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Antifungal Activities of the Marine Green and Red Macro Algae Extracts Against Some Phytopathogenic Fungi

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Abstract

Plant pathogenic fungi, in particular are the source of several plant diseases,often regarded as one of the primary causes of decreased food supply around the world. The spread of such pathogenic fungi results in considerable crop losses degrades fruits and vegetables, reduces food availability, and costs billions of dollars annually. Although fungicides made of synthetic chemicals are commonly used for managing fungal diseases, they are harmful for the environment. Marine algae present an environmentally friendly option in the search for various kinds of compounds with industrial uses. They are one of the richest marine sources with a variety of bioactive compounds. Two green algae (*Ulva flexusoa* and *Enteromorpha intestinalis*) and one red algae (*Griffithsia teges*) are explored in the current work. The three seaweeds' methanolic extracts were evaluated in *vitro* for their biological activity against *Penicillium expansum* and *Fusarium oxysporum* mycelial growth. The antifungal activity of each of the extracts employed in this study was evident. The resulting inhibition zone in mycelial growth of *Fusarium oxysporum* as affected by *Enteromorpha intestinalis* methanolic extractwas 37 mm, whereas that for *Penicillium expansum* was 32.6 mm. Gallic acid in the *Enteromorpha intestinalis* extract was 0.22 mg/ml, Chlorogenic acid was 0.41 mg/ml, Daizein was 0.16 mg/ml, Querectin was 0.64 mg/ml, Cinnamic acid was 0.05 mg/ml, and Hesperetin was 0.14 mg/ml, according to the results of the phenolic analysis carried out by HPLC-LC1620A.Also *Ulva flexusoa*contain high amount of Querectin 0.26 mg/mland *Griffithsia teges* contain high amount of Gallic acid 0.81 mg/ml .These results suggest that the seaweeds employed in the study are a preferred and natural source of bioactive chemicals. Such natural products may have applications in the management of fungal diseases in organic farming and other sustainable agricultural practices.

Keywords: Marine algae,seaweed extracts, natural substance, antifungal activity, plant pathogens.

Introduction

Phytopathogenic fungi pose a serious harm to certain plant varieties, colonizing a diverse spectrum of the host plants. (**Narayanasamy, 2011**) and (**Brauer, *et al.*, 2019**). The diseases that they have are especially concerning in plants grown for human consumption as they have the potential to decrease the amount of food available to fulfill human nutritional needs (**Brauer *et al.*, 2019).** Phytopathogenic fungi are an ancient and recurring issue that has been intensively researched to develop efficient strategies to control their global spread. Fungicides made of artificial chemicals are commonly employed using traditional agriculture to manage diseases of plants . However, as modern civilization becomes more aware, the environmental hazardous dangers posed by making frequent use of such fungicides pose health risks (**Kim *et al.*, 2009**). Additionally, pathogens are capable of developing pesticide resistance.

Since the previous century, researchers have been looking into a viable substitute made of organic macro algal compounds (direct application of extracts or dry powder). Several researchers have tested the potential of metabolites as antifungals, such as mycelial inhibition, and have identified the use of organic solvents, which have a strong preference for phenolic and lipid compounds, as the most effective method for obtaining macro algal extracts with antifungal activity (**Masuda *et al.*, 1997; Malini *et al.*, 2014**).Macro algal compounds are related with strong antifungal action (**Khan *et al.*, 2017**).The fungal membrane being disrupted by bioactive algal extracts is the most frequently described mechanism for this antifungal action (**Pohl *et al.*, 2011**) .This induces conformational abnormalities and damages the electron transport chain, increasing membrane permeability and leading to the outflow of critical cytoplasmic componentscausing the death of fungal cells (**Avis and Bélanger, 2001**) and (**Desbois and Smith, 2010**).

