Research Article

In Vitro Antifungal Activity of Seaweed Extracts Against *Sclerotium rolfsii*: A Causal Agent of Root Rot Disease on Sugar beet (*Beta vulgaris* L.)

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ABSTRACT

Evaluate the ability of the seaweed extracts to inhibit in vitro *Sclerotium rolfsii*: a plant pathogenic fungus induced root rot on sugar beet. 54 organic extracts and 18 aqueous extracts of 18 seaweed collected from the coast of El Jadida were evaluated for their ability to inhibit in vitro *Sclerotium rolfsii* the root rot pathogen. The mycelium growth was monitored on agar media amended with these seaweed extracts at the concentration of 1mg/ml. The seaweed extracts inhibited mycelial growth of *Sclerotium rolfsii* at various degrees. The maximum inhibition was obtained with organic seaweed extracts; it was recorded with *Plocamium cartilagineum* extract in dichloromethane (89%), followed by *Ellisolandia elongata* extract in methanol (62%). For aqueous seaweed extracts the highest inhibitory effect was obtained by *Ellisolandia elongata* (81%) followed by *Plocamium cartilagineum* (62%). In addition, aqueous extract of *Ellisolandia elongata* give a complete inhibition of germination of sclerotia. Organic extract of *Plocamium cartilagineum* and aqueous extract of *Ellisolandia elongata* showed an important effect against the root rot pathogen on sugar beet and could be a promising source of novel bioactive compound that can offer protection against this plant disease.

Keywords: Seaweed, *Sclerotium rolfsii*, Antifungal activity, Coast of El Jadida.

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is the most important sucrose producing crop in temperature gions covering almost 40% of the world's sucrose production. However, during the last decades, this production has gradually decreased, due to infections by various plant pathogens [1]. In Morocco, the root rot pathogen *Sclerotium rolfsii* causes damage to sugar beet mainly in the irrigated region of Doukkala [2]. Disease out breaks occurs mainly in July and August on mature roots, which are partially or completely destroyed. The pathogen survives in the soil in the form of sclerotia and the disease incidence in a sugar beet field was found to be correlated with the density of these fungal structures in the soil [2].

The root rot pathogen *Sclerotium rolfsii*is a major constraint to sugar beet production mainly in the irrigated region of Doukkala (mid-west Morocco) [1, 3], the losses can be up to 50% of the root production [4, 2]. Its geographical distribution is world wide[5]. It is the most destructive soil borne fungus [6, 7], it is prevalent in warm temperate and subtropical regions of the world [8] it can cause several types of damage, including damping-off, crown, and root-rot as well as dry rot canker in older plants [9, 10] and can affecting a large number of agricultural plants [11, 12] of over 500 different plant species [10]. The large number of sclerotia produced by *Sclerotium rolfsii* and their ability to persist in the soil for several years, as well as the profuse growth rate of the fungus make it well suited facultative parasite and a pathogen of major importance throughout the world [13] which are difficult to control [14]. The optimal temperature of manifestation of the disease is between 25 and 35°C [5, 15].

To control this soil-borne plant pathogen, many strategies have been tested. One of them, which has become more important in recent years, is biological control [1]. The biological control of plant pathogensis a potential non chemical means for plant disease control [13].Preventive methods such some cultural practices [early harvesting and crop rotation] and soil solarization have been proposed as means of control in many studies [16], but there is no economically effective chemical control against root rot on sugar beet [17] because the management of the plant diseases incited by soil borne pathogensis not achievable chemically, due to the widespread host range, abundant growth of the pathogen and its capability of producing excessive sclerotia that may persist in soil for several years [7].

Morocco known by its particular geographical position : the Mediterranean sea and the Atlantic ocean, which givesit a great bio ecological diversity, there are 451 species present on the two marine façades including green seaweed, red seaweed and brown seaweed [18, 19].

