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Antibacterial Activity of Seaweed Extracts against Plant Pathogenic Bacteria

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Abstract

Chemical pesticides have a major impact on biological diversity, alongside habitat loss and climate change with the need to replace the use of these hazardous chemicals with ecologically sound alternatives. Four seaweed species collected along the southeast coast of Ireland were tested against nine plant pathogens using the disk diffusion assay. The species used in this present study include two Phaeophyta (*Fucus serratus* and *Ascophyllum nodosum*), one Rhodophyta (*Polysiphonia lanosa*) and one Chlorophyta (*Ulva lactuca*). The seaweeds were found to exhibit a broad spectrum of activity, except for *Ulva lactuca*, which showed no activity against any of the bacterial strains. Methanol was found to be the optimum solvent for extracting antimicrobial compounds from the seaweed species. *P. lanosa* showed activity against the majority of the tested organisms; particularly the methanol extracts which proved the most potent with an inhibition zone of 15.83 ± 0.41mm exhibited against *Xanthomonas arboricola*. The tested extracts were found to demonstrate better activity against Gram-negative bacteria as opposed to Gram-positive bacteria. The minimum inhibitory concentration of the most effective extract, the methanol extract of *P. lanosa*, was determined to be 6.25mg/mL with the same concentration also found to exhibit antibiofilm activity against *Xanthomonas fragariae* in a dose response manner. These present findings therefore revealed that the methanol extract of *P. lanosa* contained the potential antibacterial compounds to control this destructive phytopathogen and lead to new alternatives over the copper-based solutions currently in use with resultant environmental benefits.

Keywords: Seaweed extracts; Antibacterial activity; Antibiofilm activity; *Xanthomonas fragariae*; *Xanthomonas aboricola*

Abbreviations

DAFM: Department of Agricultural, Fisheries and the Marine; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; ATCC: American Type Culture Collection; SD: Standard Deviation; PBS: Phosphate Buffered Saline; CFU: Colony Forming Units; MBEC: Minimum Biofilm Eradication Concentration

Introduction

Bacterial plant pathogens can cause widespread devastation in the agricultural sector. This is a critically important sector to many economies including Ireland where in 2016; the agri-food sector generated €13.6 billion [1]. Losses in crop yields due to diseases must be reduced in order to meet increasing global food demands because of the growing population [2]. To combat such crop challenges, intensive applications of synthetic pesticides is used, but this current control strategy is losing its efficacy due to the development of resistance by pathogens to these chemicals [2]. More pressingly, chemical pesticides are having significant effects on the environment, such as their effect on non-target species and soil fertility. For instance, the neonicotinoid insecticide treatment on seeds has been reported to exhibit a negative impact on the inter annual reproductive potentials of both wild and managed bees, including honeybees, across certain countries [3]. Consequently, pesticides can lead to the destruction of biodiversity [4,5] and potential chronic health effects. [6-9]. Therefore, there is a well-recognized need for the development of alternative measures to reduce chemical application. The use of natural products is receiving interest as a source of potential alternatives, particularly marine algae due to their potential as a novel source of secondary metabolites [10]. Such metabolites are believed to be synthesized by seaweeds as a defense mechanism against microbes [11]. Harder [12] was the first pioneer to observe the antimicrobial potential of these metabolites in seaweeds. Since then, many seaweed species have been found to exhibit a wide variety of biological activities including anti-inflammatory [13], antioxidant [14], antifouling [15] and antiviral [16]. Seaweeds also have a long history of safe use, being used in the habitual diet in many countries [17,18] and as a source of medicines in many coastal areas for several centuries [19].

Therefore, these potentially novel bioactive compounds could aid in protecting plants against pathogenic microbes [10]. These compounds also overcome other problems associated with synthetic pesticides such as exhibiting biodegradable properties, giving them a potentially low environmental impact and reducing the likelihood of pathogens forming resistance against treatment [20]. In addition to this, pesticides derived from natural products are less toxic and generally only affect the target pest and closely related species [21]. Although a lot of literature is available on the antibacterial capacity of seaweeds against human pathogens, their bioactive potential

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Table 1: The general plant hosts, symptoms and control strategies of the nine bacterial plant pathogens supplied by the Department of Agricultural, Fisheries and the Marine (DAFM) to be tested.