In this investigation, the phytopathogenic fungi used were*Penicillium expansum* and *Fusarium oxysporum*. Typical among soil-borne pathogens is *F. oxysporum*,It penetrates the roots, spreads throughout the tissues, colonizes and metastasizes in xylem vessels, lives in the soil for a long time as chlamydospores, and causes systemic yellowing, wilting, and death in plants.According to **Xiao and Boal (2009)**, The most prevalent and important postharvest fruit rot pathogen causing blue mould is Penicillium expansum.Blue mould, caused by Penicillium expansum and other Penicillium spp., is one of the most prevalent and significant postharvest fruit rot diseases (**Zhong *et al.*, 2018**). *Penicillium expansum* is thought to cause soft rot or blue mould on a wide variety of vegetables and fruits worldwide. Because it produces the cancer-causing toxin patulin in infected fruits and causes significant damage during storage, this species is the most significant postharvest pathogen of vegetables and fruits **(Vehapi *et al.*, 2020)**.Therefore, finding novel bioactive compounds that are environmentally acceptable, biodegradable, and naturally occurring with the potential for biorational activity are essential (**Korde *et al.*, 2020).** Also, new management strategies and antifungal alternatives that are more potent, less hazardous, more accessible, and that inhibit these resistance mechanisms are desperately needed.

Macro algae are now a great resource to investigate and utilize in many parts of the world due to their natural compounds that have broad-spectrum antifungal properties (**Harman *et al.*, 2004**). Macro algae, often known as seaweeds, are generally present in aquatic environments such as oceans and seas, while a few species may thrive and proliferate in other freshwater ecosystems (**Ibraheem *et al.*, 2014**). Such chemicals are isolated from various macro algae familiescomprising red, brown, and green algae, with an estimated 40,000 compounds (**Raven *et al.*, 1992**). Hydrocolloids like agar, carrageenan, and alginates are made from seaweed. It has also been discovered that marine algae extracts possess potent antiviral, antibacterial, and anti-inflammatory qualities (**Kuda *et al.*, 2005**). Recently, it has been discovered that several marine algae extracts possess antibacterial and antioxidant qualities (**Kuda *et al*., 2005**; **Ely *et al*., 2004**).

A number of *Ulva rigida* (formerly known as *U. armoricana*) extracts in methanolic, acetone, diethyl ether, and ethanolic forms and *Cystoseira mediterranea* showed positive antifungal activity, according to **Tuney *et al.* (2006)**. An efficient fungicide against *Ulocladium botrytis*, *A. brassicicola*, *A. alternata*, *Fusarium oxysporium*, and *Botryotricum piluliferum*is *Codium fragile* algal extract (**Galal *et al*., 2011**). Further research by **Ammar *et al.* (2017)** revealed that the bioactive substances in *Sargassum vulgare's* methanolic extract, flavonoids and phenolic acids, may work as potent antifungal agents against *Pythium aphanidermatum*, reducing pathogen mycelial growth by around 51%. Therefor , The goal of this study was to assess the antifungal properties of methanolic crude extracts from three different local seaweeds (*Ulva flexusoa*, *Enteromorpha intestinalis*, and *Griffithsia teges*) collected from the Balteem coastline against some phytopathogenic fungi (*Fusarium oxysporum* and *Pinicillum expansum*).

Materails And Methods

Algal Collection

Macroalgae were collected in November of 2022. Algal samples were manually collected at low tide from Baltem Mediterranean coastal region at Kafer Elsheikh, Egypt (Fig. 1). To get rid of macroscopic epiphytes and sand particles, the obtained algal samples were completely washed with seawater and hard brush. The salt that had adhered to them was then rinsed off with tap water. To remove extra water, the algal samples were blotted and allowed to air dry for six days at room temperature. The mechanical grinder was used to turn the dried seaweed samples into fine powder, which was then stored in plastic tubes for subsequent examination.

Fig. 1. Collected Algal samples

Preparation of methanolic extract from algae

The algal powder was extracted using methanol as a solvent.,In 250 ml conical flasks, 25 grams of each algal powder sample were immersed for 24 hours in 100 ml of the solvent and incubated at 30°C on a rotator shaker (150 rpm).Whatman filter paper number one was used to filter the final crude extracts.By using a rotary evaporator, Separation of the filtrate and solvent occurred.The obtained residues, or crude extracts, were then dissolved in 10% DMSO to yield 10 mg/ml. For the antimicrobial experiment, each extract was kept in an airtight glass bottle at -20 °C**(Cho *et al.*, 2007).**

Fungal isolates
Isolation, purification, and identification of*Fusarium oxysporum*