Seaweed extracts can offer protection against such plant diseases [20, 21] and [22]. In this context, and trying to reduce pesticides input to control phytopathogens [23], several studies have reported antimicrobial effect in vitro and in vivo of seaweed extracts on phytopathogens. The aim of the present study was to evaluate the antifungal activity of seaweed extracts to control the root rot pathogen of sugar beet caused by *Sclerotium rolfsii*.

MATERIALS AND METHODS

Seaweed species

The seaweeds were collected at Sidi Bouzid, located on the Moroccan Atlantic coast south of the city of El Jadida (Lat 32° 15' to 33° 15'; Long 7° 55' to 9° 15') and were washed thoroughly and packaged in polyethylene bags. Then, they were dried and crushed to a fine powder. These species belong to three groups of seaweed: Chlorophyceae (*Codium elongatum, Ulvafasciata, Ulva*lactuca); Phaeophyceae (*Bifurcaria bifurcata, Fucus spiralis, Laminaria ochroleuca, Sargassummuticum, Sargassum vulgaris*) and Rhodophyceae (*Hypnea musciformis, Plocamium cartilagineum, Gelidium pulchellum, Bornetia secundiflora, Gelidium corneum, Gracilaria cervicornis, Osmundea pinnatifida, Halopitys incurvus, Ellisolandia elongata, Corallina officinalis*).

Extraction procedures

Organic extracts

The powder of 18 seaweed was extracted in three solvents, methanol, dichloromethane /methanol (v/v) and dichloromethane as described by Caccamese and Azolina (1979), and they concentrated to dryness in a rotary evaporator under reduced pressure until a crude extract. They were kept in a dry place before use.

Aqueous extracts were adapted from the protocol of Zirihi and al., (2003). 1g of the fine powder obtained was macerated in 10ml of hot distilled water in a mortar, and then kept in a refrigerator (4°C) for 24h until utilisation.

Fungal pathogen

The pathogen *Sclerotium rolfsii* used in this study was obtained from infected sugar beet tubers collected from the Doukkala region and identified in mycology laboratory at the Department of Phytopathology [3, 2], Agronomic and Veterinary Institute Hassan II, Rabat- Morocco.

Inhibitory effect on mycelia growth

Sclerotium rolfsii

The organic extracts were added at concentration of 1mg/ml to the PDA medium. 15 ml of amended media was poured into 9cm diameter petri dishes and another set of untreated PDA plates was used as control. All plates were inoculated using 6mm plugs of agar and mycelium taken from actively growing cultures of fungus on PDA. Petri plates were incubated in the dark at 29°C. Two orthogonal measurements of colonies were taken using the control plates as a reference after 72h of incubation. Percentage of growth inhibition relative to the control was calculated:

$$\text{Inhibition of growth (\%)} = \frac{X - Y}{Y} \times 100$$

X: Mycelia growths of pathogen in the absence of the seaweed extract (control)

Y: mycelia growth of pathogen in the presence of seaweed extract.

Discs impregnated with standard antibiotics such as Amphotericin B were used at 200µg/ml as reference in the test of antifungal activity. In addition, control disks were prepared with each solvent used. All tests were at least triplicate

Production of sclerotia of *sclerotium rolfsii*

Mycelial disks (6mm in diameter) obtained from a 5 day old PDA culture were transferred to agar plates containing PDA and incubated at 25°C in the dark for 3 weeks. The sclerotia formed were dislodged from the surface of the agar plates and used immediately.

Statistical analysis

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

RESULTS AND DISCUSSION

Evaluation of the inhibitory effect of seaweed extracts on mycelia growth of *Sclerotium rolfsii*

The inhibitory effect of seaweed extracts on the mycelia growth of *Sclerotium rolfsii* in the PDA culture medium is calculated after 72 hours of incubation at 29°C (table1 to table 3).

Red seaweed

Organic extracts of 10 red seaweed were evaluated for their ability to inhibited the mycelial growth (figure 1).

The maximum inhibition was obtained with *Plocamium cartilagineum* extract in the three solvent (methanol, dichloromethane and methanol/dichloromethane (v/v)): 84.1%, 89.3% and 82.9%, which is superior to the inhibition effected by Amphotericin B at 200µg/ml (82.14%) (table 1).

