

Antifungal Activity and Seasonal Variation of Green Alga (*Ulva lactuca*) Extracts

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ABSTRACT--- *The present study was undertaken to explore the inhibitory effect of seaweed extracts of Ulva lactuca (green alga) with five solvents (methanol, Ethanol, Methylene chloride, chloroform and hexane) and water at different concentrations of (100, 200, 250, 500, 1000, 1500, 2000, 4000 8000 ppm) along with control against the mycelial growth of Fusarium solani, Rhizoctonia solani, Sclerotinia sclerotiorum, Alternaria solani, Phytophthora infestans and Botrytis cinerea by poison food technique. Result revealed that extract of Methanol showed the highest antifungal activity against all the tested fungi. While water extract showed a moderate antifungal activity against all the tested plant pathogenic fungi. Phytophthora infestans was most sensitive than the other tested fungi to all solvent extracts of Ulva lactuca. The results also showed that the highest antifungal activity was recorded in winter season while the lowest one was in summer. GC-MS analysis of methanolic extract of Ulva lactuca was also recorded.*

Keywords--- *Ulva lactuca* (green alga), antifungal activity, seasonal variation, Chemical analyses, Gas chromatography-mass spectrometry

1. INTRODUCTION

Chemicals largely used as pesticides in crop protection could be environmental pollutants and have undesirable biological effects on animals and human beings. The toxic effect of synthetic chemicals can be overcome, only by persistent search for new and safer pesticides accompanied by wide use of pest control methods, which are ecofriendly and effective [1]. One source of potential new pesticides is natural products produced by algae. Marine algae represent a great source of a vast variety of complex natural products and could be a promising source of a novel bioactive compound that can help plant survival by offering protection against stress imposed by pathogens [2], [3]. The antifungal activity of seaweed extracts and isolated compounds has not been extensively studied, mainly because in the past few years more attention has been paid to pathogenic bacteria, which is, by far, more explored [4]. Nevertheless, there are some studies suggested that macro-algae contain compounds with different chemical properties which could be considered for controlling specific plant pathogens [5], [6], [7], [3], [8], [9], [10] and [11].

The genus *Ulva* (Phylum Chlorophyta, Class Ulvophyceae, Order Ulvales, Family Ulvaceae) was first identified by Linnaeus in 1753 [12]. The green algae with less than 300 known compounds are the least producers of natural compounds when compared to the red (Rhodophyta) and brown algae (Phaeophyta) [13]. Anyhow, a wide range of compounds, predominantly terpenes, polyphenols and steroids, have been reported in various marine green algae [14]. The chemical composition of these macro algae was found to vary depending on geographical distribution and seasons and the principal environmental factors affecting the composition being water temperature, salinity, light, nutrients and minerals availability [15], [16], [17] and [18].

Present investigation was undertaken to evaluate different solvents extracts (methanol, Ethanol, Methylene chloride, chloroform and hexane) beside water extract with different concentration from 100 to 8000 $\mu\text{g ml}^{-1}$ for their antifungal activity against the plant pathogenic fungi *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Alternaria solani*, *Phytophthora infestans* and *Botrytis cinerea*. Also, evaluate the effect of seasonal variation on the antifungal activity of methanol extract besides identify the content of alga extract by using GC-MS.

2. MATERIALS AND METHODS

2.1 Collection and identification of algal material

Samples of *Ulva lactuca* (green alga) were collected monthly from September 2012 to September 2013 from Abu-Qir and El-Kalaa of Alexandria, where this alga predominates on the Mediterranean coast of Egypt. Samples were air dried under shade for 2 weeks, the dried algal material was ground. The alga was identified according to [19].

2.2 Preparation of alga extracts:

Solvent extract: According to [20], the powdered alga was extracted successively with methanol, Ethanol, Methylene chloride, chloroform and hexane using Soxhlet apparatus. After 6 hours of extraction the solvents were evaporated from crude extract by rotary evaporator.

2.3 Water extract:

100g of powdered seaweed was mixed with distilled water in the ratio of 1:10 ratio and autoclaved at 150 lb pressure for 1hour. The extract was filtered immediately through a muslin cloth. Then the extract was concentrated by rotary evaporator at 60°C, then measured, labeled and stored in bottles which were kept in a refrigerator. This extract was taken as 100% seaweed concentrate (SWC) described by [21].