Sections were taken from the roots of citrus plants that had wilt and root rot. Following the cutting of fragments (5.5 mm) of symptomatic tissues from the leading edges of lesions, the surfaces were surface-sterilized for 20 seconds in a 10% sodium hypochlorite solution, followed by 30 seconds in 70% ethanol, and then three times in sterile water. After being dried in sterile filter paper, tissue fragments were added to 2% potato dextrose agar (PDA) supplemented with 100 µg/mL streptomycin and 100 µg/mL penicillin (PDA-PS), and cultured at 25°C until distinct Fusarium colonies appeared. The process of transplanting a single conidia to new PDA produced pure cultures. (**Sandoval-Denis *et al.*, 2018**). In accordance with **Booth (1985),** pure cultures were kept at 5 °C for later use. The isolated fungus was recognized by microscopy as well as cultural and morphological characteristics**.** The identification was made by the plant pathology lab, college of agriculture, Mansoura University, identified as *Fusarium oxysporum***.**

Isolation, cultivation and Identification of *Penicillium* species

Samples of Valencia orange citrus fruit that was both wounded and infected were collected and covered in sterile plastic bags to prevent contamination and moisture loss. Using a sterile needle, the surface of diseased citrus fruits was used to collect the spores of developing Penicillium species.To encourage the growth of Penicillium sp., a small number of spores were taken from the infected spot (which was colored blue) was placed in the middle of the Potato Dextrose Agar "PDA" medium, which is a 9-cm petridish dish. A bacteriostatic substance called chloramphenicol, 250 mg per liter, was added to the medium (**Dawson *et al.*, 2001**). After inoculating with Penicillium spores, three replicates of each previous medium were used. All plates were sealed with cellophane to keep out other microorganisms, and they were kept at 27°C for a week before being transferred to the refrigerator to stop the growth of fungi. (**Kreuawab *et al.*, 2007**). Under a light microscope, pure growing mold colonies were used for making temporary slides. We looked closely at the morphological traits of the Penicillium formations' mycelia and spores. A textbook and specialized reference were used to identify it (**Domsch *et al.*, 1980**). The identification was made by the plant pathology lab, Faculty of Agriculture, Mansoura University.identified as *Penicillium expansum* .

Extraction and Estimation of phenolic content of macro algae extracts

The detection and determination of polyphenols were conducted using liquid chromatography with high performance (HPLC- LC1620A). The extraction operation was conducted according to the methodology outlined by **Shindy and Smith (1975).**

Mineral analysis of the collected macro algae

Macro and microelements in dried samples of the collected macroalgae (Ulva flexusoa, Enteromorpha intestinalis, and Griffithsia teges) were determined at the Environmental and Food Biotechnology Lab., GEBRI, University of Sadat City, using inductively coupled plasma-atomic emission spectroscopy ICP multi elements standard solution IV (MERCK KGaA) 1000 mg/l (23 elements in diluted nitric acid):potassium (K) Aluminium (Al), Boron(B), Barium (Ba), Bismuth (Bi), calcium (Ca), magnesium (Mg), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), Gallium (Ga), Indium(In), Silver (Ag), Lithium (Li),Cadmium (Cd) , Manganese (Mn), Sodium (Na), Nickel (Ni), lead (Pb), Strontium (Sr), Thallium (Tl), zinc (Zn) ,according to the procedure described by **Barrera-García et al. (2012)**. The findings are given as mg kg-1 dry weight (d.w.).

Antifungal assay

In *vitro*, the Agar well diffusion method was used to assess the antifungal activity. **(Magaldi *et al.*, 2004**).On PDA agar plates, inoculates of *Fusarium oxysporum* and *Penicilliumexpansum* (10^8 cells/ml) were applied then left to air dry at room temperature.. Wells were then drilled into the surface of the agar medium using a cork borer (6 mm) . Using a micropipette, 50µl of algal crude extract was placed into each well of the plate. Plates were kept in an incubatorfor six days at 26°C. The sizes of the inhibition zones were determined in millimeters when the incubation process was complete (**Karabay-Yavasoglu *et al.*, 2007).** The mean results of each assay were determined independently in triplicates, and dimethyl sulfoxide (DMSO) was utilized as a control .

