude that the algae is a source of bioactive compounds with potential applications in controlling undesired microorganisms in the field of agriculture. This may encourage the use of natural products for substituting chemical preservations in food systems.

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