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Antifungal bio-efficacy of the red algae *Gracilaria confervoides* extracts against three pathogenic fungi of cucumber plant

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ABSTRACT

In this study, the potential of the macroalgae *Gracilaria confervoides* red algae extracts and powder were evaluated as a bioagent source of the three soil-borne pathogenic fungi of cucumber namely; *Rhizoctonia solani*, *Fusarium solani* and *Macrophomina phaseolina* in Egypt. Five organic solvents; Ethyl acetate, Methanol, Acetone, Benzene, and Chloroform, in addition to water were used for the extraction to evaluate their bioeffecinecy on mycelium growth reduction of the three fungal pathogens on potato dextrose agar (PDA). Radial growth reduction of the pathogens was noticed in *R. solani* and *F. solani* with all solvents and water extraction. In case of the fungus *M. phaseolinae* all solvents and water extractions malformatted the fungal growth (aerial mycelium and no microsclerotia). The highest reduction (100%) obtained on *R. solani* when chloroform extraction was used followed by ethyl acetate extraction (50%). In the greenhouse experiment, macroalgae powder was used to evaluate its effect on disease incidence which indicated about (70%) decrease for diseases. The highest total yield (133.2g) was obtained from plants infected with *M. phaseolinae* compared with Vitavax (66.9g) which indicated that the malformation of pathogen growth limited its pathogenicity more than the reduction of cucumber plants growth. Analysis of the extractions was done by infrared spectroscopy (IR) to identify the chemical groups, such as C-O (Ether), C-F (Alkylhide) etc. Gas chromatography-mass spectrometry (GC-mass) was used to identify substances within test samples, such as, Cyclononasiloxane, Iron monocarbonyl obtained from extraction by Chloroform. Finally, the macroalgae *G. confervoides* could serve as a new bioagent source for biological control of soil fungi.

Keywords: Seaweeds, macroalgae, *Gracilaria confervoides,* biocontrol, fungal pathogens, cucumber.

Introduction

Plant diseases particularly caused by plant pathogenic fungi are considered one of the main principle factors for decreasing food production all over the world (Saharan *et al*., 2015). Current strategies to control fungal diseases consist of preventing wetness on leaf surface during long periods of time, development of host plant resistance and application of fungicides. Synthetic chemical fungicides are widely used in conventional agriculture to control plant diseases.. However, environmental toxic hazards caused by excessive use of those fungicides pose health problems as a modern society is becoming more conscious (Kim *et al*., 2009). In addition, pathogens can derive resistance against fungicides (Namanda *et al*., 2004; Kirk *et al*., 2005). Accordingly, there is an urgent need to find anti-fungal substitutes and new control strategies are more effective, less toxic, easily obtainable and inhibit these resistance mechanisms. Nowadays, marine plants and macroalgae constitute a richness to explore and exploit in several regions of the world due to its natural products bearing broad-spectrum antifungal activities (Harman *et al*., 2004). Moreover, macroalgae are also known to help and stimulate the growth of vegetables, fruits, and other crops through their metabolites. Such compounds are extracted from different macroalgae families, like green, brown, and red algae which have been estimated at approximately 40,000 compounds (Raven *et al*., 1992). There is a considerable dataset on marine macroalgae that they could play a major role in plant

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protection and improvement (Hamed *et al*., 2017). Seaweeds extracts offer a novel approach to pest management. The macroalgae *Ulva lactuca* is a common species found in green tides, which grows abundantly in the coastal water of Egypt. This alga has been reported to prosses antioxidants and antibacterial properties. A wide range of results showed antifungal of extracts of green algae and diatoms (Mostafa *et al*., 2014). Compounds with antioxidants, antiviral, antifungal and antimicrobial activities have been detected in brown, red and green algae. The different species of macroalgae collected from Mediterranean seashores showed a variety of antifungal activities which make them interesting for programs of screening for natural products (Abdel – Khaliq *et al*., 2014). Several species of *Gracilaria* macroalgae have significant commercial value and are harvested or cultivated for application in industry as food or feed and in medicine (Ruixue Tu *et al*., 2016). Further work is required to fractionate, identify and characterize the bioactive compounds of crude algal extracts that are responsible for the antifungal action.