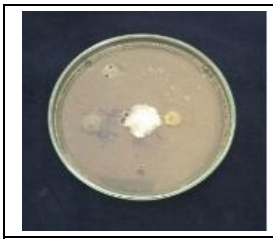
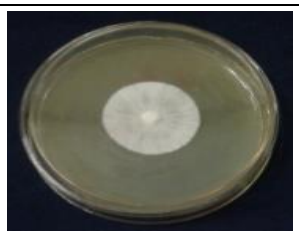
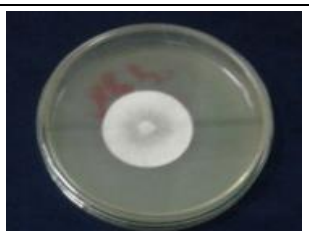
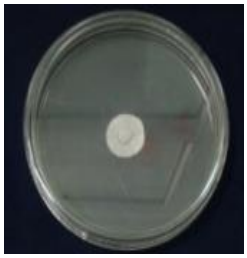
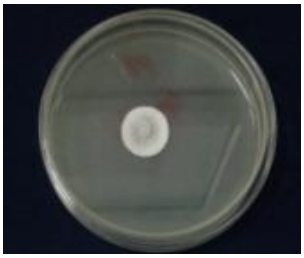
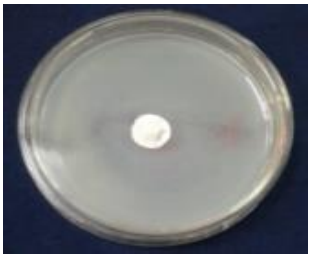
Brown seaweed

Mycelial growth inhibition was determined from 5 brown seaweed extract in the three solvent methanol, dichloromethane and methanol/dichloromethane v/v (table 2).

For brown seaweed the highest value of inhibition was obtained with *Bifurcaria bifurcate* extract in dichloromethane (51.6%). The percentage of inhibition against *Sclerotium rolfsii*i slower for all extracts than that affected by Amphotericin B (82.14%).

Green seaweed

Green seaweed extract were tested for inhibition of *Sclerotium rolfsii* growth (figure 2), only 3 extracts in dichloromethane have notable effect (56±0) which is less than percentage of inhibition recorded by Amphotericin B (82.14%).

			
Control (+)	Control (-)	*Ellisolandia elongata* (Meth)	*Corallina officinalis* (Meth / Dich)
			
Hypnea musciformis (Dich)	*Plocamium cartilagineum* (Meth)	*Plocamium cartilagineum* (Meth/Dich)	*Plocamium cartilagineum* (Dich)

Meth: Methanol, Meth/Dich: Methanol/Dichloromethan, Dich: Dichloromethan) Control (+): Amphotericin B at 200µg/ml

Figure 1: Mycelial growth of *Sclerotium rolfsii* in presence of red seaweed extracts

Table 1: Effect of red seaweed extracts on in vitro growth of *Sclerotium rolfsii*

Seaweed	Percentage of ihnibition(%)		
	Meth	Dich	Meth/Dich
Bornetia secundiflora	16.3±0.4	23.4±1.05	4.8±1.35
Ellisolandia elongata	62.3±0.4	31.7±0.36	44.8±0.8
Coralina officinalis	26.2±1.2	50.4±1.6	60.7±0.69
Gelidium pulchellum	0	27.4±1.38	13.5±0.4
Gelidium corneum	23±1.44	33.3±0.69	17.1±0.4
Gracilaria cervicornis	36.5±1.6	39.3±0.69	45.6±3.53
Halopitys incurvus	11.9±1.38	21±2.77	51.6±0.8
Hypnea musciformis	33.7±0.8	61.9±1.2	56.7±2.19
Osmundea pinnatifida	12.3±1.05	14.3±0.69	36.1±1.05
Plocamium cartilagineum	84.1±1.05	89.3±0.69	82.9±0.4
Amphotericin B (200 µg/ml)	82.14±0.4		

