



Control of Post-harvest Citrus Green Mold using *Ulva lactuca* Extracts as a Source of Active Substances

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Abstract

Penicillium digitatum, the causal agent of citrus green mold, is responsible for 90% of post-harvest losses. Chemical fungicides are responsible for damage to human health and the environment. The exploitation of green algae as a source of pigments and carbohydrate polymers deserves a special attention. In this study, we evaluated the ability of *Ulva lactuca* extracts collected from Lebanon and China, to develop products for the biocontrol of post-harvest citrus green mold. Seaweed aqueous extracts (SAE), organic extracts (SOE) and chlorophylls and Ulvans fractions of *Ulva lactuca* are analyzed by chromatography and spectroscopy methods. The chemical compositions in functional molecules (pigments, organic acids, and monosaccharides) from extracts are compared for the samples. It was found that Lebanese *Ulva lactuca* is particularly rich in ulvans and other polysaccharides, while Food grade *Ulva lactuca* has the highest content in lipophilic molecules. Four approaches were used to evaluate their antifungal properties: inhibition of the conidia germination of *P. digitatum*, fungus viability, adhesion of spores to epidermal orange cells, and the disk diffusion method. For the different seaweed liquid extracts (SLEs), inhibitions of spore germination were observed between 70 and 87%. Moreover, the samples exhibited inhibitory activities towards the adhesion of *P. digitatum* spores. More particularly, SOE showed inhibitions of spore multiplication between 85 and 97%. According zone diameters, Ulvan fractions showed maximum growth spore inhibitions. These findings are interesting to select extracts for safe and effective formulations dedicated to citrus fruit post harvesting protection.

Keywords: *Ulva lactuca*, green extraction, *Penicillium digitatum*, anti-fungal activity

1. Introduction

During storage, citrus fruit is frequently exposed to numerous post-harvest diseases, generally caused by pathogenic molds, which usually infect the host after harvest, during handling and/or processing (Karim et al., 2017). Green mold mainly caused by *Penicillium digitatum* is the most significant post-harvest disease that affects citrus fruit produced in Mediterranean climate countries, leading to possible financial losses

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during refrigeration, transport, and marketing (D'Aquino et al., 2013). To control this post-harvest disease, several chemical fungicides have traditionally been used, including imazalil (IMZ), thiabendazole (TBZ) and sodium o-phenylphenate (SOPP) (Moretto et al., 2014). These fungicides are efficient in decreasing losses due to the deterioration of food, but they also generate health and environmental concerns mainly due to their cumulative toxic effects. Even though these products were successfully used over more than 25 years, the global tendency seems to be shifting towards a minimal use of chemical fungicides. Hence, there is a need to pursue safer and more eco-friendly alternatives for reducing the decay loss in the harvested commodities (Sharma et al., 2009; Lai et al., 2012). In fact, the intensive use of chemical fungicides promotes the development of resistant isolates of *Penicillium* spp. (Kinay et al., 2007). Moreover, increasing concerns regarding the residues of fungicides in the fruit, as well as the risks associated with their continued use, have prompted a search for safe and effective alternative strategies (Spadaro and Droby, 2016).

The objective is also to comply with the restrictions on the use of pesticides such as described by the European regulations EC No 1107/2009 concerning the placing of plant protection products on the market and EC No 543/2011 about the labelling of post-harvest substances used on fruits and vegetables. Among the new strategies, the action of natural substances found in the marine macroalgae could be used on a large scale to struggle against the plant-infecting fungi (Ramkissoon et al., 2017). The green macroalgae have received increasing attention in the area of post-harvest pathogen management (Hamed et al., 2018). The natural substances, which are present in green macroalgae, are pigments, polysaccharides, proteins, polyphenols and lipids. The potentiality of these substances to be used for an ecofriendly pest management deserves further investigations (Briand et al., 2010; Zhao et al., 2017).

Seaweeds have a wide diversity in their biochemical compositions, which is related to their physiological and biochemical characteristics (Holdt and Kraan, 2011). There are more than 15,000 primary and secondary metabolites such as polyphenols (Godos et al., 2017). The main component in green seaweeds cell wall are ulvans (sulfated polysaccharides) which represents up to 29% of the algal dry weight (Lahaye and Robic, 2007). Ulvans have already demonstrated significant biological activities such as cytotoxicity (Thanh et al., 2016), antioxidative (Yuan et al., 2018) and antiviral activities (Chiu et al., 2012). In fact, the antifungal activity of *Ulva lactuca* extracts against *P. digitatum* was not yet studied. But, a few studies reported the potential effect of green algae extracts in the fungi inhibition process. For instance, anti-fungal activities are reported for organic extracts of *Ulva lactuca* towards *Penicillium purpurescens* (Kosanic et al., 2015). Shobier et al. (2016) studied the antifungal activity against *Aspergillus flavipes* of ethyl acetate extracts of *Ulva lactuca* from Egypt.

The green seaweed *Ulva linza* showed a protection against the post-harvest green mold *Penicillium digitatum* (Chbani et al., 2013).