Statistical Analyses

It was decided to use the average of the three determinations. Using SPSS software version 16 (**SPSS Inc., 2007),** a one-way analysis of variance (ANOVA) was carried out, and the least significant difference at $p \leq 0.05$ was identified.

Results

Identification of tested algal samples

Collected macroalgae were identified by Prof. Mervat Hosny, Prof. of algae, Faculty of Science, El-Mansoura University, in accordance with (**Jha *et al.*, 2009**) as green algae *{Ulva flexusoa* (Fig. 2) and *Enteromorpha intestinalis* (Fig. 3)} belong to the family: Ulvaceae, and red algae {*Griffithsia teges}* belongs to Family: Ceramiaceae (Fig. 4).

Fig. 2. *Ulva flexusoa*

Fig.3.*Enteromorpha Intestinalis*

Fig. 4. *Griffithsia teges*

Macro and microelements in macro algae extracts

The data reported in Table 1 illustrates the presence of macro and microelements in the three studied macro algae. The Iron, zinc, copper, manganese, magnesium, phosphorus, and potassium, sodium, and 15 other element levels were measured and expressed as $mg.kg^{-1}$ dry weight.

Collected macro algae contains different levels of essential macro and micro elements (Table 1). Presented data reveals that the three studied macro algae contain high levels of sodium (ranging from 106.1 in *G. teges* to 148.1 $mg.kg^{-1}$ in *U. flexusoa)* and calcium (ranging from 60.9 in *G. teges* to 105.3 in *U. flexusoa).* They also demonstrate moderate magnesium and manganese concentrations, as Mg ranged from 20.7 to 49.8 mg.kg-1 and Mn ranged from 14.4 to 18.2 $mg.kg^{-1}$. In contrast, the P, K, Cu, Zn and Fe levels are relatively low.

Total phenolic content of macro algae extracts

The chromatogram results for standard solutions of different phenolic substances are shown in Fig. 5 and Table 2. The chromatograms of total phenolic contents in liquid extracts of the three studied macro algae are illustrated in Fig.6 (A, B, and C), and their concentrations are summarized in Table 3.

The data presented in Table 3 shows that each of the three macro algae under study included a variety of phenolic components. The extract of *Ulva flexusoa* contains Gallic acid (0.07 mg/ml), Chlorogenic acid (0.18 mg/ml), Ferulic acid (0.06 mg/ml), Daidzein (0.20 mg/ml), Querectin (0.26 mg/ml) and Apigenin (0.19 mg/ml), (Fig. 6:A)**.**

The analysis of the phenolic components in the *Enteromorpha intestinal* liquid extract (Fig. 6:B) revealed the presence of six different phenolic compounds: Gallic acid (0.22 mg/ml), Chlorogenic acid (0.41 mg/ml), Daidzein (0.16 mg/ml), Querectin (0.64 mg/ml), Cinnamic acid (0.05 mg/ml), and Hesperetin (0.14 mg/ml).

Regarding the phenolic components of the *Griffithsia teges* extract, it contains the highest concentration of Gallic acid (0.81mg/ml), in addition to a small amount of Chlorogenic acid (0.29mg/ml), Catechin (0.25mg/ml), Ferulic acid (0.06mg/ml), Daidzein (0.06mg/ml), Apigenin (0.2 mg/ml), and Hesperetin (0.2 mg/ml). These findings are illustrated in Fig. 6:C and summarized in Table 3. The findings indicate that the highest amounts of phenolic components were Gallic acid (0.81 mg/ml) in *G. teges* as well as Querectin (0.46 mg/ml) and Chlorogenic acid (0.41 mg/ml) in *E. intestinalis*. Catechin was exclusively found in *G.* teges; however, Cinnamic acid and Hesperetin were only detected in E. intestinalis.

Table 1. Macro and microelement contents ($mg.kg^{-1}$ dry weight) in three macroalgae of *Ulva flexusoa*, *Enteromorpha intestinalis* and *Griffithsia teges*.