2.4 Fungi

The six plant pathogenic fungi species used *Fusarium solani*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Alternaria solani*, *Phytophthora infestans* and *Botrytis cinerea* were obtained from the Fungicide Bioassay Laboratory, Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University. The fungi were maintained during the course the experiments on potato dextrose agar medium at 25°C.

2.4.1 Antifungal assay

The antifungal activity of alga extracts was tested using the radial growth technique method [22]. Appropriate volumes of the stock solutions of the alga extracts either in dimethyl sulfoxide (DMSO) for solvent extracts or in distilled water for water extracts were added to molten nutrient agar (potato dextrose agar Medium, PDA) to obtain range of concentrations (100, 200, 250, 500, 1000, 1500, 2000, 4000 and 8000 $\mu\text{g ml}^{-1}$) immediately before pouring into the Petri dishes (9.0 cm in diameter) at 40-45°C. Each concentration was tested in triplicate. Parallel controls were maintained with DMSO mixed with PDA. The discs of mycelial felt (0.5 cm diameter) of the plant pathogenic fungi, taken from 8-day-old cultures on PDA plates, were transferred aseptically to the centre of Petri dishes. The treatments were incubated at 25°C in the dark. Colony growth diameter was measured after the fungal growth in the control treatments had completely covered the Petri dishes. Percentage of mycelial growth inhibition was calculated from the formula: Mycelial growth inhibition = $[(DC-DT)/DC] \times 100$ [23], where DC and DT are average diameters of fungal colony of control and treatment, respectively. The concentration of extract that inhibiting the fungi mycelial growth by 50% (EC_{50}), was determined by a linear regression method [24].

2.5 Seasonal variation of methanol algae extract.

All Samples of *Ulva lactuca* (green alga) were distributed into four Seasons, Autumn (season 1, Septmeber-December), Winter (season 2, December-March), Spring (season 3, March-June), and Summer (season 4, June-September). Every season was evaluated for its antifungal activity as mentioned above.

2.6 Chemical analyses

Gas chromatography- mass spectrometry was used for Identification of crude extract. The GC-MS were performed in electronic impact ionization mode using a Varian GC (CP-3800) interfaced with a Varian 1200L single quadrupole mass spectrometer. The GC was equipped with a DB-5 column (30 m \times 0.25 mm i.d.; Agilent, Santa Clara, CA). The carrier gas was ultra-high purity He. The injector and detector temperatures were maintained at 300°C. Samples (1 μ) were injected in split (1:15) mode, and the oven program was identical to that of the GC-FID analysis. Ion source and transfer line were kept at 300°C.

3. RESULTS AND DISCUSSION

3.1 Antifungal activity of *Ulva lactuca* extracts

3.1.1 Solvents extracts.

The antifungal activities of methanol, Ethanol, Methylene chloride, chloroform and hexane extracts of *Ulva lactuca* in terms of radial growth inhibition are summarized in Table 1, 2 and table 3. Methanol extract of *Ulva lactuca* showed the highest antifungal activity against all the tested fungi especially *Phytophthora infestans*, *Sclerotinia sclerotioru* and *Alternaria solani* with EC₅₀ values of 1149, 1225 and 1330 µg ml⁻¹, respectively. Methylene chloride extract exhibited the strongest antifungal effect against *Phytophthora infestans* with EC₅₀ values of 891 µg ml⁻¹. *Phytophthora infestans* was most sensitive than the other tested fungi to all solvent extracts of *Ulva lactuca*. Chloroform extract of *Ulva lactuca* showed weaker antifungal activity against all the tested fungi. Concerning to the effect of ethanol extract, it gave a moderate effect against most of the tested fungi while it was the most effective one against *Botrytis cinerea* with EC₅₀ value of 1017.8 µg ml⁻¹. The same conclusion was reported for the ethanol extract of the seaweed *Sargassum myricocystum* the plant pathogenic fungi *Colletotrichum falcatum* [11]. Our results showed low antifungal activity of all the solvents extracts against the plant pathogenic fungi *Fusarium solani* in all tested concentration except methanol extract which gave a moderate toxicity against this fungi with EC₅₀ values of 3441 µg ml⁻¹, the same result was also recorded against *Fusarium oxysporum* [10]and [11].Most of the results obtained supported by many of the previous research on [25], [8] and [26] Also [27] reported that *Ulva lactuca* methanol and ethanol extracts had antifungal activity against *Aspergillus niger*, *Penicillium digitatum* and *Rizoctonia solani*. Hexane, chloroform and methanol extracts of of *Ulva lactuca* and *Ulva Fasciata* inhibited the growth of *F. oxysporum* f. sp. *Vasinfectum* [28].