Pathogen Name	ATCC	Susceptible plant species	Symptoms of infection	Control strategies	Ref
Xanthomonas arboricola pv. *Pruni*	19316	Prunus species such as peach, apricot, cherry and plum.	Lesions on leaves, twigs, fruit and steam cankers.	Preventive applications of copper-based bactericides. Use of disease resistant plants and infected plants destroyed.	[46,47]
Xanthomonas hyacinthi (Wakker)	19314	*Hyacinthus orientalis, Scilla tubergeniana, Eucomis autumnalis* and *Puschkinia scilloides*.	Infected bulbs planted, yellow discoloration of the vascular tissue and surrounding parenchyma. If the plant manages to develop leaves will turn black and wither.	Bulb heat treatment, treatment with alkyl dimethyl benzyl ammonium chloride (if permitted) on leaves showing disease symptoms and infected bulbs and plants are destroyed.	[26]
Clavibacter michiganensis subsp. *nebraskensis* (Vidaver and Mandel)	27794	Maize - Goss wilt (*Zea mays* L.).	Foliar blight lesions and vascular wilt symptoms, e.g. internal orange discoloration of the vascular bundles and by the external water-soaked and slimy appearance of the stalk.	Crop rotation, use of disease resistant plants and infected plants destroyed (no chemical treatments labeled for maize against this pathogen).	[48,49]
Clavibacter michiganensis subsp. *michiganensis*	7433	Main host of economic importance is tomatoes - Bacterial canker disease (*Solanum lycopersicum*) but reported to infect other *Lycopersicon* spp. and *Capsicum annuum*, *Solanum douglasii*, *S. niigrum* and *S. triflorum*.	Under glass house conditions: Wilting on leaves and eventually the whole plant desiccation. In the field, desiccates of the edge of the leaflets observed and the plant slowly desiccates.	Healthy seeds acid extracted. Chemical treatments such as copper hydroxide to reduce incidence. Chemical activation of the hosts defence system e.g. salicylic acid treatment. Use of biocontrol agents e.g. *Bacillus subtilis* and infected plants destroyed.	[50,51]
Clavibacter michiganensis subsp. *tessellarius* (Carlson and Vidaver)	33566	Wheat - bacterial mosaic (*Triticum aestivum*).	Leaf freckles and leaf spots.	Infected plants destroyed and development of more resistant genotypes.	[52]
Xanthomonas fragariae Kennedy and King emend. Van den Mooter and Swings	33239	Cultivated strawberries (*Fragaria ananassa*) main host of concern.	Angular leaf spot.	Use of healthy of plant materials and avoidance of conditions favoring disease. Treatments with copper containing products and infected plants destroyed.	[53]
Xanthomonas campestris (Pammel) Dowson pathovar uppalii	11641	Vegetable Brassica crops such as broccoli, cabbage, chinese cabbage, cauliflower, brussel sprouts and a number of other cruciferous crops, ornamentals and weeds.	V-shaped yellow lesions starting from the leaf margins and blackening of the veins commonly known as black rot.	Use of pathogen-free planting material, crop rotation and the elimination of other potential inoculum sources such as destruction of infected plants. Seed treatments including hot water, antibiotics, sodium hypochlorite, hydrogen peroxide but not fully effective.	[54]
Ralstonia sp.	N/A	Wide host range of over 200 species including model plant Arabidopsis thaliana and important crops such as potato, tomato, and banana.	Wilting and discoloration of the leaves, dark streaking in the vascular tissue of the infected stems.	Use of pathogen-free planting material. No effective biological or chemical control available. Infected plants must be destroyed.	[55]
Erwinia amylovora (Burrill)	N/A	Maloideae sub-family of Rosaceae such as apples, pears and ornamental Rosaceae species.	Infects all host tissues with flowers, leaves, shoots and fruits dark coloured or blackish.	Integrated management: pruning, tree nutrition, use of disease resistant plants and infected plants destroyed. Chemical controls: spraying plants with streptomycin in North America or in Europe testing the use of flumequine a biological control.	[56,57]

against bacterial plant pathogens is a relatively new concept. Kulik [22] was one of the first researchers who investigated the possible use of cyanobacteria and algae against plant pathogenic bacteria and fungi. Since then, studies have been completed on a wide range of bacterial pathogens including *Xanthomonas* sp. [23], *Clavibacter michiganensis* subsp. *sepedonicus* [24] and Pseudomonas *syringe* [25] with very encouraging results.

This present study evaluated the antibacterial potential of extracts from four seaweed species collected from the Irish coast against nine quarantine bacterial pathogens (information on each pathogen is presented in Table 1) that affect a wide range of crops and trees worldwide with no effective treatment currently available. It is estimated that plant diseases can affect 30% of the crop harvest if not managed correctly and efficiently [26]. The four seaweed species used in this investigation were selected based on being indigenous to the southeast coast of Ireland and with demonstrated antimicrobial activity in previous studies [27-31]. These seaweeds were extracted in four different solvents including water, methanol, ethanol and acetone as these solvents have shown their effectiveness in extracting antibacterial compounds in previous studies [32-36].

Once the extract with the best antibacterial activity was identified, the next step was to determine their antimicrobial efficacy through the determination of the Minimum Inhibitory Concentration (MIC) value for that particular extract. MIC is defined as the lowest concentration of the antimicrobial compound that will inhibit the visible growth of the bacteria after overnight incubation [37,38]. However, this concentration may not actually kill the bacteria and once the compound is removed, the bacteria will start to grow again.

Table 2: Bacterial cultures and their respective incubation temperatures and growth media.

Bacteria	Gram positive/negative	Incubation temperature	Growth media
X. arboricola	Gram negative	26°C	Nutrient agar
X. hyacinthi	Gram negative	26°C	Nutrient agar
E. amylovora	Gram negative	26°C	Nutrient agar
X. campestris	Gram negative	26°C	Nutrient agar
X. fragariae	Gram negative	27°C	Nutrient agar + 1% glucose
C. michiganensis subsp. Nebraskensis	Gram positive	26°C	Brain heart infusion agar
C. michiganensis subsp. michiganensis	Gram positive	26°C	Brain heart infusion agar
C. michiganensis subsp. Tessellarius	Gram positive	26°C	Brain heart infusion agar
Ralstonia sp.	Gram negative	30°C	Trypticase soy agar

Therefore, a simple test following MIC that is typically carried out is the Minimum Bactericidal Concentration (MBC), which determines the lowest concentration of the antimicrobial compound that will prevent (≥ 99%) the growth of the bacteria when sub-cultured on fresh media plate [37].