Therefore, the present study intended to estimate the variation in the biochemical constituents of extracts from the chlorophyta *Ulva lactuca* collected from Lebanese coastal zones or from China. Green solvents including biobased solvents have been selected to perform an ecofriendly extraction. *In vitro* tests have been implemented to assess the antifungal activities of *Ulva lactuca* liquid extracts (SLEs) and their fractions of chlorophylls and ulvans against *Penicillium digitatum*. A new methodology has been implemented to test the adhesion properties of spores, as their adhesion to orange cells is one of the first stages of the colonization and can be considered as the starting point of the infectious process. The objective is to evaluate the ability of these extracts to be an alternative to chemical fungicides in the process of controlling post-harvest phytopathogens in fruit.

2. Materials and Methods

2.1. Materials

Three different samples of *Ulva lactuca* were studied. Lebanese *Ulva lactuca* (Leb Ul.) was freshly and manually collected from a coastal zone of the Mediterranean, El Mina (34 ° 26 'N–35 ° 50' E) in Tripoli, Lebanon, on 1 May 2017. The algae were cleaned with seawater to remove unwanted impurities, then cleaned three times with distilled water and dried at room temperature until the mass remained constant. The samples were then stored hermetically at room temperature. Two other samples of dried *Ulva lactuca* in powder Food and Feed quality (Ch Ul. PFo, Ch Ul.PFe) were purchased from Fujian, Mainland China (Fuzhou Wonderful Biological Technology Co., Ltd.).

2.2. Preparation of seaweed liquid extracts (SLEs)

The seaweed liquid extracts (SLEs) were prepared using water or green organic solvents of different polarities (Figure 1). Two fractions rich in ulvans and in chlorophylls were also isolated.

2.2.1. Seaweed aqueous extracts (SAE)

SAE were prepared according to a previous study (Chbani and Mawlawi, 2014), based on a protocol adapted from Jimenez et al. (2011). A volume of 100 ml of boiling Milli-Q water was added to five grams of dried algae put on a flask. The medium was stirred magnetically for one hour at room temperature. The solid residue was then filtered through a double layer of sterile muslin filter. The filtrate was dried at 60 °C during 5 hours and stored at 4 °C until analysis.

2.2.2. Seaweed organic extracts (SOE)

Five grams of algal material were macerated in 100 ml of an organic solvent (acetone, ethanol or ethyl acetate) at room temperature in dark for a period of three days under magnetic stirring. A centrifugation at 10 000 g was carried out for 5 min. If necessary, the medium is filtered and the organic solvent



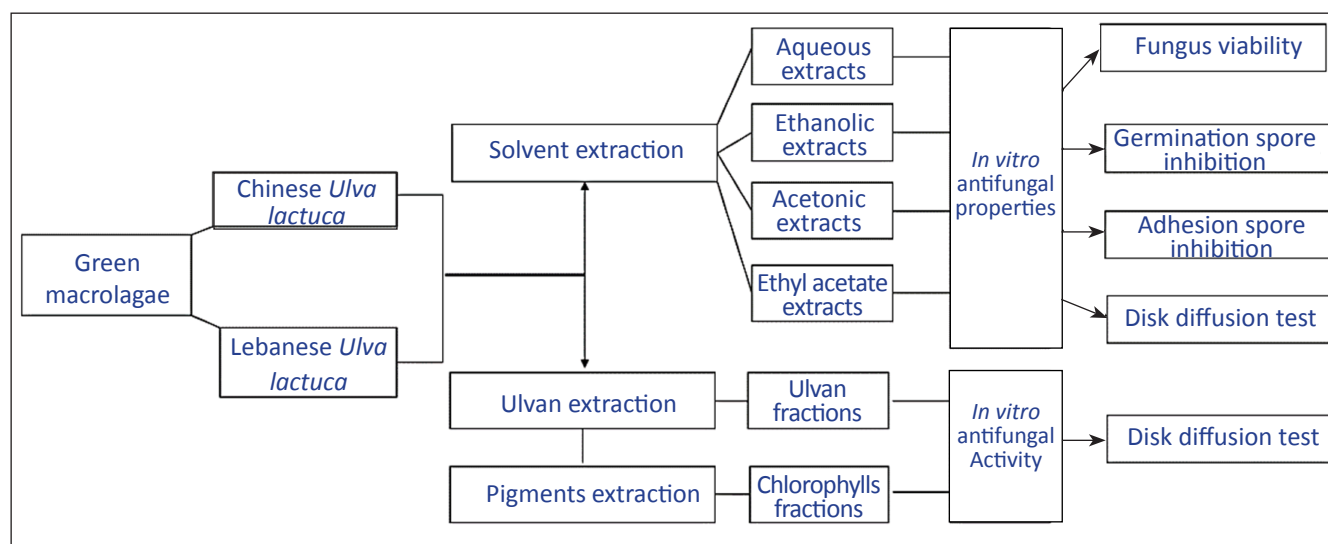


Figure 1: Strategy to generate *Ulva lactuca* extracts

evaporated under vacuum at 45 °C, and the extract stored at 4 °C until analysis.