Table (1): Fungicidal activity of methanol and ethanol extracts of green alga (*Ulvalactuca*against six plant pathogenic fungi.

Fungus	EC ₅₀ (µg ml ⁻¹)	95% Confidence limits		Slope
		Upper	Lower	
Methanol extract				
<i>Phytophthorainfestans</i>	1149	1391	950	1.2
<i>Alternariasolani</i>	1330	1651	1072	1.1
<i>Sclerotiniasclerotiorum</i>	1225	1432	1048	1.5
<i>Botrytis cinerea</i>	2210	2767	1331	1.3
<i>Rhizocotoniasolani</i>	6308	11170	3584	0.8
<i>Fusariumsolani</i>	3441	5188	2291	0.88
Ethanol extract				
<i>Phytophthorainfestans</i>	6382.3	15975.1	2584.8	0.7
<i>Alternariasolani</i>	2184.3	3601.4	1332.2	0.76
<i>Sclerotiniasclerotiorum</i>	2081	2866.5	1514.3	1.2
<i>Botrytis cinerea</i>	1017.8	1265.8	819.1	1.2
<i>Rhizocotoniasolani</i>	5734	11006.9	3005.2	1.06
<i>Fusariumsolani</i>	>8000	-	-	-

Table (2): Fungicidal activity of methylene chloride and chloroform extracts of green alga (*Ulvalactuca*) against six plant pathogenic fungi

Fungus	EC ₅₀ (µg ml ⁻¹)	95% Confidence limits		Slope
		Upper	Lower	
Methylene chloride Extract				
<i>Phytophthora infestans</i>	891	1084	732.4	1.3
<i>Alternaria solani</i>	5170.9	10391	2593.5	0.87
<i>Sclerotinia sclerotiorum</i>	6710	15028	3025	0.86
<i>Botrytis cinerea</i>	2733.1	3866	1935.9	1.3
<i>Rhizoctonia solani</i>	4161.4	7188	2421.1	1.03
<i>Fusarium solani</i>	>8000	-	-	-
Chloroform extract				
<i>Phytophthora infestans</i>	>8000	-	-	-
<i>Alternaria solani</i>	6319	11933.8	3358.7	1.4
<i>Sclerotinia sclerotiorum</i>	>8000	-	-	-
<i>Botrytis cinerea</i>	7839.42	18121	3423.6	0.9
<i>Rhizoctonia solani</i>	>8000	-	-	-
<i>Fusarium solani</i>	>8000	-	-	-

Table (3): Fungicidal activity of hexane extract of green alga (*Ulvalactuca*) against six plant pathogenic fungi.

Fungus	EC ₅₀ (µg ml ⁻¹)	95% Confidence limits		Slope
		Upper	Lower	
Hexane Extract				
<i>Phytophthora infestans</i>	2933	5011.5	1726.7	0.84
<i>Alternaria solani</i>	3983.9	7069.5	2258.1	0.9
<i>Sclerotinia sclerotiorum</i>	1458.2	1788.95	1189.6	1.5
<i>Botrytis cinerea</i>	3072.2	4294.1	2201.5	1.6
<i>Rhizoctonia solani</i>	>8000	-	-	-
<i>Fusarium solani</i>	4356.3	8340.9	2292.18	0.8

3.1.2 Water extract

Water extract showed a moderate antifungal activity against all the tested plant pathogenic fungi while it gave a highly antifungal action against *Phytophthora infestans* and *Fusarium solani* with EC₅₀ values of 1382.1 and 2222.8 µg ml⁻¹, respectively, (table 4). Our results in this point were supported by the results of [29], [9] and [3]. In general, using

organic solvents are able to extract a large quantity of lipophilic compounds (glycolipid, phenolic terpenoids, unsaturated-fatty acids and hydroxylated unsaturated-fatty acids), [30].