Meth: Methanol; Dich: Dichloromethane

Table 2: Effect of brown seaweed extracts on the in vitro growth of *Sclerotium rolfsii*

Seaweed	Percentage of inhibition (%)		
	Meth	Dich	Meth/Dich
Bifurcaria bifurcata	23.4±2.62	51.6±1.44	50±1.2
Fucus spiralis	0	49.2±1.05	15.9±0.4
Laminaria ochroleuca	0	17.5±1.03	50.8±0.4
Sargassum muticum	29.4±0.8	24.2±0.4	0
Sargassum vulgare	27.4±1.2	26.6±0.8	22.6±0.69
Amphotericin B (200µg/ml)	82.14±0.4		

Meth: Methanol; Dich: Dichloromethane

Control (-)	Control (+) (Amphotericin B at 200µg/ml)	*Ulva lactuca* (Dich)

Figure 2: Mycelial growth of *Sclerotium rolfsii* in presence of *Ulva lactuca* (Dich)

No significant inhibition observed for other extracts (table 3).

Table 3: Effect of green seaweed extracts on the in vitro growth of *Sclerotium rolfsii*

Seaweed	Percentage of inhibition (%)		
	Meth	Dich	Meth/Dich
Codium elongatum	0	36.5±0.8	0
Ulva fasciata	15.5±1.38	29±0.4	32.1±1.16
Ulva lactuca	0	56±0	0
Amphotericin B (200 µg/ml)	82.14±0.4		

Meth: Methanol; Dich: Dichloromethane

Evaluation of the inhibitory effect of 18 aqueous seaweed extracts on the mycelia growth of *Sclerotium rolfsii*

Aqueous extracts from 18 seaweed were tested for their effect on the in vitro mycelia growth of the *Sclerotium rolfsii*. The percentage of inhibition was determinate after 72h of incubation (table 4).

According to the figure 3, *Ellisolandia elongata* extract has an important inhibition 81.3%, followed by *Plocamium cartilagineum and Corallina officinalis* which have successively: 62.3% and 58.7%. No notable inhibition was observed with the other extracts.

After 4 weeks of incubation, we noticed in the petri dishes containing the extracts of *Ellisolandia elongata* and *Plocamium cartilagineum* that the mycelium was developed until the dishes were filled. While in the petri dish which contains the aqueous extract of the red alga *Ellisalandia elongata* the mycelial growth remains unchanging.

Evaluation of the effect of aqueous extracts on the germination of sclerotia

The previous test showed that there are 6 aqueous seaweed extracts who have a percentage of inhibition >40% against the mycelia growth of *Sclerotium rolfsii*. Based on this result, these extracts were tested on the germination of sclerotia. After 5 days we observed the results and calculated the % of inhibition (table 5).

Table 4: Antifungal activity of aqueous seaweed extracts against *Sclerotium rolfsii*

	Seaweed	Percentage of inhibition (%)
Red seaweeds	*Bornetia secundiflora*	29.8 ± 3.43
	Ellisolandia elongata	81.3 ± 1.4
	Corallina officinalis	58.7 ± 0.4
	Gelidium pulchellum	0
	Gelidium corneum	0
	Gracilaria cervicornis	22.6 ± 0.69
	Hypnea musiformis	17.9 ± 0.66
	Osmundea pinnatifida	0
	Plocamium cartilagineum	62.3 ± 1.05
Brown seaweeds	*Bifurcari abifurcata*	23 ± 1.44
	Fucus spiralis	0
	Halopitys incurvus	10.3 ± 1.44
	Laminaria ochroleuca	0
	Sargassum muticum	0
	Sargassum vulgare	0
Green seaweeds	*Codium elongatum*	43.7 ± 7.37
	Ulva fasciata	44.8 ± 1.02
	Ulva lactuca	44.4 ± 2.19
	Amphotericin B (200µg/ml)	82.14±0.4