However, for a bio pesticide to be effective against bacterial pathogens it must exhibit antibiofilm activity since the predominant mode of growth of bacteria is through biofilm formation. Biofilms are defined as a tightly-packed, multispecies population of cells in a self-produced polymer matrix that is attached to a tissue or surface [39,40]. Biofilm formation in bacterial plant pathogens protects the bacteria from desiccation, UV radiation, other environmental stresses as well as the plants immune system [41]. This ability to form biofilms also provides enhanced protection against chemical antimicrobial treatments making it very difficult to eradicate such infections. Therefore, biofilm formation is a major issue [42] with all nine bacteria in this study exhibiting this property.

At present, the use of bio pesticides is only observed in speciality and niche agricultural and horticultural circumstances [43], with bio pesticides accounting for only 2.5% of the total world pesticide market [44], demonstrating the novelty of this work. This low uptake is as result of bio pesticides been found to exhibit a short shelf life and field persistence leading to repetitive applications required for the effective eradication of a pest [45]. This increases the costs of using bio pesticides as an integrated pest management strategy making it non-competitive economically in comparison to synthetic pesticides.

Therefore, this work aims to evaluate the antibacterial activity of four seaweed species collected from the southeast coast of Ireland against quarantine plant pathogens in order to find alternative means as well as a promising source of novel bio pesticides. The most promising seaweed extract(s) from the initial screen study were assessed for their MIC, MBC and antibiofilm activity.

Materials and Methods

Collection of seaweeds

The seaweed species, *Fucus serratus*, *Ascophyllum nodosum, Polysiphonia lanosa* and *Ulva lactuca* were harvested from the southeast coast of Ireland (52°11'53.68"N, 6°49'34.64"W) in June 2017. The seaweeds were thoroughly washed with distilled deionized water to remove any macroscopic epiphytes and sand particles. The seaweeds were frozen at -20°C and freeze-dryer to remove all water. The seaweeds were then powdered in an electric blender, sieved to approximately 850 µm, and stored in polyethylene bags under a nitrogen atmosphere at -20°C until required for further analysis.

Production of seaweed extracts

The crude seaweed extracts were generated using a pre-optimised liquid extraction method for 2h under continuous stirring. The seaweeds were extracted in four solvents of varying polarity including; acetone (99.8%), ethanol (96%), methanol (99.8%) and distilled deionized water. Approximately 1 g of the seaweed powder was extracted with 50mL of the respective solvent (1:50 w/v). The solution was filtered under vacuum with what man No. 1 filter paper to remove the solid particles. The solvent was removed via rotary evaporation, at temperatures no higher than 30°C, to yield dried extracts. The dried extracts were stored at -20°C for further analysis. All extractions were carried out in triplicate with the % yield for each extraction calculated.

Bacterial strains

The antibacterial activity of the seaweed extracts was evaluated using nine bacterial plant pathogens provided by DAFM. Three strains of Gram positive (*Clavibacter* species) and six strains of Gram negative (*Xanthomonas* species, *Ralsonia* spp. and *Erwinia amylovora*) bacteria were studied. Details of the growth conditions for each bacterial strain can be found in Table 2. The cultures were stocked in sterile broth containing 25% glycerol and stored at -20°C. A master stock of each bacteria was stored at -80°C.

Antibacterial assay

The disk diffusion assay was the test method used in this study and is the standard protocol recommended by the Clinical and Laboratory Standard Institute [46-50]. The dried extracts were re-dissolved in their respective solvent to give a concentration of 100mg/mL. Sterile paper disks (6mm) were loaded with 50µL of each extract at a rate of 10µL at a time to give a final concentration of 5mg/disk. Disks loaded with 50µL of the respective solvent served as the negative control, and disks containing 10µg/disk of chloramphenicol served as the positive control for all bacterial pathogens except for the *Clavibacter* species, where 10µg/disk of streptomycin was used instead. The disks were allowed to dry under sterile conditions.

A suspension of each bacterial culture was prepared according to the 0.5 MacFarland standard and was lawned on the specific agar as outlined in Table 2 to produce the bacteria field. The impregnated

Table 3: Extraction yields for the four seaweed species collected in June 2017, extracted using solvents of varying polarity for 2 h.

Solvent	Average % yield			
	F. serratus	*A. nodosum*	*P. lanosa*	*U. lactuca*
Water	31.57 ± 0.86[a]	27.32 ± 0.05[b]	16.58 ± 1.61[d]	19.61 ± 0.53[c]
Ethanol	11.35 ± 1.17[a]	11.32 ± 1.18[a]	-	-
Methanol	13.45 ± 2.11[b]	28.11 ± 1.45[a]	4.12 ± 0.74[c]	2.67 ± 0.52[c]
Acetone	6.34 ± 0.88[b]	11.20 ± 1.22[a]	-	-

Data (n=3) is presented as the mean ± SD; Data that do not share a common superscript are statistically different in terms of yield for that particular solvent depending on seaweed species (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test). Note; - = too small a yield to test.

disks were placed on the bacterial field by a sterile forceps. The plates were incubated under their optimum growth conditions (Table 2) and the zones of inhibition were measured as a clear zone of no bacterial growth around the disk after 24 h. The antibacterial activity of the seaweed extracts were tested in triplicate and repeated in duplicate.