Table (4): Fungicidal activity of water extract of green alga (*Ulva lactuca*) against six plant pathogenic fungi.

Fungus	EC ₅₀ (µg ml ⁻¹)	95% Confidence limits		Slope
		Upper	Lower	
Water extract				
<i>Phytophthora infestans</i>	1382.1	1670.5	1144.3	1.6
<i>Alternaria solani</i>	4917.3	9059.4	2684.6	1.00
<i>Sclerotinia sclerotiorum</i>	>8000	-	-	-
<i>Botrytis cinerea</i>	3961.7	6261.9	2514.1	1.3
<i>Rhizoctonia solani</i>	2222.8	3205.5	1545.6	1.00
<i>Fusarium solani</i>	7926.7	22125.5	2885.3	0.7

3.2 Seasonal variation and antifungal activity of methanol alga extract

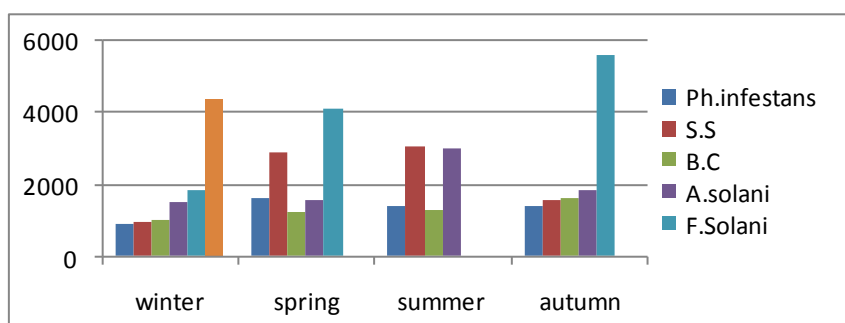
Seaweeds are exposed to seasonal variations of abiotic factors that influence their metabolic responses (photosynthesis and growth rates) and Levels of proximate constituents [31]. Concerning to the antifungal activity of methanol extract during the four seasons, the results in table (5) and Figure (1) showed that the highest antifungal activity was recorded in winter season while the lowest one was in summer. Our results in this point were in-agreement with [32] who observed the seasonal variation in antimicrobial activity of *Ulva pertusa* against *Gardnerella vaginalis*, and showed no activity during summer and autumn, and showed antibacterial activity from winter to spring. Also, [18] who found that bromophenol at high level was recorded in January to May and drastically decreased after that. Also, [33] observed good growth and high content of polysaccharides content of *Ulva Fasciata Delile* during September to March and rest of season was unfavorable for the growth of the plant. The protein content of red seaweeds, *Catenella repens* showed highly seasonal variation with higher values during November, [34], [35] recorded the highest antibacterial activity from the crude extracts of seaweeds collected during monsoon (November) and post-monsoon (February), whereas seaweeds collected during summer and pre-monsoon (August) showed low antibacterial activity. Our results showed that *Phytophthora infestans* was the most sensitive fungi to the methanol extract in all the four seasons while, *Rizoctonia solani* was the least one with EC₅₀ values of 887 and >8000 µg ml⁻¹, respectively (table 5).

Table (5): Seasonal variation and antifungal activity of methanol alga extract.