In the present study, we aimed to assess the antifungal potentiality of some red macroalgae against some phytopathogenic fungi infecting cucumber plants.

Materials and Methods

Macroalgae Collection

Macroalgae were collected during April to September 2013 from Port Said and Damietta coast, Egypt. The collected algae were immediately washed under running fresh water to remove the epiphytes, sand and other extraneous matter. The collected algae drained and wiped with a blotting sheet then air-dried at 45˚c for 5 days in a hot air oven. The completely dried material was then weighed and grounded finely in a mechanical grinder according to (Aseer, *et al*., 2009).

Pathogenic fungi

Three plant pathogenic fungi namely *Fusarium solani*, *Rhizoctonia solani* and *Macrophomina phaseolina* were obtained from the culture collection of Mycology Research and Diseases Survey Department, Plant Pathology Institute, Agriculture Research Center, Egypt.

Macroalgae Identification

The collected isolates were morphologically identified in Marine Science Department, Faculty of Science, Suez Canal University, Ismailia, Egypt. (Madkour and El-shoubaky, 2007).

Preparation of algae extracts

Five solvents were used, methanol, ethyl acetate, acetone, benzene, and chloroform in addition to water. Extraction was carried out by adding 50g of algae powder to 200ml of each solvent (W/V). Mixtures were shaken for 10 days on an arbitral shaker at lap temperature (25˚C) then filtered using cheesecloth followed by Whatman paper No.2 (Kumar, *et al*., 2008).

Assay of growth reduction

On PDA medium (*in vitro*)

Dual culture plates with PDA medium were used to study the reduction effect of the tested algae against *R. solani, M. phaseolina*, and *F. solani*, as described by Dennis and Webster (1971). In each plate, two wells (5 mm in diameter) were made 4cm apart. One well was inoculated with a disk (5 mm) of each pathogen (4 days-old culture). The opposite well was inoculated with (100µl) of each tested algae extract. Three plates were used for each treatment. Plates inoculated only with each of the pathogenic fungi served as a control. All inoculated plates were incubated at 25 ± 2° C for 6-10 days. When mycelial growth covered all the medium surface in the control plates, all plates were then examined and the linear growth of the pathogens was measured. The growing cultures were observed visually and microscopically for evidence of a reduction. Percentage of reduction in mycelial growth of the fungal pathogens was calculated using the following formula:

$$X = [G_1 - G_2 / G_1] \times 100$$

Where: X: % of reduction in growth G_1: linear growth of pathogenic fungus in control plates

G_2: linear growth of pathogenic fungus in dual plates with algae

Greenhouse experiments (In- vivo)

The algae powder was used in this experiment and tested for its potentiality as biocontrol agents against the three tested pathogenic infested cucumber seeds fungi. Plastic pots (25 cm in diam) filled with autoclaved sterilized sandy-loam soil were infested with 3% (w/w) sorghum grain inoculum of each pathogen/ kg soil. Infested pots were irrigated and kept for 7 days to ensure fungi dispersal in the soil before seed sowing. The infested soil was amended with dry algae powder in the ratio (1g powder: 1 Kg soil Kg soil) w/w. The control treatment carried out by the chemical fungicide (Vitavax) as seed coating 3g vitavax /Kg seeds before sowing. Seeds of cucumber were obtained from the commercial sector in Egypt, and surface sterilized by soaking in 2% sodium hypochlorite for 2 min before sowing (Sultana, *et al*., 2011).Three seeds were sown in each pot, 4 replicates were used for each treatment. Treated pots were kept in the greenhouse, watered daily to allow the decomposition of organic substrates. Disease incidence was evaluated after (60 days) by measuring number of dead plants, dry weight, and total yield.

Analysis of crude extracts (Ethyl acetate and Chloroform) by IR and GC-mass

The infrared (IR) absorption spectrum of the purified fraction was evaluated to determine the possible functional groups in the algae extracts responsible for bio-reduction for fungal pathogens, this work was estimated at the National Center for Research (He *et al*., 2016).

