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Antimicrobial Properties of *Fucus Vesiculosus* and *Porphyra Dioica* Collected From the Irish Coast.

Cover Page Footnote

Special thanks to Annabel Higgins Hoare and Emma O'Keefe, both PhD students, who helped me around the completion of this project. I would like to thank you Waterford Institute of Technology for allowing me to complete this project on seaweeds of my selection.

Antimicrobial Properties of *Fucus Vesiculosus* and *Porphyra Dioica* collected from the Irish Coast.

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Abstract

Background

Algae play an important role in entire ecosystem and have could play an important potential role in the search for biologically active compounds with miscellaneous properties; such as antioxidant, antiviral and antimicrobial. The objective of this research was to study the antimicrobial properties of two seaweeds, *Fucus vesiculosus* and *Porphyra dioica*.

Methods

Water content analysis was performed on both seaweeds and each seaweed was determined to have a high water content (approximately 80%), with higher water content found in *Porphyra dioica* compared to *Fucus vesiculosus*. Extraction of active metabolites was performed in solvents with various polarities (diethyl ether, methanol and water) at a concentration of 1/100 w/v. *Porphyra dioica* also additionally extracted in ethyl acetate and a methanol:water mixture at concentrations 1/100 w/v and 1/30 w/v, respectively.

Results

Seaweed crude extracts were tested against one active pathogenic clinical strain of Methicillin Resistant *Staphylococcus aureus* (MRSA; WIT-676) obtained from University Hospital Waterford. The effect of various extracts concentrations (1/40, 1/60, 1/80 and 1/100 w/v in methanol) was examined using extracts of *Fucus vesiculosus*, followed by antimicrobial screening against the same pathogen to determine the optimal concentration of active metabolites in relation to the screening and which would then require further separation.

Conclusions

The highest antimicrobial activity from an extract of *Porphyra dioica* was found in ethyl acetate and the solvent mixture methanol:water (1:1) compared with *Fucus vesiculosus*, which exhibited the highest antimicrobial activity in water and methanol extracts. Further analysis for separation and characterisation of the crude extracts would be required for future use of those extracts as antimicrobials.

Keywords: Seaweed, Antimicrobial, MRSA, bioactive extraction.

1. Introduction

The Earth's surface is covered by 71% water and provides various important resources (Visbeck, 2018). Various types of algae play an important role in the entire ecosystem and they have a potential role in the continuous search for biologically active compounds with various bioactivities, including antimicrobial properties (Scheuer, 1990). In recent times, the misuse of β -lactam antibiotics supported the development of bacterial antibiotic resistance to them (Kong, et al., 2010). Increasing resistance of methicillin resistance *Staphylococcus aureus* (MRSA) to β -lactam antibiotics lead to the need to investigate the antimicrobial properties of various alternative natural sources. Marine sources, especially algae, appear as a potential alternative and valuable rich source of primary and secondary metabolites (Mostafa, 2012). The selection of algae species in nature is limited by factors, such as temperature, seabed condition, pH of water, salinity, life stage and reproductive state and age of seaweed. Seasonality and geographic location also play an important role in the production of a variety of bioactive compounds (Pérez, et al., 2016).

Red seaweeds (*Rhodophyta*) contain a higher diversity of secondary metabolites compared to brown seaweeds (*Phaeophyta*) and green seaweeds (*Chlorophyta*). Red algae provide the highest proportion of secondary metabolites, which are biologically active molecules with identified miscellaneous therapeutic properties (Kasanah & et al 2, 2015). Three of the most common types of red seaweeds; *Gracilaria vermiculophylla*, *Porphyra dioica* and *Chondrus crispus*, have been extensively studied and we observed to possess antimicrobial activity against multiple pathogens when cultured in both the wild and in an integrated multi-tropic aquaculture system (Mendes, et al., 2013). Compounds found in seaweeds include; phenolic compounds possessing complex polymer structures or simple molecules dependant on the type, carrageenans, which are one of the main compounds contained in red seaweeds cell walls, galactans, laminarans, fucoidans and many others (Pérez, et al., 2016), (Cardoso, et al., 2014). Laminarin, which can be found mostly in brown algae, is one of the main polysaccharides (Chojnacka, et al., 2012). Dieckol was identified as the main compound in *Ecklonia stolonifera* possessing antimicrobial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) (Dae-Sung Lee, Min-Seung Kang, Hye-Jin Hwang, 2008). Recent studies showed that the average dry mass of *Fucus vesiculosus* contains 47.8% carbohydrates; comprising 5.9% laminarin, 12.3% mannitol, 14.4% alginate, 12.4% fucoidan, 2.8% cellulose, 10.5% polyphenols, 17.5% minerals, 10% proteins, 4.8% lipids and 9.4 % other constituents (Hahn, et al., 2012), (Obluchinskaya, et al., 2002). Previous investigations into the antimicrobial activity of *Fucus vesiculosus* concluded that polyhydroxylated fucophloethol was responsible for the observed antimicrobial activity against Gram-positive and Gram-negative bacteria (Sansdalen, et al., 2003). Extracts from *Stocheospermum marginatum*, *Padina tetrastromatica*, and *Grateloupia lithophila* showed strong and moderate susceptibility against multidrug resistance strains (Mnikandan, et al., 2011). Furthermore, 44 different types of seaweeds belonging to red, brown and green algae were screened for antimicrobial activity in the Canary Islands (González del Val, et al., 2001). The diversity of bioactive molecules depends on many factors, including both natural factors and chemical factors such as species tested, temperature conditions, solvent used for extraction and time of the year of harvesting (Pérez, et al., 2016) (Salvador N., et al.1, 2007). It was suggested in a study carried out by (Deveau, et al., 2016) conducted on *Ulva lactuca* for antimicrobial activity with findings from a study by (Tan, et al., 2012), showing that antimicrobial activity of *Ulva lactuca*, could be unique to *Staphylococci* strains.