Determination of Minimum Inhibitory Concentration (MIC)

The methanol extract of *P. lanosa* was found to be the most effective extract exhibiting strong antibacterial activity at 5mg/disk and, therefore. Had its MIC determined using the standard microbroth dilution assay as recommended by the CLSI [51-58] and Srikong et al. [59]. The MIC was determined for the bacterial pathogen *X. fragariae* as this was the bacteria that extracts of *P. lanosa* exhibited strong activity against. The extracts were initially sterilized through autoclaving and re-dissolved in their respective growth media for *X. fragariae* to give a final concentration of 10mg/200µL. For the MIC procedure, 200µL of the stock seaweed extract was added into a sterile 96 well micro titre plate. A series of twofold dilutions were performed on the seaweed extracts with their respective broth serving as the diluent. The resulting concentrations ranged from 5 mg per well to 0.156 mg per well. The negative control wells contained the respective broth and the positive control well contained 5µg/mL chloramphenicol solution.

The wells were inoculated with 100µL of the adjusted (0.5 MacFarland standard) bacteria, except for the extract controls, which contained only extract, and broth to ensure complete sterility of the seaweed extract had been achieved. The plates were incubated at 27˚C overnight. After the incubation period, the turbidity of each well was measured at 620 nm using a BioTek EL×800 Absorbance Microplate Reader (Biotek, VT, USA). The experiment was completed in triplicate, on three separate days. A reduction of more than 80% of bacterial growth was considered valid [60], and was calculated using equation 1.

Equation 1: $$\%\text{reduction}=\frac{Abs_{(bacteria)}-Abs_{(sample)}}{Abs_{(bacteria)}}\times 100\%$$

Determination of Minimum Bactericidal Concentration (MBC)

The MBC method used in this study was adapted from CLSI [61] and Indira et al. [62]. MBC was performed by spreading the contents of the wells from the MIC assays on fresh growth medium with incubation of the plates overnight. The concentration at which there was no visible bacterial growth (≥ 99%) after incubation was regarded as the MBC value.

Antibiofilm properties of the seaweed extracts

Biofilm prevention assay: The biofilm disruption assay is conceptually similar to the micro broth dilution assay described in the CLSI document M07-A9 with several modifications [58]. Dried methanol extracts of *P. lanosa* were autoclaved and dissolved in sterile broth (nutrient broth supplemented with 1% glucose) to a starting concentration of 50mg/mL. 100µL of the extract solution was added in triplicate to a 96-well micro titre plate. Serial twofold dilutions were then carried out on the extracts with sterile broth. 100µL of sterile broth served as the negative and media only controls. 100µL of 0.5mg/mL chloramphenicol solution was loaded and served as the positive control.

A 1% inoculation of *X. fragariae* was prepared in BHI and incubated overnight at 27˚C. The subsequent cells were washed in triplicate in MRD and adjusted to the 0.5 M McFarland Standard of 10^7-10^8 colony forming units per ml (CFU/mL) as described in section 2.4. The adjusted bacteria were diluted 1:100 in broth and 100µL was loaded into the extract containing wells to give a final concentration of 5 – 0.3125mg /200µL. The negative and positive control wells were made up to a final volume of 200µL.

Following overnight incubation, the supernatants were transferred to a new 96-well micro titre plate. The original wells were carefully washed in triplicate with Phosphate Buffered Saline (PBS) to remove any planktonic cells without affecting the biofilm formed at the bottom of the plate. This was followed by the addition of 110µL of MRD into the wells. The bacteria/biofilms in the wells were scraped into the MRD solution with the use of a 20 – 200µL pipette tip. Serial tenfold dilutions were performed on the extracts and negative control wells and were plated neat – 10-7 for the purpose of plate counts. Positive and media only controls were plated neat (without any dilutions). Plate counts for the supernatants were performed in the same manner. Biofilm prevention was calculated as a percentage against the negative control using equation 2. The assay was repeated in triplicate on three different days.

Equation 2: $$\%\text{prevention}=100-\left(\frac{sample\ cfu/ml}{negative\ control\ cfu/ml}\times 100\right)\%$$

Biofilm disruption assay: The biofilm disruption assay is also similar to the microbroth dilution assay described in the CLSI document M07-A9 with several modifications [58]. A 1% inoculation of *X. fragariae* was prepared in BHI and incubated overnight at 27˚C. The subsequent cells were washed in triplicate in MRD and adjusted to the 0.5 M McFarland Standard of 10^7-10^8 CFU/mL as described in section 2.4. A 1:100 dilution of the adjusted bacteria was prepared in nutrient broth supplemented with 1% glucose and 100µL of this bacterial stock was then loaded into a 96-well microtitre plate. Three rows remained empty and were loaded with 100µL of broth to serve as the media only controls. The microtitre plate was incubated for 48

Table 4: Antibacterial activity of crude *P. lanosa* extracts using the disk diffusion assay at 5mg/disk against nine bacterial plant pathogens.