2.3. Extraction of selective fractions

2.3.1. Ulvans extraction

Twenty grams of dried seaweed were treated at room temperature with a 4:2:1 MeOH-CHCl₃-H₂O mixture to remove the colored matter. After filtration, the defatted algal biomass (Thanh et al., 2016) was used to obtain Ulvans following the method of Mao et al. (2006). The resulting alga was placed into 400 ml of water. This solution was continuously stirred and kept at 80–90 °C in a hot-water bath for 2 h. The medium was centrifuged, and the liquid supernatant was filtered. The water extract was concentrated by a rotary evaporator to reduce the volume and precipitated with 3 vol. of absolute ethanol. The precipitate was separated, then re-dissolved in distilled water, and dried in a vacuum oven at 40 °C to obtain 3, 7 g of purified ulvan from Lebanese *Ulva lactuca*.

2.3.2. Pigments extraction

Five grams of *Ulva lactuca* were homogenized in acetone (100 ml, 80%) and allowed to stand overnight in dark at 4 °C for complete extraction followed by centrifugation at 10 000 g for 5 min.

2.3.3. Fractionation of pigments by TLC

The pigments present in the acetonic extracts (cf. 2.3.2) were separated by the thin layer chromatography on 10×20 cm² silica gel plates (Sigma Aldrich). 5 µl of *Ulva lactuca* extracts were applied to 1 cm of the base of plate and developed with hexane/ acetone (75:25 v/v) (Bhagavathy et al., 2011). The separated compounds were located and identified by visualizing plates stained with a UV lamp at 254 nm and 365 nm. The R_f values of pigments were measured and compared with the reports available for standard pigments.

2.3.4. Separation of chlorophyll a and b

The separation of chlorophylls a and b was carried out by

the method of differential solubility of pigments in organic solvents (Prat, 2007).

2.4. Characterization of fractions

2.4.1. Pigments analysis with high pressure liquid chromatography coupled with UV detector (HPLC-UV)

The acetonic extracts of *Ulva lactuca* (obtained by protocol 2.2.2) were analyzed by an external calibration method using a Dionex-Refractometer diode (UltiMate 3000). The samples were diluted with methanol and filtered using a micro-filtration cartridge Polyray 0.45 micron to eliminate cell residues. The column (SunFir Watess) C₁₈ used was an apolar column (5 µm, 4.6×150 mm²) with a pre-column. The temperature of the furnace was 50 °C, the injection volume was 20 µl and λ = 430 nm. The mobile phase was a mixture of solvent A (H₂SO₄ 5 mM), B (acetone), C (H₂O) and D (pure methanol), according to a step gradient, lasting 30 min, which started from 55% B, changing at 62% in 1 min, rising up to 100% B at 15 min before ending with 25% D. Then, the mobile phase composition was kept constant until the end of the analysis. Total acquisition time was 30 min. The analyzed compounds were identified by comparing their elution times with those of the standards.

2.4.2. Spectrophotometric scanning for chlorophylls fractions

The chlorophyll fractions were scanned using a Thermo Scientific spectrophotometer. Triplicate scanning was performed in a 3 ml-quartz cell in the range 400-700 nm. The degree of purity was defined as the relative percent of chlorophyll area peak to the total area of peaks determined either by a spectrophotometric scanning from 300 to 700 nm or by HPLC using absorbance measurements (430 nm).

2.4.3. Monosaccharide and disaccharide analysis by High Pressure Liquid Chromatography (HPLC)

Five concentrations (5 mg l⁻¹, 15 mg l⁻¹, 30 mg l⁻¹, 60 mg l⁻¹, and 100 mg l⁻¹) of standards (glucose, galactose, xylose, arabinose,

rhamnose, fucose, mannose, mannitol, and sucrose) were dissolved in 1000 ml of Milli-Q water, and then 125 µl of fucose standard was added. One milliliter of aqueous extract was dissolved in 25 ml of Milli-Q water, and then 3 mL of the solution with 125 µl of fucose standard was added to the vial, after filtration with the micro-filtration cartridge Polyray 0.45 micron.

Monosaccharides were analyzed by ion exchange chromatography with a CarboPac PA1 column (Dionex). The eluent was potassium hydroxide, at a flow rate of 1 ml.min⁻¹. The following concentration gradient was used: 2 mM for 39 minutes, increasing to 10 mM for 2 minutes, 100 mM for 8 minutes and 100 mM for 3 minutes. The temperature was set to 25 °C. The injection volume was 25 µl. External standards were used for polysaccharide identification. Fucose was used as an internal standard for polysaccharide quantification.

2.4.4. Anion and organic acid analysis by high pressure ion chromatography (HPIC)

Chromatographic analysis was performed using a Dionex apparatus with an AS 11-HC (4 mm) anion-exchange column and an AG11-HC precolumn. 25 µl of each sample was injected into the loop of the chromatograph. The flow rate was 0.5 ml min⁻¹ with a gradient between phase A (ultra-pure water) and phase B (a solution at 2 mM KOH) during 50 min.