The Gas chromatography-mass spectrometry analysis for derivative of ethyl acetate extracted filtrate of *Gracilaria confervoides* by using an Agilent 6890series II gas chromatograph. An Agilent 5973 mass spectrometer with electron ionization, mode (EI) generated at 70 eV (ion source at 230˚C and transfer line at 280˚C). The GC was performed using a HP5-MS capillary column (30mx 0.25mm, the film thickness of 0.25µm). Operating conditions were as follows carrier gas, helium with a flow rate of (1 ml min-1). The initial temperature was programmed from 80˚C to 280˚C (at 8˚C min-1). And maintained at 280˚C for 5 min. all compounds were identified by comparison of both the mass spectra (Wiley and Nist library) (Liu, *et al*., 2008). At the Biotechnology laboratory, plant pathology research institute, Agriculture Research Center.

Statistical analysis

The results of all experiments were statistically analyzed using one-way analysis of variance (ANOVA) to test for significance, and the Fisher test was used for mean separations by M-state computer package system. Means were compared by least significant difference test (LSD was at 0.01for *vitro* experiment while it was at 0.05 for *vivo* experiment) (Duncan, 1955).

Results and Discussion

Effect of algae extracts on fungal growth reduction (*In vitro*).

The antifungal activity of six crude extracts; aqueous, ethyl acetate, methanol, acetone, benzene and chloroform of *G. confervoides* against the three pathogenic fungi were determined by dual culture plates with PDA. As shown in Table 1, radial growth of all pathogenic fungi was significantly reduced at different levels. Extraction by chloroform completely inhibited the radical growth of *R. solani* while lowered it to only 3.7cm in case of *F. solani* compared with the control (8.0cm). On the other hand, malformation of the fungus *M. phaseolina* (areal mycelium and no formation of microsclerotia) was noticed in all treatments. Fig (1) shows the effect of this variation was related to the great variety of different secondary metabolites with a broad spectrum of biological activities

(Corderio, *et al*., 2006; Pal and Gardener, 2006; Cox, *et al*., 2010; Senthilkumar, *et al*. 2014). Percentages of mycelia reduction is presented in fig (2), which emphasizes that the presence of bioactive metabolites in marine algae, which can be soluble in solvents, could be related to the high and low effect of organic extracts against microorganisms (Kolanjinathan and Stella, 2009; Omar, *et al*., 2012).

Effect of algae powder on disease incidence under greenhouse Experiments

Plastic pots with amended soil by the powder of *G. confervoides* were used to evaluate disease incidence by estimating the percentage of dead plants, dry weight (g) and total yield (g). The overall treatments showed different significant degrees in reducing the percentage of dead plants compared with the controls as shown in table (2). The chemical fungicide vitavax gave the lowest percentage of dead plants 8.3, 8.3 and 16.7% infection with R. *solani, M. phaseolina*, and *F. solani,* respectively. It was interesting to notice that highest total yield (133.2g) was obtained in plants infected with *M. phaseolina* compared with all controls. It could be explained by the malformation of this fungus as mentioned before which limited its population and virulence. Also, comparing the high total yield (133.2g) with non-treated control (59.4g) could prove that algae *G. confervoides* secondary metabolites had a bioactive substance of the plant growth like amino acids, nutrients, phytohormones, enzymes and vitamins (Klarzynski, *et al*. 2006; Faten, *et al*. 2009).

Our results are in agreement with other reports demonstrating the efficient red algae extracts as unique antiprotozoal and anti-mycobacterial agents plus increasing plant yield production(Jimenez, *et al*. 2011; Sultana, *et al*. 2011).

Table 1: Effect of *Gracilaria confervoides* macroalgae extracts by different solvents on the mycelial growth reduction of *Rhizoctonia solani*, *Macrophomina phaseolina* and *Fusarium solani* of cucumber on PDA medium.