Solvent	Antibacterial activity								
	X. arboricola	*X. Hyacinthi*	*E. amylovora*	*X. campestris*	*X. fragariae*	*C. nebraskensis*	*C. michiganensis*	*C. tessellarius*	*Ralstonia* sp.
Water	+++	-	+++	-	+++	++	-	-	-
Methanol	++++	+	-	+	++++	++++	++	+++	-
Positive control[a]	+++++	++++	+++++	+++++	++++	+++++	++++	++++	+++++
Negative control[b]	-	-	-	-	-	-	-	-	-

[a]Positive control was chloramphenicol antibiotic disk (10µg/disk) with streptomycin antibiotic disks (10µg/disk) used for the Clavibacter species; [b]Negative control was 50µL of the respective solvents; Inhibition zones are reported as clear zones (including 6mm diameter of blank disks); - indicates no activity; + indicates zone of inhibition of 6 – 8mm; ++ indicates zone of inhibition of 8.1 – 10mm; +++ indicates zone of inhibition of 10.1 – 13mm; ++++ indicates zone of inhibition of 13.1 – 16mm and +++++ indicates zone of inhibition of >16mm.

Table 5: Antibacterial activity of crude *A. nodosum* extracts using the disk diffusion assay at 5mg/disk against nine bacterial plant pathogens.

Solvent	Antibacterial activity								
	X. arboricola	*X. hyacinthi*	*E. amylovora*	*X. campestris*	*X. fragariae*	*C. nebraskensis*	*C. michiganensis*	*C. tessellarius*	*Ralstonia* sp.
Water	+	-	-	-	+++	-	-	+	+
Methanol	-	-	-	-	+	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-	-
Acetone	-	-	-	-	-	-	-	-	-
Positive control[a]	+++++	++++	+++++	+++++	+++++	++++	++++	+++++	++++
Negative control[b]	-	-	-	-	-	-	-	-	-

[a]Positive control was chloramphenicol antibiotic disk (10µg/disk) with streptomycin antibiotic disks (10µg/disk) used for the *Clavibacter* species; [b]Negative control was 50µLof the respective solvents; Inhibition zones are reported as clear zones (including 6mm diameter of blank disks); - indicates no activity; + indicates zone of inhibition of 6 – 8mm; ++ indicates zone of inhibition of 8.1 – 10mm; +++ indicates zone of inhibition of 10.1 – 13mm; ++++ indicates zone of inhibition of 13.1 – 16mm and +++++ indicates zone of inhibition of >16mm.

h at 27°C to allow the formation of a biofilm.

Following incubation, the old media was removed from the microtitre plate carefully without disrupting the biofilm formed and 100µL of fresh media was added. The treatments/samples were prepared by dissolving the autoclaved methanol extracts of *P. lanosa* to a starting concentration of 50mg/mL in broth. Serial twofold dilutions were then performed on these stock treatments in broth and 100µL of each sample dilution was loaded into three bacteria containing wells producing final extract concentrations of 5, 2.5, 1.25, 0.625 and 0.3125 mg/200µL. Bacteria wells loaded with 100µL of broth served as the negative control and bacteria wells loaded with 100µL of 0.5mg/mL chloramphenicol solution served as the positive control. Therefore, all wells contained a final volume of 200µL. The microtitre plate was incubated for a further 18-20 h at 27°C.

Subsequent to the incubation period, the supernatant containing media, samples and planktonic cells was removed and the wells were washed in triplicate in PBS and then loaded with 110µL of MRD. The bacteria/biofilm containing wells were then carefully scraped with the use of a 20-200µL pipette tip. Serial tenfold dilutions were carried out on the bacteria containing extracts and the negative control. These dilutions were then plated from neat (undiluted) to 107 to achieve plate counts. The positive control and media only controls were plated neat. Biofilm disruption was calculated as a percentage against the negative controls using equation 3 in order to determine the minimum biofilm eradication concentration (MBEC50 and MBEC90) which is the minimum concentration of seaweed extract capable of inhibiting the mature biofilm by 50 and 90% [63]. The method was performed in triplicate and repeated on three separate occasions.

Equation 3: $\%\text{distruption}=100-\left(\frac{sample\ cfu/ml}{negative\ control\ cfu/ml}\times 100\right)$

Statistical analysis

Results are presented as the mean ± Standard Deviation (SD) of three replicates. The statistical analysis was performed using Minitab 18 Statistical Software. Data obtained were analysed statistically to determine the degree of significance with repeated measures using one-way ANOVA followed by a post-hoc analysis using Tukey's multiple comparison tests at a 5% statistical significant level ($\rho<0.05$).

Results and Discussion

The extraction yields obtained for the four seaweed species are illustrated in Table 3 and, as expected, different solvents yielded different amounts of crude extracts with water achieving the highest yields. This was as a result of seaweeds consisting of a large quantity of polar-constituents including carbohydrates, which can account for almost 20% of the dry weight, followed by proteins and lipids [64,65].