2.4. In vitro antifungal activity

2.5.1. Preparation of pathogen inoculum

Protocol was adapted from Li Destri Nicosia et al. (2016). Fungal isolates of *Penicillium digitatum* were obtained from infected oranges and then cultivated on malt extract agar (MEA, Sigma–Aldrich) in order to obtain single conidia colonies. For inoculum growth, cultures were kept at 22 °C for 7–10 days. Obtained spores were then collected using a sterile spatula, diluted in sterile distilled water, and then vortexed for 1 min to ensure uniform mixing. The concentration was fixed to 10⁸ spores ml⁻¹ using a densitometer (Biosan DEN-1B, McFarland densitometer). This solution was then diluted to two solutions; S1= 10⁶ spores ml⁻¹ was used for the study of extract effects on spore germination in liquid medium and on germ tube elongation, and S2= 2x10³ spores ml⁻¹ was used to study the extract effects on fungus viability in solid medium.

2.4.2. Effects of SLEs on spore germination and germ tube elongation

The solution S1 (12.5 µl at 10⁶ spores ml⁻¹) were transferred to Eppendorf tubes to which 25 µl of SLEs. Then 12.5 µl of potato dextrose broth (PDB, Sigma–Aldrich) were added. Sterile ultrapure water, organic solvent (acetone, ethanol, ethyl acetate) and Nystatin were used as negative and positive controls, respectively. The tubes were then mixed and incubated for 20 hours at 22 °C followed by a vortex in order their homogenization. Afterward, 2 µl of spore suspension were transferred to microscope slides, mixed with 2 µl of Lactophenol blue than observed at a magnification of 40 for

spore germination and tube elongation. For each slide, three observations were randomly done.

2.4.3. Effects of SLEs on fungus viability

In order to evaluate the SLEs effects on pathogen viability, 0.5 ml of solution S2 (2x10³ C ml⁻¹) was transferred to Eppendorf tubes containing 0.5 ml of SLEs. Sterile ultrapure water, organic solvent (acetone, ethanol, ethyl acetate) and Nystatin were respectively used as negative and positive controls. Tubes were gently mixed and then incubated for 20 hours at 22 °C. Afterwards, the tubes were mixed and 100 µl of each mixture were spread on MEA culture medium containing ampicillin and streptomycin. Finally, cultures were incubated at 25 °C and the number of colony-forming units (CFU) was counted after 3–4 days.

The CFU percentage of each tested extract was then calculated by comparing it with water, used as a negative control.

2.4.4. Effects of SLEs on the adhesion of spores to the epidermal orange cells.

Protocol was adapted from Kimura and Pearsall, (1978) viable fungi adhered much better than did nonviable fungi, and this adherence was greater at 37 than at 25 degrees C. Viable yeasts, preincubated in saliva for 90 min at 37 degrees C before being washed and mixed with epithelial cells in phosphate-buffered saline, adhered better than nonviable yeasts or yeasts preincubated in phosphate-buffered saline. Enhanced adherence in saliva appeared to be associated with germination of the yeast cells. Conditions permitting germination (growth in tissue culture medium 199 at 37 degrees C but not at 25 degrees C. The orange cells were collected in the middle ripening stage, by scratching its outer peel. These cells were then washed in PBS (1 ml tube⁻¹), centrifuged at 2500 g for 10 min, and finally re-suspended in 1 mL PBS for counting. *Penicillium digitatum* spores were taken from an infected orange and cultivated on MEA for 4 days at room temperature. Spores were then suspended in PBS. Spores and oranges cells' concentrations were evaluated using a densitometer (Biosan DEN-1B, McFarland densitometer). Spores and oranges cells' solutions were diluted to obtain concentrations of 10⁷ cells ml⁻¹ and 10⁵ cells ml⁻¹ respectively; 1 mL of orange cells suspension was brought into contact for 1 h with 1 ml of crude extracts solutions. After 1 h of incubation, 1 ml of *Penicillium* spores was added to the various mixtures and the whole mixture was stirred for 2 h at room temperature. After 2 h of incubation, the preparation was washed 3 times in PBS; for a clean wash, 0.5 ml of PBS solution was added each time and the mixture was centrifuged at 2500 g for 10 minutes. The aim of these centrifugations is to eliminate the remaining free spores. The cells being in suspension, the test reading was carried out by the examination of fresh pellet reconstituted in 0.1 ml PBS. The adhesion index is defined as the number of *Penicillium digitatum* spores fixed per orange cell. An index greater than 25 is the indicator of a strong adhesion, whereas, for an index below 10, the adhesion is low



(Lin, 1986). An average of the number of spores that have adhered to epithelial cells were calculated from triplicate.

2.4.5. Disk diffusion test for SLEs, Ulvans and chlorophylls

The determination of the antifungal properties of the extracts of *Penicillium digitatum* was carried out by a Disk diffusion test on Malt Extract agar, previously inoculated by flooding with *P. digitatum*. The microorganisms were grown on MEA (Sigma Aldrich) plates at 24 °C for 48 hours prior to seeding into MEA. One or several colonies of a similar morphology of each *P. digitatum* were transferred into the sterile distilled water and adjusted to the 0.5 McFarland turbidity standards (10^7 C ml⁻¹ suspension). Inoculates of the *P. digitatum* were seeded on MEA. Each sterile disc was impregnated with 25 µl SLEs obtained according to protocol 4.2 or 25 µl Ulvan fractions (500 mg in 10 ml distilled water) or 25 µl chlorophyll fractions (500 mg in 10 ml acetone). The sterile filter discs, 6.4 mm in diameter, were placed on inoculated agar medium and incubated at 24 °C for 48 hours. Nystatin (100 U/disc) was used as a positive antifungal control. The results were determined from the presence or absence of growth and the size of the inhibition zone. The diameter (mm) of the growth inhibition halos caused by the different extracts was measured. All the assays were carried out in triplicate.