Fungus	EC ₅₀ (µg ml ⁻¹)	95% Confidence limits		Slope
		Upper	Lower	
Winter				
<i>Phytophthora infestans</i>	887	1065	738	1.33
<i>Alternaria solani</i>	951	1795	1274	1.3
<i>Sclerotinia sclerotiorum</i>	979	1146	789	1.28
<i>Botrytis cinerea</i>	1512	1145	837	1.5
<i>Rhizoctonia solani</i>	4361	6104	3122	0.9
<i>Fusarium solani</i>	1809.3	2192	1493	1.2
Spring				
<i>Phytophthora infestans</i>	1614	1878	1387	1.55
<i>Alternaria solani</i>	2901	1881	1317	1.22
<i>Sclerotinia sclerotiorum</i>	1248	3651	2307	1.4
<i>Botrytis cinerea</i>	1574	1419	1097	2.1
<i>Rhizoctonia solani</i>	>8000	-	-	-
<i>Fusarium solani</i>	4089	5933	2825	0.81

Summer				
<i>Phytophthorainfestans</i>	1413	1598	1250	1.9
<i>Alternariasolani</i>	3041	3439	2658	2.2
<i>Sclerotiniasclerotiorum</i>	1282	3931	2356	1.03
<i>Botrytis cinerea</i>	3023	1498	1098	1.6
<i>Rhizocotoniasolani</i>	>8000	-	-	-
<i>Fusariumsolani</i>	>8000	-	-	-
Autumn				
<i>Phytophthorainfestans</i>	1397	1675	1165	1.2
<i>Alternariasolani</i>	1581	2220	1503	1.2
<i>Sclerotiniasclerotiorum</i>	1619	1884	1327	1.3
<i>Botrytis cinerea</i>	1826	1833	1431	1.9
<i>Rhizocotoniasolani</i>	>8000	-	-	-

Figure (1): Seasonal variation and antifungal activity of methanol alga extract



Ph. Infestans: Phytophthora infestans, S.S: Sclerotinia sclerotiorum, B.C: Botrytis cinerea, A. solani: Alternaria solani, F. solani: Fusarium oxysporum and R. solani: Rhizoctonia solani.

3.3 GC analysis of methanolic extracts of *Ulva lactuca*.

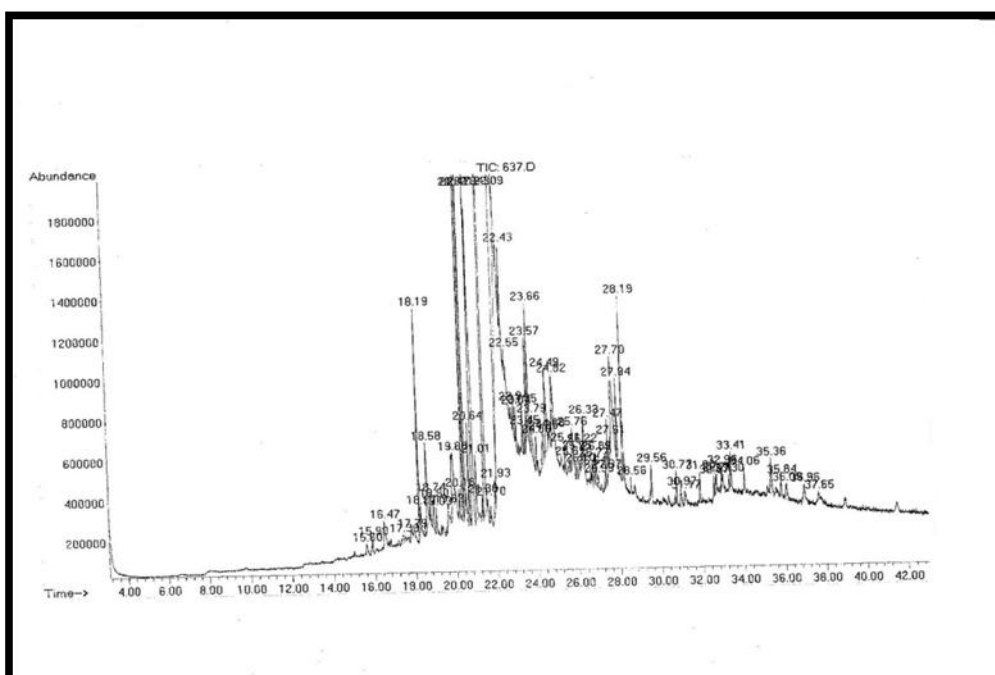
The Spectra of compound were matched with Wiley library. Their structures were identified by percentage similarity values. GC-MS analysis of methanolic extract of *Ulva lactuca* showed that the major compound have twelve peaks indicating the presence of twelve compounds shown in table (6) and figure (2). Antifungal compounds from algae extracts are form a wide range of chemical classes, including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons [36], [37] and [38] Thus, GC-MS methods were used to analyzed the alga methanol extracts that showed higher antifungal activity from the *ulva lactuca*, mainly fatty acids, lipid ,alkanes, phenols. GC-MS analysis of methanolic extract of *Ulva lactuca* showed phytol (2-hexadecen-1-ol, 3,7,11,15-tetramethyl) and neophytadiene, these results were in-agreement with [36] and identified lipids composition such as Hexadecanoic acid methyl ester, 8 heptadecene and Bis 2(ethyl hexyl) phthalate, these results were also in-agreement with [39], [40] and [27]. fatty acid compound including Palmitic acid and oleic acid agreement with [39], [15], [27] and [41] these compounds have antifungal activity, another compounds identified such as-(-) loliolide;2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-6hydroxy-4,4,7 trimethyl were also found in the results of [42] and volatile halogenated hydrocarbon such as P-Floro-nitro-benzene was also identified in [43] and [44]. GC- analysis of these extractives revealed the presence of many compounds which know to have antifungal activity. [18] were able to detect 2, 4, 6-tri-bromophenol from the crude extract of *U. lactuca*. Later, 3-*O*-β-D glucopyranosylstigmasta-5,2,5-diene was isolated from the methanol extract of *U. lactuca*, collected from the coast of Alexandria, Egypt[44] and [15]. Using High Performance Liquid Chromatography (HPLC), the Chinese group was able to isolate five *nor*-isoprenoids: (3*S*,5*R*,6*S*,7*E*) 3,5,6-trihydroxy-7-megastigmen-9-one (3*R*,5*R*,6*R*,7*E*) 3,5,6-trihydroxy-7-megastigmen-9-one (3*S*,6*R*) 3,6-dihydroxy-4,7-megastigmadien-9-one ,grasshopper ketone ,and isololiolide,, from the methanol extract of *U. lactuca*, collected off the coast of Bohai in China [42]. **In conclusion**, the present results clearly indicate that the extracts of *Ulva lactuca* (green alga) able to affect fungal