These newly generated extracts were investigated for their antibacterial potential against bacterial plant pathogens using the disk diffusion assay at an extract concentration of 5mg/disk. From examination of the results, it was found that the seaweed extracts were effective at inhibiting the microbial growth of all nine tested bacteria with variable potency except for the green seaweed *U. lactuca*, which demonstrated no antibacterial activity against any of the tested pathogens. The red seaweed *P. lanosa* (Table 4) demonstrated the broadest range of activity. Hellio et al. [29] also reporting this activity with the dichloromethane fractions of *P. lanosa* producing inhibition against seven sensitive strains of marine bacteria at 24µg/mL. This was further supported by Shanab [66] who reported greater antibacterial

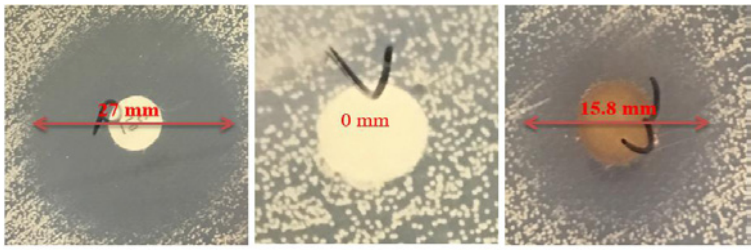

Figure 1: Antibacterial activity of crude methanol extracts of *P. lanosa* against X. aboricola using the disk diffusion assay at 5mg/disk. Positive control: 10µg/disk chloramphenicol; Negative control: 50µL of methanol.

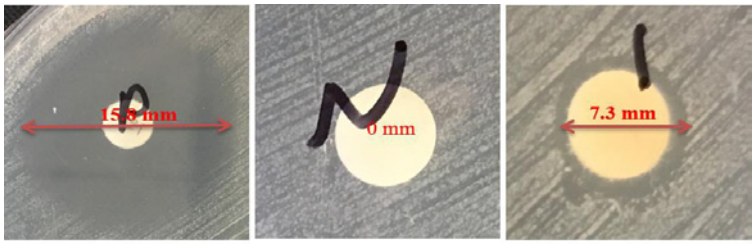

Figure 2: The antibacterial activity of crude water extracts of *F. serratus* against *X. fragariae* using the disk diffusion assay at 5mg/disk. Positive control: 10µg/disk chloramphenicol; Negative control: 50µL of water.

Table 6: Antibacterial activity of crude *F. serratus* extracts using the disk diffusion assay at 5mg/disk against nine bacterial plant pathogens.

Solvent	Antibacterial activity								
	X. arboricola	*X. hyacinthi*	*E. amylovora*	*X. campestris*	*X. fragariae*	*C. nebraskensis*	*C. michiganensis*	*C. tessellarius*	*Ralstonia* sp.
Water	-	-	-	-	+	-	-	-	-
Methanol	-	-	-	-	+	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-	-
Acetone	-	-	-	-	-	-	-	-	-
Positive control[a]	+++++	++++	+++++	+++++	+++++	++++	++++	+++++	++++
Negative control[b]	-	-	-	-	-	-	-	-	-

[a]Positive control was chloramphenicol antibiotic disk (10µg/disk) with streptomycin antibiotic disks (10µg/disk) used for the *Clavibacter* species; [b]Negative control was 50µL of the respective solvents; Inhibition zones are reported as clear zones (including 6mm diameter of blank disks); - indicates no activity; + indicates zone of inhibition of 6 – 8mm; ++ indicates zone of inhibition of 8.1 – 10mm; +++ indicates zone of inhibition of 10.1 – 13mm; ++++ indicates zone of inhibition of 13.1 – 16mm and +++++ indicates zone of inhibition of >16mm.

activity in red seaweed species compared to the brown seaweed S. *dentifolium* against human pathogens including *Bacillus subtilis*, *Staphylococcus albus*, *Streptococcus faecalis* and *Escherichia coli*.

The methanol extracts exhibited the strongest range of activity as shown in Figure 1 with over a 15mm zone of inhibition against *X. aboricola*, the causal agent of bacterial spot disease of stone fruit. This pathogen can have a significant economic impact with estimated crop losses over €10,000 per hectare in epidemic years on commercial plum orchards [67]. Methanol has been reported to be the most effective extraction solvent in other studies for extracting antibacterial compounds [23,68]. Kumar et al. [25] reported that methanol extracts exhibited the broadest range of activity in controlling the phtyopathogen *P. syringae* in the medicinal plant *Gymnema sylvestre*.

A. nodosum also produced viable activity with the water extracts exhibiting an inhibitory effect against a number of these problematic pathogens as presented in Table 5. The aqueous and methanolic extracts of *F. serratus* exhibited low inhibitory activity against *X. fragariae* (Table 6) with the water extract producing an inhibition zone of 7.33 ± 0.51mm as shown in Figure 2.

The most susceptible strain was *X. fragariae*, since the majority of seaweed extracts reduced the growth of this pathogen to some extent. A concentration study was conducted on the methanol extracts of *P. lanosa* against *X. fragariae* with a dose response effect observed as shown in Table 7 and Figure 3. Kolanjinathan et al. [32] also reported this dose response effect when the ethanol extracts of *Gracilaria edulis, Calorpha peltada* and *Hydroclothres* sp. were screened against six bacterial pathogens.