Elements ($mg.kg^{-1}$ dry weight)	***U. flexusoa***	***E. intestinalis***	***G. teges***
P	7.33	10.88	10.64
K	7.79	9.95	6.93
Ca	105.3	100.4	60.9
Mg	49.8	20.7	28.4
Mn	14.4	16.3	18.2
Cu	5.74	2.23	11.1
Zn	11.9	5.77	7.84
Fe	6.10	5.30	12.7
Na	148.1	107.0	106.1
Ti	18.297	12.592	19.640
Cr	0.362	0.277	0.472
Li	0.367	0.227	0.426
B	6.052	3.833	4.295
Ga	0.312	0.261	0.633
Se	0.156	0.127	0.116
Sr	3.838	3.618	3.739
Ag	0.044	0.046	0.328
Cd	0.233	0.177	0.573
In	0.073	0.044	0.050
Al	5.490	5.228	16.158
Co	1.015	0.994	6.562
Ni	0.330	0.247	0.480
Si	1.990	1.948	3.900

T**able 2.** Phenolic analyses for the standard solutions.

No	**Phenolic Components**	**Conc.(mg/ml)**
1	Gallic acid	15
2	Chlorogenic acid	50
3	Catechin	75
4	Ferulic acid	20
5	Daidzein	35
6	Querectin	40
7	Cinnamic acid	10
8	Apigenin	50
9	Hesperetin	20

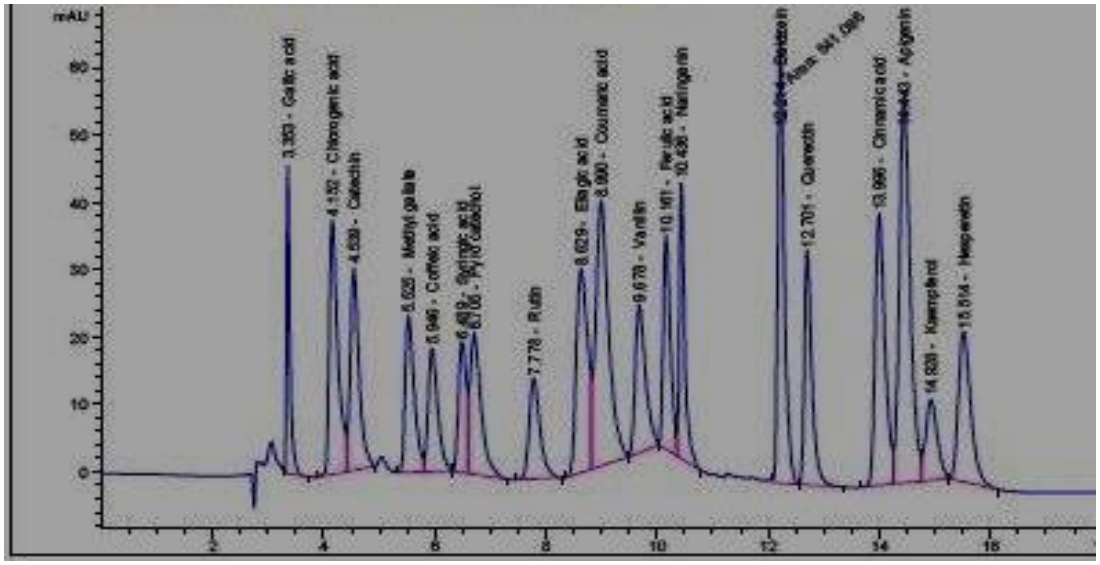


Fig. 5. Chromatogram of phenolic substances of standard solution

Table 3. Total phenolic components (mg/ml) of the extract of the three studied macro algae*Ulva flexusoa, Enteromorpha intestinalis* and *Griffithsia teges.*

No	Phenolic Components	*U. flexusoa*	*E. intestinalis*	*G. teges*
1	Gallic acid	0.07	0.22	0.81
2	Chlorogenic acid	0.18	0.41	0.29
3	Catechin	Nd	nd	0.25
4	Ferulic acid	0.06	nd	0.06
5	Daidzein	0.20	0.16	0.06
6	Querectin	0.26	0.64	nd
7	Cinnamic acid	Nd	0.05	nd
8	Apigenin	0.19	nd	0.20
9	Hesperetin	Nd	0.14	nd