G. confervoides by Different solvent	*Rhizoctonia solani*		*Macrophomina phaseolina*		*Fusarium sola*ni	
	Reduction of hyphal growth		Reduction of hyphal growth		Reduction of hyphal growth	
	cm	%	cm	%	cm	%
Ethyl acetate	3.47	56.6	8	0	4.13	48.4
Methanol	5.93	25.9	7.67	4.1	4.47	44.1
Acetone	6.3	21.2	7.07	11.6	4.47	44.1
Benzen	4.97	37.9	7.63	4.6	4.23	47.1
Chloroform	0	100	6.83	14.6	3.7	53.7
Water	6.3	21.2	8	0	6.8	15
Control	8	0	8	0	8	0
L.S.D. at 0.01	S= 0.023		F= 0.021		SxF= 0.051	

S= Solvent F= Fungi S x F= Solvent x Fungi

Fig. 1: Malformation of *M. phaseolina* by effect of solvent extracts from *Gracilaria confervoides*

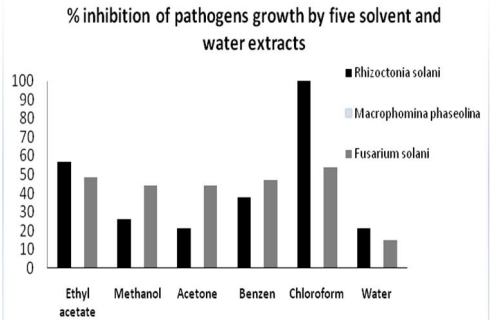

Fig. 2: Reduction percentage of *Gracilaria confervoides* macroalgae extracts by six solvent on the mycelial growth of *Rhizoctonia solani*, *Macrophomina*

Table 2: Effect of macroalgae treatments on the percentage of dead plants, total dry weight (g) and total yield (g) infested with the three pathogens and compared with vitavax fungicide 60 days after planting.

Treatment / Fungi	% of Dead plants		Total dry weight (gm)		Total yield (gm)	
	G. confervoides	Vitavax	*G. confervoides*	Vitavax	*G. confervoides*	Vitavax
R. solani	33.3	8.3	29.6	13.8	38.4	63.5
Control *R. solani*	83.3	83.3	5.9	5.9	25.3	25.3
M. phaseolina	25	8.3	28	16.2	133.2	66.9
Control *M. phaseolina*	66.7	66.7	15	15	25.2	25.2
F. solani	33.3	16.7	25.7	11.6	81.6	43.2
Control *F. solani*	50	50	19.3	19.3	25.2	25.2
Control without algae and fungus	25	25	21.3	21.3	59.4	59.4
L.S.D. at 0.05	F= 0.35 T= 0.43 FxT= 0.74		F= 1.9 T= 2.3 FxT= 4.0		F= 0.3 T= 0.7 FxT= 1.2	

F= fungi T= treatment FxT= interaction between Fungi and treatment

Analysis of algae extracts

Extraction of *G. confervoides* by the two solvent ethyl acetate and chloroform were analyzed with IR and GC-mass. Infrared spectroscopy (IR) involves the interaction of infrared radiation with matter, it can be used to identify and study chemical groups by measuring the vibration of atoms. Generally, stronger bands and light atoms will vibrate at high stretching frequency (wave number). Gas chromatography-mass spectrometry (GC-mass) is an analytic method that combines the features of gas chromatography and mass spectrometry to identify different substances within a test sample.

Data in table (3) and fig (3) showed the chemical groups obtained by IR for both solvents chloroform and ethyl acetate, which revealed a great number of chemical groups, such as C-O (Ether), N-H (Amine), C=O (Carbon), N-O (Nitro), C=C (Aromatic), O-H (Alcohol) and others. We notice some similarity in this groups obtained by the two solvents such as C-O (Ether) at wave number 1038.48 of ethyl acetate, the same group at wave number 1026.91 of chloroform. Chemical compounds of extraction by chloroform and ethyl acetate are conducted by GC-mass, are presented in tables (4, 5) and fig (4). Cyclononasilxane compound was a fraction observed in both extracts with different ratio and recorded as an antibacterial, antifungal and antiviral (Gunsena and Senarth, 2017). Also, Iron- monocarbonyl was recorded as an antifungal (Derbalah *et al*. 2012). All derivatives from extracts showed a suppression effect against microorganisms, which explain the ability of macroalgae *G. confervoides* extracts and powder to decrease the fungal growth of the three pathogens.