The MIC and MBC of the *P. lanosa* methanol extracts were evaluated against *X. fragariae* in order to determine its bacteriostatic and bactericidal properties. The inhibitory effect of *P. lanosa* extract against *X. fragariae* started at 1.25 mg per well (200µL) in a dose response manner, with the higher the extract concentration the higher the kill rate. The MIC value of the methanol extract of *P. lanosa* was calculated to be 6.25mg/mL. These results were in accordance with

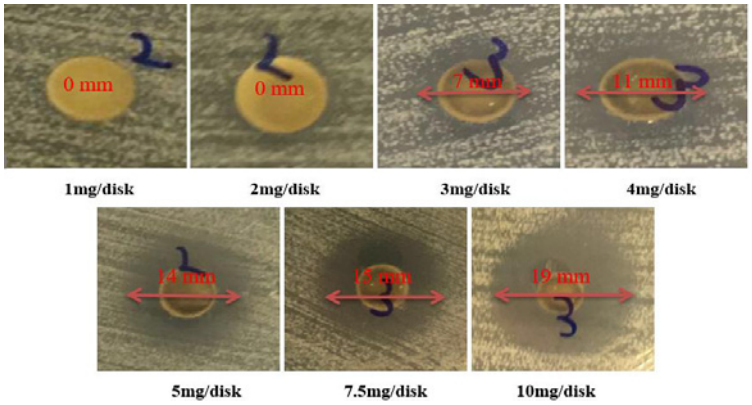

Figure 3: Dose response effect of crude methanol extracts of *P. lanosa* at 1, 2, 3, 4, 5, 7.5 and 10mg/disk against *X. fragariae*.

Table 7: Concentration study on the antibacterial activity of crude *P. lanosa* extracts using the disk diffusion assay at 1-10mg/disk against *X. fragariae*.

Extract	Antibacterial activity (mm)						
	1 mg/disk	2 mg/disk	3 mg/disk	4 mg/disk	5 mg/disk	7.5 mg/disk	10 mg/disk
P. lanosa extract	0[a]	0[a]	8.3 ± 1.2[b]	10.2 ± 0.4[bc]	14.3 ± 1.0[c]	15.7 ± 0.82[c]	18.3 ± 1.2[d]
Positive control[a]	28.5	28.5	29	29	29.5	28	28.5
Negative control[b]	0	0	0	0	0	0	0

[a]Positive control was chloramphenicol antibiotic disk (10µg/disk); [b]Negative control was 10, 20, 30, 40, 50µL of methanol respectively. Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different in terms of activity (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

those of Sasidharan et al. [69] who reported MIC values of 6.25 and 3.125mg/mL against Pseudomonas aeruginosa and Bacillus subtilis respectively, for the crude extracts from the red seaweed *Gracilaria changii*. Other studies have reported lower MIC values particularly from purified extracts. Purified fractions from the seaweed *Dictyota acutiloba* exhibited MIC values as low as 0.69-0.7µg/mL against MRSA, while the positive control methicillin (commercial antibiotic) showed an MIC value of 50µg/mL [70]. Purification of crude extracts typically has a positive effect on activity. This is not surprising since most of the non-active compounds have been removed including antagonistic compounds. Ha et al. [71] described the MIC values for crude ethanolic extracts of Ulva *pertusa* at 312µg/mL, whereas the nitrogenous compounds fractioned from the crude extracts were twice as potent with a MIC of 156µg/mL.

The MBC value of the methanol extracts against *X. fragariae* was found to be above the tested concentration range, with extract concentration above 5 mg per well or 25mg/mL required. Unfortunately solubility issues prevented the concentration of the methanol extract to be increased to this level. However, it is common to have a higher MBC compared to MIC, since a higher concentration of the antimicrobial compound is required to completely eliminate the bacteria [72]. Eom et al. [73] also found that their hexane, dichloromethane and butanol fractions generated from the brown seaweed *Eisania bicyclis* required concentrations above their MIC to achieve an MBC value against MRSA. Furthermore, these MIC and MBC concentrations can vary and depend on a number of factors including the specific antimicrobial compounds and the type of test bacteria used [72]. For instance, isolates of a particular species will have variable MIC's; sensitive strains producing low MICs, compared to the more resistant strains which will exhibit relatively higher MICs.

As mentioned previously, microbial biofilms are causing great concern in the management of bacterial and fungal infections due to their high resistance to antibacterial treatments. This resistance is as result of the planktonic bacterium irreversibly adhering to biotic or abiotic surfaces and the formation of three-dimensional extracellular matrices with regulated motility [74]. There are a number of studies which have noted the antibofilm potential of seaweed extracts against human biofilm-forming bacteria [75-77] and marine biofouling [78,79]. The vast majority of studies have been conducted on bacteria from marine environments [80-82] including bacteria symbionts extracted from the surface of seaweeds [83]. This is not surprising since bacteria produce secondary metabolites similar to seaweeds in response to external pressure including competition for nutrients or space [82] as demonstrated by coral associated bacteria against *Streptococcus pyogenes* biofilm formation [84].

Bacterial attachment and biofilm formation are regarded as critical steps in the establishment of biofilms. The crude *P. lanosa* extracts were initially assessed for their ability to inhibit biofilm formation of *X. fragariae* using the colony counting method. It was found that the crude extracts prevented biofilm formation in a dose response manner with 6.25mg/mL preventing over 80% biofilm formation (Table 8). Jun et al. [85] also reported antibiofilm prevention from marine algae against dental plaque, particularly the compound fucoidan isolated from *Fucus vesiculosus* with a concentration above 125µg/mL completely suppressing biofilm formation and planktonic cell growth. This demonstrated the high potency of purified compounds. Below the 6.25mg/mL concentration, a promotion in biofilm formation was noted. This phenomena was reported by Omwenga et al. [86] who found that the methanol extract of *Elaeodendron buchananii* and the aqueous ethanol extract of *Acacia gerrardii* did not exhibit any

Table 8: Antibiofilm activity of crude methanol extracts of *P. lanosa* against *X. fragariae*.