nd: not detected

Antifungal activities of the three studied macroalgae

The extracts of the three studied algae (*Ulva flexusoa, Enteromorpha intestinalis*, and *Griffithsia teges*) were tested for their antifungal properties against two significant fungal species (*Fusarium oxysporum* and *Penicillium expansum*). The inhibition zones (mm) resulting from the presence of methanolic extracts from the three studied macro algae are presented in Fig. 7 and summarized in Table 4. There were variable reaction patterns to algae extracts. *Fusarium oxysporum* was found to be the most sensitive isolate for all extracts. The extract derived from *Enteromorpha intestinalis* had the highest efficacy against both fungal species, exhibiting the largest inhibition zone of 37 mm and 32.6 mm in diameter for *Fusarium oxysporum* and *Penicillium expansum*, respectively. In contrast, the extract of *Griffithsia teges* had

the least impact, resulting in inhibition zones measuring 21.3 and 21.6 mm in diameter against *Fusarium oxysporum* and *Penicillium expansum*, respectively.

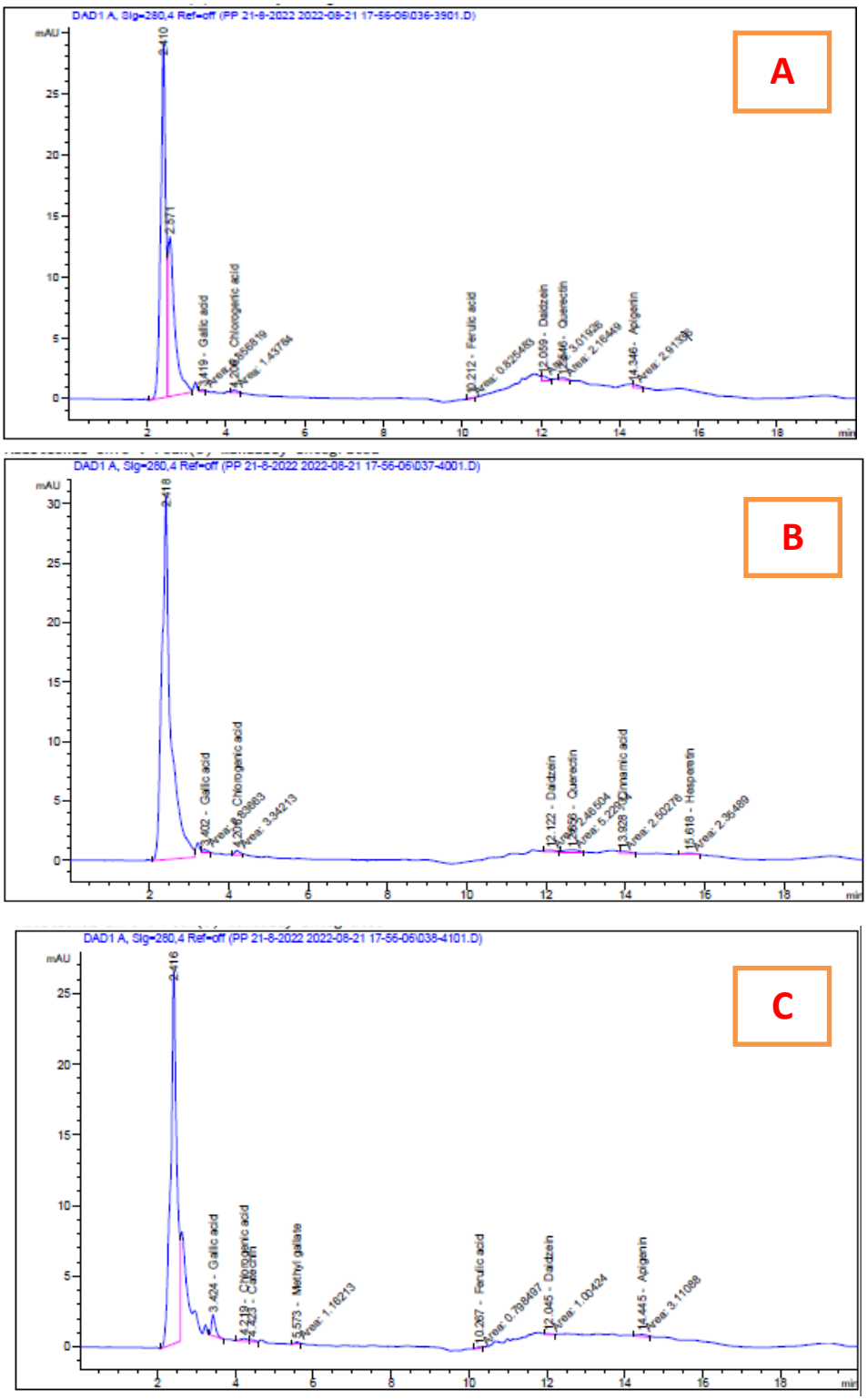
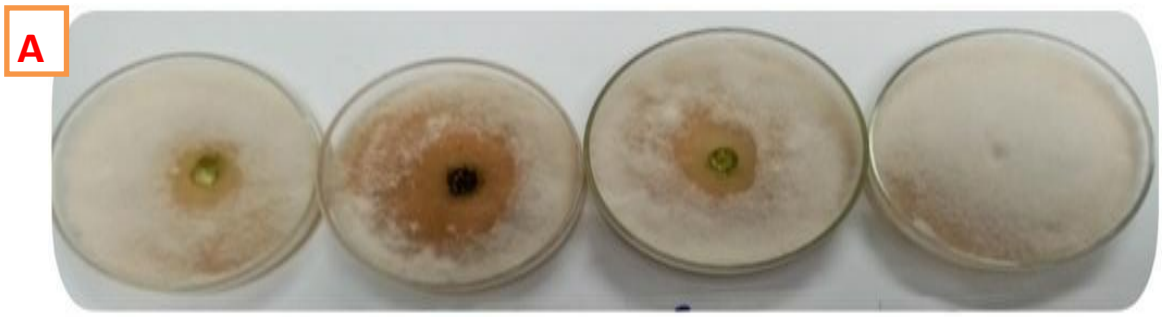