2.5. Microscopic observation

Observations at high magnification ($\times 1000$) were performed. A small quantity of cell suspension was placed on a specific plate (Nikon SMZ 1500). The images were captured under a constant exposure and illumination by a Nikon eclipse E600 camera.

2.6. Statistical analysis

All the data were subjected to variance analysis using the GLM procedure of SAS (SAS Institute, 1987, Cary, NC, USA). The mean pair wise comparisons were based on a Duncan test at 0.5% probability level.

3. Results and Discussion

3.1. Extraction yield

3.1.1. Extraction yield of SLEs

Four solvents were selected to test the effect of a variable polarity on the extract compositions and on their properties. Extraction yields for *Ulva lactuca* from different geographic origins are presented in Table 1 and were calculated according to the formula below.

$$\% \text{ extraction yield} = 100 \times \frac{\text{Final Weight of dried extract}}{\text{Initial weight of dried material}}$$

Extraction yields are expressed in % w/w dry weight (d.w).

As expected, extraction yields depend on the solvent (hydrophilic and protic degree), as the solvents have various affinities with the chemical substances to be extracted (Table 1).

We can observe that Lebanese *Ulva lactuca* contains the most

abundant hydrophilic fraction. Among the lipophilic fractions extracted with ethyl acetate, Food grade sample is the richest

Table 1: Yields in aqueous and organic extracts of *Ulva lactuca*

Type of extraction	Solvent	Macro algae		
		Lebanese <i>Ulva lactuca</i>	Chinese <i>Ulva lactuca</i>	
			Food powder	Feed powder
Aqueous extract (SAE)	Water	44±0.1	30.7±0.0	30.7±0.0
Organic extract (SOE)	Ethanol	5.7±0.1	10.6±0.3	25.6±0.2
	Acetone	25.0±0.1	33.1±0.9	21.2±1.2
	Ethyl acetate	6.5±0.2	13.2±1.2	5.8±0.1

in lipids. This sample also has the highest acetic fraction. As for Feed grade sample, it generates the most abundant ethanolic fraction.

The yield (6,5%) obtained for the lipophilic extract from Lebanese *Ulva lactuca* was close to the lipid content (7.8% w/w in dry matter) found by Yaich et al. (2014) in *Ulva lactuca* collected in Tunisia, according to AFNOR protocol (1984).

Palmitic acid was the most abundant fatty acid in the lipidic fraction from *Ulva lactuca* (Yaich et al, 2014). Bhagavathy et al. (2011) and Tabarsa et al. (2012) suggested the contribution of fatty acids in the antimicrobial activity. In fact, the presence of lipids in the extract may be significant as they form a protective matrix for bioactive compounds, thus preserving their efficiency over time.

3.1.2. Extraction yield of Ulvans

These sulfated polysaccharides have already been used as an alternative treatment for chemical fungicides (Merillon and Riviere, 2018). Extraction of ulvans showed that Lebanese *Ulva lactuca* is particularly rich in ulvans, which is in agreement with the high extraction yield obtained for its SAE. The extraction yield was 18.5 %, (± 1.2) against 6.3% (± 0.1) and 9.8% (± 0.4) for food and feed grades respectively (Chinese *U. lactuca*). *Ulva lactuca* collected from Vietnam had 15% of ulvans with similar extraction conditions (Thanh et al., 2016).

3.2. Characterization of fractions SAE and SOE

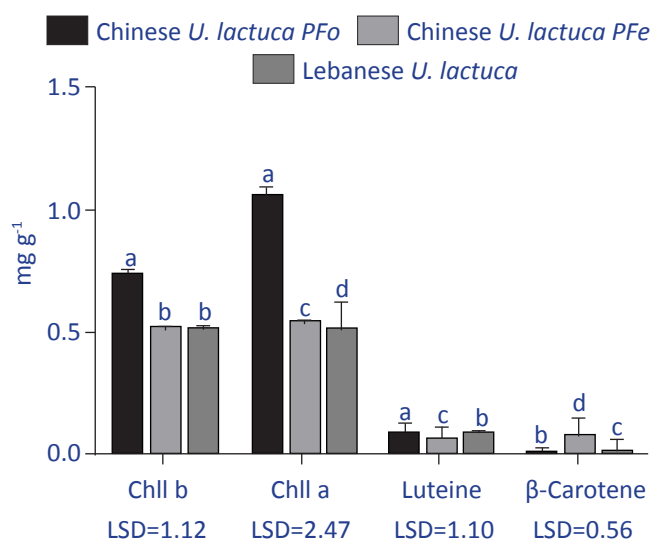
3.2.1. Pigment composition

The separation by TLC showed that acetic extracts (Protocol 2.3.3) contain three kinds of pigments (chlorophylls, carotene and xanthophylls) which were identified with their frontal ratio and their absorption maxima. HPLC-UV analyses have confirmed the three pigment categories, and showed that chlorophylls were the major pigments (Figure 2). Food grade sample was the richest one in chlorophyll. This result is in



agreement with the high yield in acetonetic extract. A similar content in chlorophyll (2.1 mg g⁻¹) was found in *U. lactuca* from the Indian coast (Chakraborty and Santra, 2008).