Extract concentration (mg/mL)	% Prevention	Supernatant	% Disruption
1.563	>88.2 ± 9.35[a]	>531.1 ± 6.03[e]	>116.0 ± 6.03[i]
3.125	>69.9 ± 10.00[b]	>110.9 ± 7.33[f]	78.2 ± 9.27[j]
6.25	98.5 ± 7.40[c]	92.7 ± 7.66[g]	100 ± 0[k]
12.5	98.7 ± 6.20[c]	99.9 ± 7.55[g]	100 ± 0[k]
25	100 ± 0[d]	100 ± 0[h]	100 ± 0[k]

Data (n=9) are presented as the mean ± SD; Data that do not share a common superscript for % prevention, supernatant or % disruption are statistically different in terms of activity (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test). Note: > indicates a promotion in growth.

antibiofilm activity but rather promoted biofilm formation. This was concluded to be as a result of the tested extracts not possessing any compounds capable of targeting and inhibiting biofilm formation such as phytochemical compounds including N-[4-(phenylamino) phenyl]-benzamide which has been reported to exhibit such activity [87]. This was not the case in this study, as up to a certain extract concentration antibofilm activity was observed. Therefore, our findings are suspected to be as result of the nutrients present in the extracts promoting the growth of the bacteria since seaweeds have been used for years as an organic fertiliser in agriculture with promotion in the growth of beneficial soil microbes reported [88-90]. A dose-response relationship categorised by opposing effects of low and high extract doses was also suspected and this is known as a hormetic response [91]. This biphasic dose-response relationship was reported by other recently published studies in which high concentrations of antibiotics eradicated bacteria, whilst at low concentrations, biofilm formation was encouraged [92,93]. Salta et al. [94] reported such a phenomenon for all three terrestrial natural products, including *Chondrus crispus* extracts, against marine biofilm forming bacteria at low extract concentrations.

The extract concentration required to inhibit biofilm growth was also found to be dose dependent with 6.25mg/mL the lowest concentration to achieve this 80% inhibition in the supernatant. This is technically the MIC against *X. fragariae* and from comparison of the MIC obtained using a colorimetric assay it can be seen that the same MIC values were recorded for both assays, demonstrating the accuracies of both methods.

For biofilm disruption assessment, the bacteria were incubated over a 48 h period to allow sufficient time for biofilm formation. The same concentrations of extract (25–1.5625mg/mL) were applied in this assay with antibiofilm disruption activity also found to be concentration dependent with 100% disruption observed above the concentration of 6.25mg/mL and falling to 78.32 ± 0.01% for 3.125mg/mL. Therefore, the MBEC50 and MBEC90 of *P. lanosa* was found to be 3.125mg/mL and 6.25mg/mL respectively. These results demonstrate the antibiofilm activity of the crude *P. lanosa* extracts against biofilms. This was a very promising result since mature biofilm communities are complex and once established are very difficult to eradicate [95]. For instance, Jun et al. [85] reported strong biofilm prevention from the compound fucoidan isolated from *F. vesiculosus* at 125µg/mL. But it was found that the fucoidan compound was unable to disrupt and eliminate the completed biofilm. A promotion in bacterial growth was also observed for the lowest tested concentration of 1.563mg/mL with such a promotion also suspected to be as a result of nutrient supplement and/or a hormesis response [96].

This study indicated the potent antibiofilm compounds present in the methanol extracts of *P. lanosa* against the plant pathogen, *X. fragariae*. This pathogen can enter the plant through penetration of the stomata into the interior air spaces of the mesophyll, where biofilms are produced consisting of a large volume of xanthans [97]. Infections in this manner results in plasmolysis and deformation of the plant cells making this international quarantine pathogen a considerable concern to strawberry nurseries and growers [98]. Strawberries are an economically and socially significant crop worldwide. In 2014, the United States produced three billion pounds of strawberries estimated at a value of $2.9 billion [99]. But *X. fragariae* has been reported to cause losses in yield from 8% up to 80% in North America [100]. Management of this pathogen in the field is typically through the foliar application of copper compounds such as copper sulphate but they have to be applied at near phytotoxic levels due to bacterial resistance [98]. Therefore, new alternatives are required to ensure future success in the management of this disease in strawberries, further demonstrating the importance of the antibacterial activity of these crude extracts.

The pesticidal activity of the *P. lanosa* extracts against a strawberry pathogen was not totally surprising since seaweed extracts had been previously applied in strawberry management concerning both their bio-stimulant effects and antimicrobial properties [101]. Although studies have demonstrated the strong antifungal potential of seaweed extracts against common fungal pathogens effecting strawberries [102,103] no such study has been found on the use of *P. lanosa* extracts against *X. fragariae* adding to the novelty of this result.

Conclusion

In the present study, the antibacterial activity of four seaweed species were evaluated against quarantine plant pathogens. The crude seaweed extracts were shown to contain potent antimicrobial compounds. Most notably the methanol extract isolated from the red seaweed, *P. lanosa* demonstrated an MIC of 6.25mg/mL against the Gram negative *X. fragariae*. The same concentration of crude extract suppressed its biofilm formation by over 80% with the MBEC50 and MBEC90 found to be 3.125mg/mL and 6.25mg/mL respectively, suggesting that the methanol extract of *P. lanosa* might be a potential antibiofilm agent capable of inhibiting biofilm formation and/or disruption of an already established biofilm for this problematic phytopathogen.

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