Meth: Methanol; Dich: Dichloromethan

Table 5: Antifungal activity of aqueous seaweed extracts on the germination of sclerotia

Seaweed	Percentage of inhibition (%)
Ellisolandia elongata	100 ± 0
Corallina officinalis	66.66 ± 8.33
Plocamium cartilagineum	0
Codium elongatum	0
Ulva fasciata	0
Ulva lactuca	0

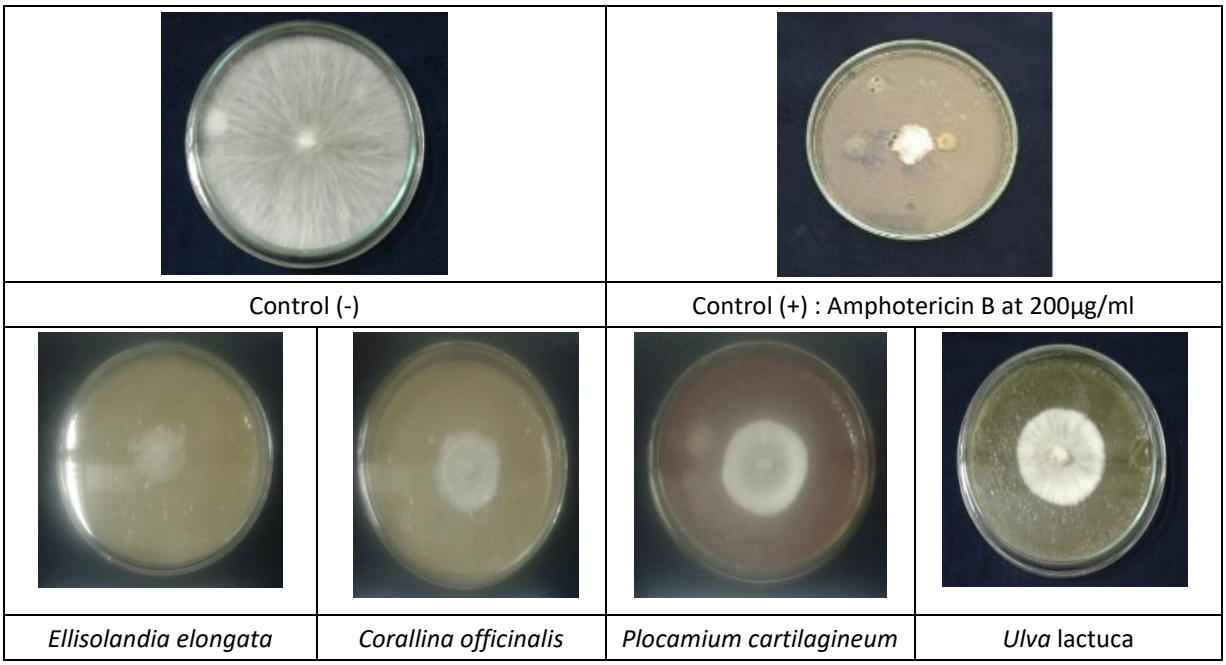


Figure 3: Mycelial growth of *Sclerotium rolfsii* in presence of aqueous extracts of seaweed

The aqueous extract of *Ellisolandia elongata* totally inhibited the germination of sclerotia, 66.66% of inhibition is recorded with the extract from *Corallina officinalis*. However, there is no inhibition from the other seaweed extracts.

In order to find an effective and inexpensive solution to combat root rot disease caused by *Sclerotium rolfsii*, a major constraint to the production of sugar beets, several researches have been carried out.

A study was conducted by Bhuiyan and al.[13], using six fungicides and *Trichoderma harzianum* to control *Sclerotium rolfsii*. The complete inhibition was obtained with Provax-200 at all the selected concentrations also with the highest concentration of tilt. The screened isolates of *Trichoderma* showed significantly variable antagonism ranging from 65.01% to 83.06% reduction of radial growth of *Sclerotium rolfsii*. Similarly, ten antibiotic-producing *Streptomyces* spp. Isolated from *Moroccan soils* were evaluated for their ability to inhibit in vitro *Sclerotium rolfsii* development. Four isolates having the greatest pathogen inhibitory capabilities were subsequently tested for their ability to inhibit sclerotial germination in sterile soil. The selected streptomyces isolated reduced significantly the disease severity and gave a significant increase in seedling development compared to the control.