Table 3: Determination of chemical compounds of partially purified extract filtrate of chloroform and ethyl acetate from *Gracilaria confervoides* by IR.

	Chloroform		Ethyl acetate	
No.	Wave number (Cm^{-1})	Functional group	Wave number (Cm^{-1})	Functional group
1	1026.91	C-O (Ether)	1038.48	C-O (Ether)
2	798.385	C-Br (Alkyl halide)	1246.75	C-F (Alkyl halide)
3	1258.32	C-F (Alkyl halide)	800.314	C-Cl (Alkyl halide)
4	2918.73	C-H (Alkan)	3424.96	N-H (Amine)
5	2958.27	C-H (Alkan)	1727.91	C=O (Carbonyl)
6	2850.27	C-H (Alkan)	2958.27	C-H (Alkan)
7	3414.35	N-H (Amine)	613.252	C=C (Alkene)
8	468.617	(Alkyl halide)	1384.64	C-H (Alkan)
9	1458.89	C=C (Aromatic)	1620.88	C-Br (Alkyl halide)
10	865.882	=C-H (Alkene)	457.047	(Alkyl halide)
11	699.069	=C-H (Alkene)		
12	523.579	C-Br (Alkyl halide)		
13	1633.41	C=C (Alkene)		
14	1539.88	N-O (Nitro)		
15	3753.76	O-H (Alcohol)		

Table 4: Determination of chemical compounds of partially purified extract filtrate of chloroform from *Gracilaria confervoides* by GC-mass.

RT.	Area%	Compound	Biological activity	Ref.
17.718 17.855 18.221 18.033 18.118	0.46 0.12 3.94 0.33 0.30	Hexadecanoic acid	Antibacterial activities	El-Din and El-Ahwany, (2016)
21.185 22.530 23.863	1.31 3.82 3.48	Iron, monocarbonyl	Antifungal activities	Derbalah, *et al*. (2012)
15.973 21.391 25.551	1.99 3.71 2.92	Cyclononasiloxane	antibacterial, antifungal and antiviral activity	Gunasena and Senarath, (2017)
17.512	2.97	Cyclodecasiloxane	Antifungal activities	Ahsan , *et al.* (2017)
14.245 18.925	1.84 2.53	1,3,5,7-Tetraethyl-1-ethylbutoxysiloxy cyclotetrasiloxane	Antifungal activities	Derbalah, *et al*. (2012)
20.213	2.70	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane	Antibacterial activity	Omoruyi , *et al.* (2014)
12.242	1.87	Cycloheptasiloxane	antibacterial, antifungal and antiviral activity	Gunasena and Senarath, (2017)
19.383 19.886 19.984	1.24 0.11 0.35	Octadecanoic acid	Antibacterial activities	El-Din and El-Ahwany, (2016)
9.999	0.66	Cyclohexasiloxane	antibacterial, antifungal and antiviral activity	Gunasena and Senarath, (2017)

Table 5: Determination of chemical compounds of partially purified extract filtrate of ethyl acetate from *Gracilaria confervoides* by GC-mass.

RT.	Area%	Compound	Biological activity	Ref.
15.973 17.518 21.391 22.530 25.546	6.62 14.78 12.05 17.86 11.48	Cyclononasiloxane	antibacterial, antifungal and antiviral activity	Gunasena and Senarath, (2017)
18.605 18.714 19.681 21.054 23.863	3.60 2.48 3.41 1.41 13.43	Iron monocarbonyl	Antifungal activities	Derbalah, *et al*. (2012)
14.239 18.25	5.93 8.51	1,3,5,7-Tetraethyl-1-ethylbutoxysiloxycyclotetrasiloxane	Antifungal activities	Derbalah, *et al*. (2012)
20.207	9.93	Benzoic acid	Antifungal activities	Derbalah, *et al*. (2012)
12.242	5.13	Cycloheptasiloxane	antibacterial, antifungal and antiviral activity	Gunasena and Senarath, (2017)
19.372 19.978	4.65 0.47	Octadecanoic acid (CAS)	Antibacterial activities	El-Din and El-Ahwany, (2016)