growth and their activity is dose- and season- dependent, and it have been identified as a new and rich source of bioactive compounds.

Table (6): GC-MS Analysis of the major compound from *Ulva lactuca* extract.

No	Compound	M.W	Formula	RT ^a (min)	Area%
1	Palmitic acid	256	C ₁₆ H ₃₂ O ₂	22.09	23.35
2	Hexadecanoic acid methyl ester	270	C ₁₇ H ₃₄ O ₂	21.43	19.16
3	P-Floronitrobenzen	141	C ₆ H ₄ FNO	20.79	8.41
4	2-Pentadecanone,6,10,14trimethyl	268	C ₁₈ H ₃₆ O	20.42	8.01
5	Neophytadiene;2-6-10 trimethyl,14ethylene-14-pentadecne	278	C ₂₀ H ₃₈	20.32	3.84
6	1,5- cyclodecadiyne	132	C ₁₀ H ₁₂	22.7	3.28
7	8-Heptadecane	238	C ₁₇ H ₃₄	18.9	2.18
8	Oleic acid: 9-Octadecenoic acid	282	C ₁₈ H ₃₄ O ₂	24.49	1.98
9	-(-)loliolide;2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-6hydroxy-4,4,7 trimethyl	196	C ₁₁ H ₁₆ O	19.88	1.78
10	Bis(2-ethylhexyl) phthalate	390	C ₂₄ H ₃₈ O ₄	28.20	1.71
11	3-Buten-2-one,4-(4-hydroxy-2,2,6trimethyl-7-oxabicyclo[4.1.0] hept-1-yl)	224	C ₁₃ H ₂₀ O ₃	18.58	1.67
12	Phytol Isomer;3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296	C ₂₀ H ₄₀ O	23.66	1.51

Figure (2): GC-MS Analysis of the major compound from *Ulvalactuca* extract



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