Liposoluble pigments of marine algae are already known for beneficial biological activities, such as antioxidant properties (Pangestuti and Kim, 2011).



Means in each compound with different letters (a-b-c-d) are significantly different (Duncan test at 0.5% probability level) Figure 2: Pigment contents (mg g⁻¹ DM) of the studied seaweeds *Ulva lactuca*

3.2.3. Monosaccharide and disaccharide analysis

Table 3 indicates the polysaccharide compositions of SAE analyzed by HPLC. The compositions in sugar differ from one sample to another. The Lebanese *Ulva lactuca* extract had the highest total content in sugar (77 mg l⁻¹) mainly due to fructose and rhamnose coming from ulvans. Sucrose was clearly the most abundant sugar in Food grade samples (Table 2).

3.2.4. Anions and organic acids composition of SAE

Organic acids and some anions were detected in SAE by HPLC (Figure 3). All the samples of *Ulva lactuca* contain succinic acid and sulfates mainly due to sulfated polysaccharides (Figure 4). Low amounts of maleic acid and phosphate compounds were found in *Ulva lactuca* extracts.

Furthermore, Lebanese *U. lactuca* provided the richest ulvans fraction in sulfates (17.8% ± 0.2), against about 8% of sulfates in ulvans from Chinese *U. lactuca*. Sulfated polysaccharides from an ulva green seaweed have already shown their contribution to struggle against fungi on plants (Paulert et al., 2009).

To conclude this part, we found that Food grade *Ulva lactuca* has the highest lipophilic fraction, rich in liposoluble pigments (chlorophyll and carotenoids) and organic acids. Moreover, the monosaccharides and ulvans contents confirmed the abundant hydrophilic fraction found for Lebanese *Ulva lactuca*. Such results confirm the potential anti-fungal activity of these extracts, hence the tests carried out with *Penicillium digitatum*.

Table 2: Monosaccharide and disaccharide compositions of *Ulva lactuca* aqueous extracts (HPLC analysis)

Algae source	Amounts (mg l ⁻¹)							Total
	Rhamnose	Galactose	Glucose	Sucrose	Xylose	Fructose	Mannitol	
Ch Ul. PFo	n.d.	1.05±0.06	1.35±0.04	52.32±0.04	n.d.	n.d.	3.95±0.04	58.68±0.04
Ch Ul. PFe	n.d.	2.31±0.03	25.23±0.01	n.d.	n.d.	37.32±0.03	n.d.	64.87±0.03
Leb Ul.	4.362±0.01	n.a.	5.98±0.02	n.d.	n.d.	66.61±0.04	n.a.	76.95±0.04

Values are expressed as the mean of three measurements ± SD

Table 3: Antifungal activities around the wells of *Ulva lactuca* extracts and fractions in relation to inhibition zone diameter (mm)

		Inhibition zone diameter (mm)						
		Extracts				Fractions		
		Aqueous	Acetonetic	Ethanolic	Ethyl acetate	Chll a	Chll b	Ulvan
Seaweeds	Ch Ul. PFo	12.5	14.1	13.1	12.5	11	10	19
	Ch Ul. PFe	11.1	13.8	14.2	12	15	10	17
	Leb Ul.	13	13.8	12.3	14	14	10	16
Control	Nystatin				13.5			
	Water				0			
	Acetone				10.2			
	Ethanol				9.3			
	Ethyl acetate				7.3			



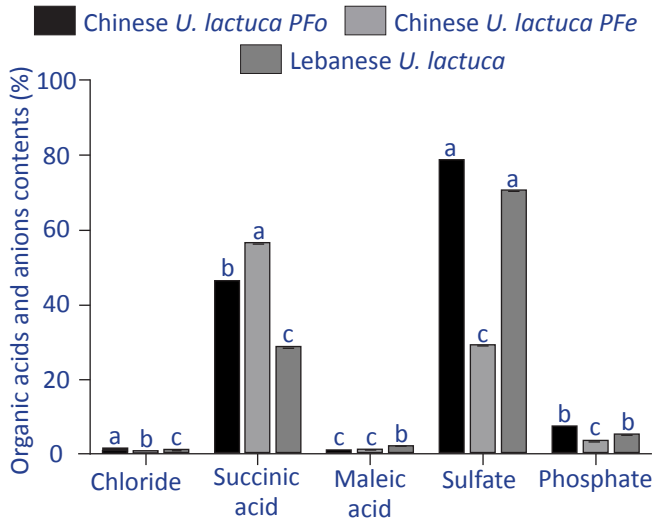


Figure 3: Organic acids and anions contents (%) in the aqueous extracts of *Ulva lactuca*. For each compound, means with different letters (a-b-c-d) are significantly different (Duncan test at 0.5% probability level)

3.3. In vitro antifungal activity of SLEs

3.3.1. Effects of SLEs on spore germination and on fungus viability

After 20 h of incubation, inhibitions of spore germination for all the extracts ranged between 70 and 87%, compared to water (0%), acetone (20%), ethanol (25%), ethyl acetate (10%) as a negative control and Nystatin (100%) as a fungicidal

standard (Figure 4A).