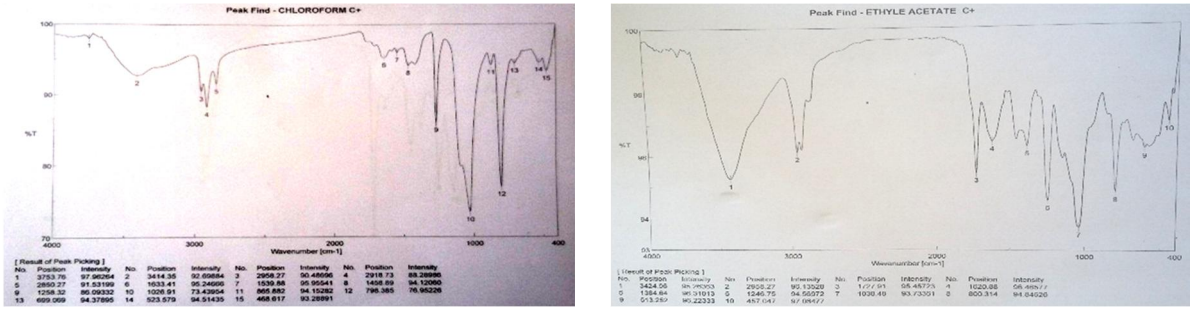

Fig. 3: Chemical compounds determination sheet of partially purified extract of chloroform and Ethyl acetate from *Gracilaria confervoides* by IR

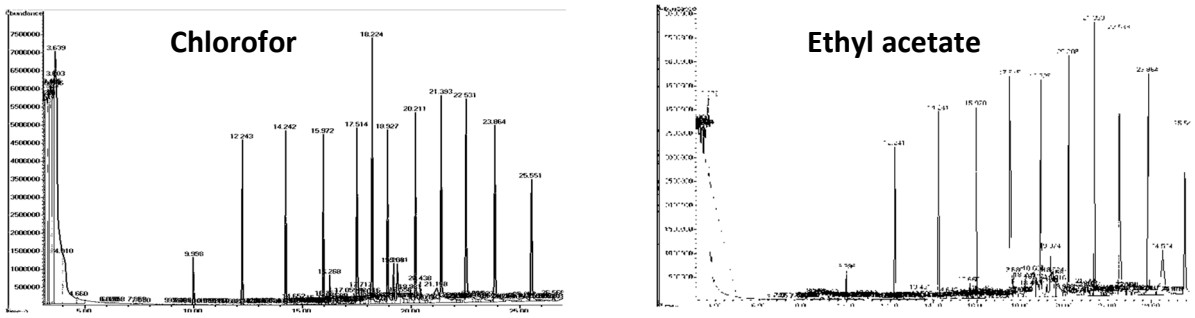

Fig. 4: Chemical compounds determination sheet of partially purified extract of chloroform and ethyl acetate from *Gracilaria confervoides* by GC-mass

On the other hand, further studies are needed to explore the function mechanism of these compounds to reduce fungal growth and their malformation factors which affect *M. phaseolina* growth as we mentioned earlier. At the same time, we can't ignore the plant growth promoting factors in these compounds.

Finally, it can be assumed that macroalgae contained various compounds with different antimicrobial properties might be responsible for the interesting results regarding the pathogens control and enhancing the plant growth.

Conclusion

The Egyptian macroalgae *Gracilaria confervoides* can be considered a good alternative bioagent with promising potential in controlling the tested pathogenic fungi which may limit the extensive use of chemical fungicides in plant disease management. Based on our results mentioned above, we suggested that macroalgae also can act as a potential source of nutrients for plant growth and improve its yield. Most importantly, the results findings proposed that the Mediterranean sea coast could be considered a rich source for different macroalgae species with unique antimicrobial properties which might be exploited in the future in a numerous diverse applications in agriculture and plant disease control, revealing their activity to control some plant pathogens.

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