According to Shahzaman Ahsan and al.[6], Soil application of maize grain based culture of *T. harzianum* at 5, 10, 15 and 20 g per pot showed significant reduction in mortality of chickpea seedlings caused by *Sclerotium rolfsii.* Maximum control of collar rot (53.33%) was recorded in treatment where *T. harzianum* was applied: 20 g per pot. Seed treatment with fungicides significantly reduced the mortality of chick pea seedlings when compared with control. The Seeds treated with Carboxin (Vitavax):2 g/kg proved most effective and showed 66.70% disease control followed by Propiconazole: 2 g/kg seed.

Mallotus oppositifolius (Euphorbiaceae) was employed for testing in vitro, by using double dilution method on PDA medium, the sensitivity of two fungal strains *Sclerotium rolfsii*. MIC and MFC of the growth of spawn were obtained at 100mg/ml and 200mg/ml for the aqueous extract and 12.5mg/ml and 25mg/ml for ethanol extract. The MIC of the sclerotia's germination was 6.25mg/ml and the MFC was 12.5 mg/ml for the two strains.[24] In the same way, the efficacy of pseudo-banana trunk sapis tested on *Sclerotium rolfsii* fungus cultures as a culture medium and under cover on tomato plants inoculated with *S. rolfsii*. The results show that the rate of withered plants treated with the saps was 4.15%, 16.55% and 33.33% against 49.95% in the control treated with distilled water. The mulch from the three banana varieties did not give any tomato plant wilt against 41.5% of withered plants in the controls [25].

In addition, the effects of caraway and peppermint extracts were evaluated at concentrations of 2, 4, 6, 8 and 10%, respectively on the radial mycelia growth of *Sclerotium rolfsii*. All treatments showed a significant reduction in disease incidence compared with the control treatment [26]. Likewise, aqueous extract of twenty different plant species were invitro evaluated for their inhibitory effect on mycelia growth of *Sclerotium (Athelia) rolfsii*. Generally, all plant species inhibited mycelia growth of the pathogen but maximum inhibition was recorded by *Azadirachta indica* (73.8%) [15].

Another study was conducted on the extracts (essential oils and powders of fruits and leaves) of *Xylopia aethiopica* (Dunal), *A. Rich* (Annonaceae) and two synthetic fungicides (Banko-plus and mancozebe) to test their antifungal potency of *Sclerotium rolfsii*. Fifteen

isolates of *Trichoderma* sp. Are also used for quality antibiotic and fertilizer for crops. At the end of the *in vitro* tests, it was revealed that the essential oil of fruits was higher than the other extracts on mycelial growth of *Sclerotium rolfsii* inhibition rate. Four isolates with the best attitudes to mycelial growth were used for *in vivo* testing. After *in vivo* assays, the essential oil of fruits and the 9th isolate of *Trichoderma harzianum* showed a good return on the growth parameters of tomato. The powdered fruit of *Xylopia aethiopica* also showed a good response to the incidence of the disease. The impact of the higher disease was 10.66 for the fruit powder and 5.96 for the essential oil of the fruit. This study offers the possibility of using essential oils and powdered fruit of *Xylopia aethiopica* in strategies to control *Sclerotium rolfsii*[27].