After an incubation at 25 °C for 3–4 days, the number of colony-forming units (CFU) was recorded. The tested extracts (Figure 4B) led to inhibition percentages of CFU ranged between 70% and 97%, compared to the negative controls with lower percentages of inhibition (water 0%, acetone 23%, ethanol 31% and ethyl acetate 17%). The extracts SOE in particular, inhibit the development of the fungal pathogens, with inhibitions of spore multiplication between 85 and 97%.

When SLEs inhibited the germination of *P. digitatum* and its multiplication, it means that the infectious life cycle can be stopped.

3.3.2. Effects of SLEs on the adhesion of spores to the epidermal orange cells.

The adhesion of *Penicillium digitatum* spores to the epidermal cells of oranges can be considered as the first main step in the inhibition of the infectious cycle of this pathogen. Moreover, the adherence of fungus on the host cells is known to be a prerequisite for the attachment, colonization, and infection of the cells by the pathogen (Sebastian and Keerthi, 2013).

Figure 5 shows the adhesion of *P. digitatum* spores to the epidermal cells of oranges in the presence of the SLEs. Indexes of adhesion for tested extracts were lower than 10, which, according to Linas (1986), indicates a low spore adhesion. A high adhesion was observed with water (AI=26), low adhesions were observed for organic solvents used as negative controls. In these conditions, the results clearly show that all tested

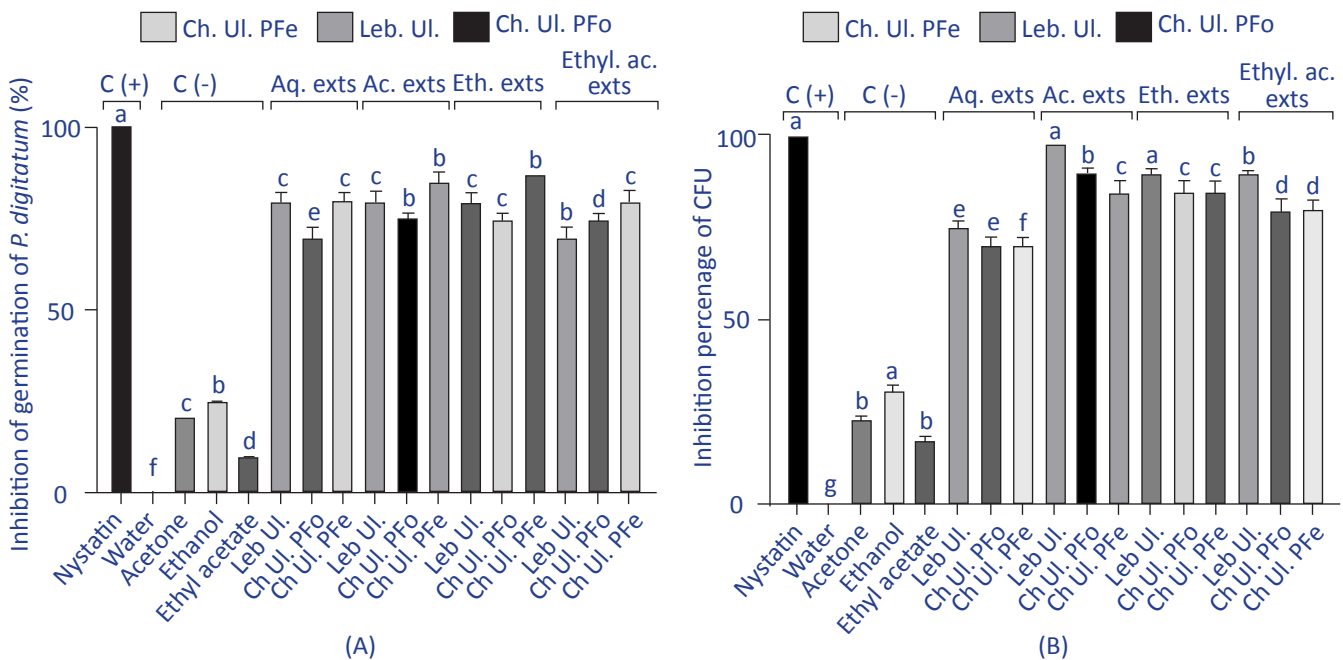


Figure 4: Inhibition percentages of spore germination (A) for Lebanese (Leb) and Chinese (Ch) *Ulva lactuca* (Ul.) Food powder (PFO), Feed powder (PFe), aqueous extracts (Aq.exts), Acetonic extracts (Ac. exts), Ethanolic extracts (Eth. Exts) and Ethyl acetate extracts (Ethyl ac. exts.): Inhibition percentage of colony-forming units (CFU) of fungal pathogens (B) on MEA medium incubated for four days with SLEs (B). Means with different letters (a-b-c-d-e-f) are significantly different (Duncan test at 0.5% probability level).