Fig. 6. Chromatograms of phenolic content in liquid extracts of *Ulva flexusoa* (A), *Enteromorpha intestinalis* (B) and *Griffithsia teges* (C).

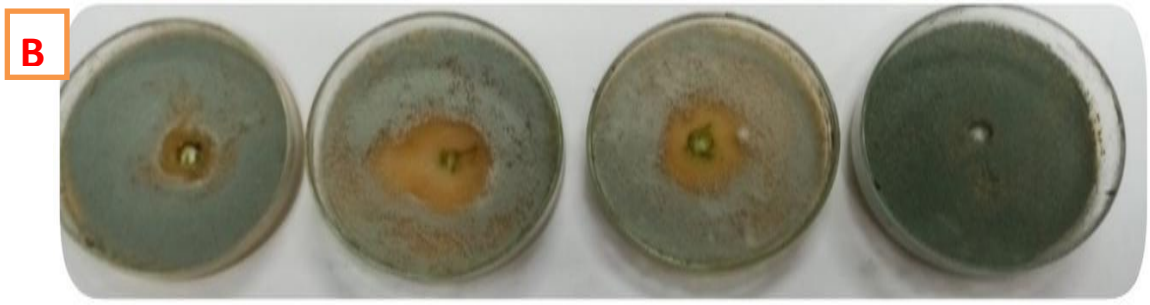
Table 4. Inhibition zone (mm) in the presence of methanolic extracts of the three examined macroalgae (Ulva flexusoa, Enteromorpha intestinalis, and Griffithsia teges) in compared to the control (DMSO).

Treatments	Inhibition zone (mm)	
	Fusarium oxysporum	***Penicillium expansum***
U. flexusoa	28.0 ^{b}	25.3 ^{ab}
E. intestinalis	37.0^{a}	32.6 ^{a}
G. teges	21.3^{c}	21.6 ^{ac}
Control	0.00 ^{d}	0.00 ^{d}

All data represent the average of three replicates. Means with the same letter in the same column show no significant difference ($p \leq 0.05$).



Fusarium+ extract(1) Fusarium+extract (2) Fusarium+ extract (3) Control



Penicillium+ extract(1)Penicillium + extract (2)Penicillium + extract (3) Control

Fig. **7.** The effect of methanolic extracts of three macroalgae (*Ulva flexusoa*, *Enteromorpha intestinalis*, and *Griffithsia teges*) in comparison to the control ((DMSO) against two fungal species **(A)**: *Fusarium oxysporum* and **(B)** *Penicillium expansum*).