The antifungal activities of numerous seaweed species have been reported by many researches, presenting an extended spectrum of action against fungi attacking many cultures [28]. In the present study, we showed the effective ness of organic and aqueous extract treatment from different seaweed in order to reduce root rot disease. This is the first published report use natural seaweed extract to fight against *Sclerotium rolfsii*: a causal agent of root rot disease on sugar beet. Direct inhibition of pathogen growth was observed, the seaweed extracts inhibited mycelia growth of *Sclerotium rolfsii* to various degrees. The organic seaweed extracts showed the maximum inhibition, it was recorded with *Plocamium cartilagineum* (red seaweed) extract in dichloromethane (89%), followed by *Ellisolandia elongata* (red seaweed) extract in methanol (62%). The highest inhibitory effect of aqueous seaweed extracts was obtained by *Ellisolandia elongata* (81%) followed by *Plocamium cartilagineum* extract (62%). In addition, treatment of sclerotia with the aqueous seaweed extract resulted in a complete inhibition (100%) of germination of sclerotia with *Ellisolandia elongata* extract and a significant effect inhibitory (66%) was recorded to *Corallina officinalis*.

Our results are in agreement with the study conducted in vitro [23], which shows that three brown seaweed extracts, namely *Cystoseiramyrio phylloides*, *Laminaria digitata* and *Fucus spiralis*, have a significant inhibition of growth with methanolic seaweed extracts against fungal and bacterial disease of tomato and significantly reduced disease severity in the greenhouse. The same result was showed [21] which the red seaweed *Cystoseira humilis* has an important inhibition against *Fusarium culmurum* (29±0.2 mm) and the highest activity against *Alternaria alternat* was obtained with the brown seaweed *Bifurcaria bifurcata* (22 ± 0.3 mm). Likewise, [29], was evaluated six seaweed extracts include green and red seaweed to control disease of tomato caused by fungus *Alternaria solani*. Five of them exhibited biocidal activity against *Alternaria solani* and the highest inhibition was 94%.

The effect inhibitory of seaweed extracts from *Rhodomela confervoides* (red seaweed) and *Padina pavonica* (brown seaweed) was tested [30] against *Candida albican*s and *Mucor ramanianus*. They have a significant antifungal activity which the highest inhibiting effect was noted (26mm: diameter of inhibition zone).A similar study conducted [31] proved that algal extracts showed an important anti fungal activity. It is correlated with the study done [32], which showed that the extracts of marine brown seaweed tested has a significant anti fungal activity.

Begum and al.[33] reported that the seaweed extract from *Tubinaria conoides* (brown seaweed) has a significant antifungal activity against *Fusarium oxysporum*. As well, the results of the study done [34], who showed that different extracts of marine macro seaweed have a significant anti fungal activity. The minimum inhibitory concentration (MIC) was0.03µg/ml within *M. canis* and the largest inhibition halo in *T. rubrum* (25mm) through the use of the methanol extract. The ethanol extract was shown to be the best inhibiting fungi growth, chloroform and hexane fractions of *H. musciformis* inhibited the growth of all fungus species, yielding the conclusion that a polar extracts obtained from seaweed presented the best activity against important pathogenic fungi. The same results are confirmed [35], showing that *S. zonale*, *L. dendroidea*, *P. canaliculata*, *S. muticum*, *A. nodosum* and *F. spiralis* extracts significantly inhibited the *C. lagenarium* growth, causing damage to the crop of cucumber.

Also, Sujatha and al.[36], evaluated the anti fungal activity of seaweed extract against soil borne pathogens in pulses. The results revealed that seaweed extracts have a significant effect inhibitory specially the methanol extract of *Sargassum myricocystum*. The same result was proved by Manigandam and al.[37] and also by Kumar and al.[38]

Seaweeds extract are producers of biologically active compounds. Such ability was developed as defense against several organisms[39]. To date, many chemical compounds of marine seaweed having various biological activities have been isolated, and some of them are under investigation and are being used to develop new product.

CONCLUSION

These studies show that the organic seaweed extract from *Plocamium cartilagineum* has the most effect inhibitory (89%), also the treatment of Sclerotia with the aqueous seaweed extract from *Ellisolandia elongata* inhibited completely the germination of Sclerotia. Other studies are being conducted to evaluate their effective ness in vivo, also might be effective to control in vivo the damping-off caused by *Sclerotium rolfsii.*

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