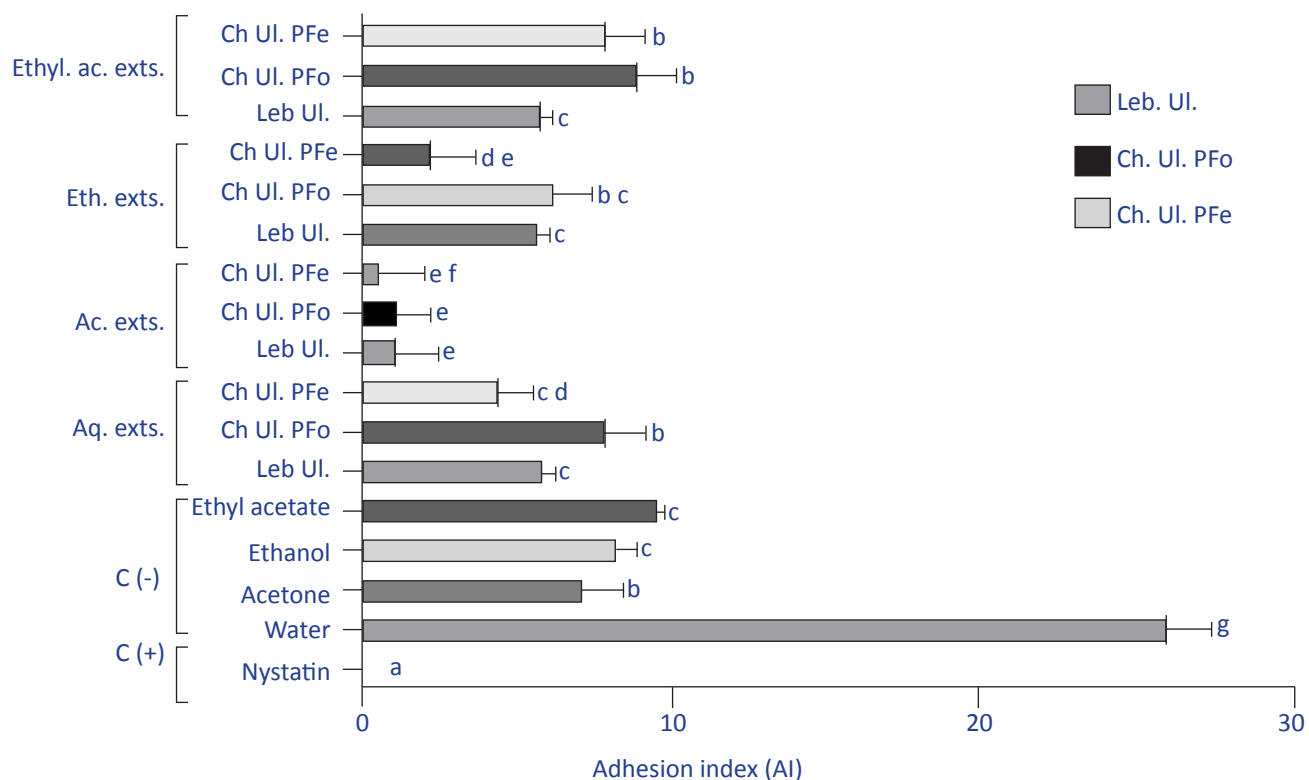


Figure 5: The adhesion index of *P. digitatum* to the orange epidermal cells in the presence of SLEs. Means with different letters are significantly different (Least significant difference LSD= 1.8, Duncan test at 0.5% probability level)

Ulva lactuca liquid extracts (SLEs) have an inhibitory activity towards the adhesion of *P. digitatum* spores.

3.4. Disk diffusion test

The antifungal activities of different extracts were assessed against the fungal strain *Penicillium digitatum* by evaluating the inhibition zone diameter (mm). According to Table 3, extracts had inhibition zones ranged from 11 mm to 16 mm, above the limit value defined by Drouhet and Dupont (1976) for a positive sample (10 mm). The different extracts (SAE, SOE and fractions) exhibited higher antifungal activities than their standard solvent. The ulvans fractions showed the highest inhibition zone diameters for the three samples of *U. lactuca*. Acetonic extracts showed higher inhibition zones than for the separated chlorophyll fractions, meaning that the detected activity is due to other molecules than chlorophylls.

Finally, antifungal activities were detected for the extracts from *Ulva Lactuca*; the inhibition of the spore adhesion was found in particular for acetonic and ethanolic extracts, while the inhibition of spore multiplication was shown more particularly for aqueous extracts. We can assume that active compounds are present in the different extracts, these molecules acting on one of the fungi development steps.

4. Conclusion

Despite their different compositions, the extracts (SAE or SOE) from *Ulva lactuca* exhibited antifungal activities linked to

their contents in polar or low-polar molecules, among them, pigments, polysaccharides, lipids and organic acids. The levels of inhibition observed towards the fungi species *Penicillium digitatum* give promising indications for the development of alternative formulations to chemical fungicides, involving marine natural sources.

5. Further Research

Further research focuses on *in vivo* tests with formulated extracts sprayed on citrus peels, in order to check the performances and to promote their industrial development. Moreover, additional studies aiming to elucidate the mechanisms for the inhibition of the germination of *P. digitatum* are in progress.

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