Discussion

In our investigation, methanolic extracts from the three species of algae (*Ulva flexusoa, Enteromorpha intestinalis* and *Griffithsia teges*) inhibited the growth of the tested fungi, *Fusarium oxysporum* and *Penicillium expansum*. Our results are consistent with those of (**Zovko *et al.*, 2012**), who found similar results against

fungus strains of algal extracts that are very active against *Cadida albicans*. The three algal extracts were screened against some phytopathogenic fungal species, the most sensitive isolate was *Fusarium oxysporum*. Numerous species of macro algae, red, green, and brown have been studied for their ability to combat *Fusarium* species, and this ability has been seen in in vitro tests (**Ehteshamul- Haque *et. al.*, 2013**). In thepresent study methanol extracts of (*Ulva flexusoa* and *Enteromorpha*) showed higher activity against *Fusarium oxysporum* than *Penicillium expans*um This activity is attributed to the secondary metabolites found in algae species.

Compounds produced from macro algae have been reported to have antibacterial (**Nirupama, 2014**) and antifungal activities (**Soliman *et al.*, 2018**). Previous study has shown that algal extracts contain chemicals that cause fungal cells to break down, make hyphae and grow less effectively, and create more intracellular holes (vacuolization). (**Komari *et al.*, 2017).** Additionally, the current findings were consistent with those of (**Sheikh *et. al.*, 2018**), who discovered that *Chlorophyta* had the strongest antifungal impact against *Candida albican*, *Aspergillus flavus*, and *Aspergillus niger*, followed by *Rhodophyta*.Additionally, our findings were consistent with those of (**Saleh and Al-Mariri, 20**17), who discovered that marine seaweeds *Jania rubens* (Rhodophyta) and *Ulva lactuca* (Chlorophyta) prevent two fungus strains (*Candidaalbicans* and *Aspergillus niger* utilizing algal extracts.

The extracts of the three algal species (*Ulva flexusoa, Enteromorpha intestinalis*and *Griffithsia teges*) contained a broad range of various components, such as alkaloids and phenolic compounds (**Malini *et al.*, 2014**). Some of these compounds, like phenols, are frequently said to have antifungal propertiesagainst the *Fusarium* genus' phytopathogenic fungus (**Bennamara *et al.*, 1999; Belattmania *et al.*, 2016**).These findings corroborated those of (**Osman *et al.*, 2010**) who discovered that *Ulva fasciata* (*Chlorophyceae*) was the most effective seaweed against all examined microbes Following one strain of the yeast *Candida albicans* and the gram-negative bacterium *Bacillus subtitis*, Rhodophyta is the next in line. The results from the present study also agreed with those from (**Kandhasamy and Arunachalam, 2008**), who found that the studied algae (*Rhedophyceae* and *Phaeophyceae*) had lower antibacterial activity than theChlorophyceae.

Our results indicated that the HPLC analysis of *Enteromorpha intestinalis* extract is abundant in polyphenolic chemicals when compared with other tested algal extracts.It is already widely known that phenols, a type of phytochemical, may play a role in the antifungal action of algae extracts. According to (**Ambika and Kandasamy Sujatha, 2015**), Phenolic compounds probably have an impact on fungal growth and metabolism. Phenolic substances may have an impact on the development and metabolism of fungus. **Perry *et al*., 1991**).

Conclusion

The findings demonstrated that algal extracts from Chlorophyta (*Ulva flexusoa* and *Enteromorpha intestinalis*) and Rhodophyta (*Griffithsia teges*) have promising antifungal properties. It has been proposed that the active antifungal chemicals in seaweeds are of interest. Our experimental results show that the alga inhibits plant-pathogenic fungal development in vitro. Thus, this alga could be exploited to create an environmentally acceptable, dependable, and cost-effective antifungal treatment to control *Fusarium oxysporum* and *Penicillium expansum*. For the control of plant

diseases, this may be an effective alternative for extremely dangerous chemical fungicides. The Egyptian macro algae (*Ulva flexusoa*, *Enteromorpha intestinalis*, and *Griffithsia teges*) can be considered a promising alternative bio agent for controlling the investigated pathogenic fungi, potentially limiting the widespread use of chemical fungicides in plant disease management.

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