The present study is focused on the determination of the antimicrobial activity of methanol, water, ethyl acetate and diethyl ether extracts from two different algae *Fucus vesiculosus* (brown alga) and *Porphyra dioica* (red alga), which could be used in the potential development

of a novel antimicrobial seaweed wound dressing. Antimicrobial dressings are commonly used for infected MRSA wounds treatment (Tan, et al., 2012). *Fucus vesiculosus* has had previously confirmed antimicrobial properties, but *Porphyra dioica* was investigated for the first time in this Institute in order to elicit its antimicrobial potential.

2. Materials and Methods

Material preparation

Fucus vesiculosus and *Porphyra dioica* were handpicked in the intertidal zone, during low tide in Tramore, Co. Waterford, Ireland (Latitude 52.15947, longitude -7.14889) in the middle of January, with water temperature around 10 °C. Healthy seaweeds were carefully plucked, washed in salt water and placed into the cooler box for transportation to prevent temperature stress and desiccation. The seaweeds were collected from a variety of rocks to obtain a representative sample from the whole site of collection. Once in the lab, the collected seaweeds were washed in distilled deionised water to remove necrotic parts, epiphytes and sand. Samples were frozen overnight at -20 °C and subsequently freeze-dried in freeze-drier (FreeZone 2.5). Those samples were prepared (blended and sieved) and stored under nitrogen in plastic bags for further analysis. Blending was done in common kitchen blender. Each seaweed was blended maximally for 10 s repeatedly to avoid heat production and sieved in 850µm sieve size.

Water content analysis

After samples preparation water content analysis was carried out on both seaweeds, *Porphyra Dioica* and *Fucus vesiculosus*. In brief, 5 g of each fresh sample was weighed on a top pan balance (Pioneer-Ohaus) and samples were allowed to dry in an oven (Memmert –Germany) at 100 °C for 5 days, followed by cooling to room temperature in a desiccator for 3 h. Each seaweed analysis was carried out in triplicate. Water content was expressed as a percentage and was calculated according Equation 1:

$$Mn = \frac{(Mw - Md)}{Mw} \times 100 \quad \text{Equation 1}$$

where Mw is the mass of wet sample, Md is mass of dry sample and Mn is moisture content expressed as a percentage.

Extractions

The prepared *Fucus vesiculosus* and *Porphyra dioica* according were extracted in three different solvents of differing polarities at room temperature in a ratio of 1:100 w/v. *Porphyra dioica* was separately extracted in ethyl acetate of same concentration also. The method used for extraction was developed in WIT by Tan and co-workers (Tan, et al., 2012). All solvents used during extractions were of HPLC grade and were as follows; methanol (log P= -0.764; 99.5% Fischer Scientific), water (log P= -1.380; 99.9% Honeywell), diethyl-ether (log P=0.870; 99.5% Honeywell) and ethyl acetate (log P= 0.70, 99.5% Honeywell). Solvents were carefully chosen based on prior research (Tan, et al., 2012), (Rajauria, et al., 2012), (Mendes, et al., 2013), (Moubayed, et al., 2016), (Tuney, et al., 2006).

Soxhlet extraction was performed only on the *Porphyra dioica* seaweed sample according to the method of Mendes and colleagues (Mendes, et al., 2013) with some method modifications and due to the different capacity of glassware for Soxhlet apparatus. In brief, 5 g of seaweed was extracted in a mixture of 132 ml polar solvents methanol:water HPLC grade (1:1; 99.5% Fischer Scientific, 99.9% Honeywell). The sample was placed in the paper thimble and extracted under Soxhlet apparatus for 2 h, in triplicate.

An extra experiment on the effect of the solvent during extraction on antimicrobial activity was carried out, but only with one seaweed species *Fucus vesiculosus*. This brown seaweed was extracted at four different concentrations of 1:100 w/v, 1:80 w/v, 1:60 w/v and 1:40 w/v in methanol only, at room temperature with continuous stirring for 2 h.

Preparation of algae extracts for antibacterial screening

Methanol and diethyl ether extracts were separated by vacuum Buchner filtration and using the vacuum rotary evaporator, at low temperatures with a maximum temperature of 40 °C. Water extracts, due to their viscosity, were separated by centrifugation at 1494xg (4500 rpm) for 4 min. Water was removed from the sample by freeze-drying. Samples were frozen at -20 °C after separation until further use.

Yield of extraction

Dried extracts of each extraction solvent were re-dissolved in < 8 ml of same extraction solvent. Crude re-dissolved extracts were transferred to the pre-weighed glass bottles with aluminium foil lids followed by drying under nitrogen gas to the dry state and reweighed to obtain the yield of extraction in grams and percentage as shown in Equation 2:

$$Y_E = M_E - M_b \quad \text{Equation 2}$$

where Y_E is the yield of extraction (g), M_E is the mass of the sample bottle plus sample, and M_b is the mass of the sample bottle.

The percentage yield was obtained according to the equation 3:

$$\% \text{ yield} = \frac{Y_E}{\text{actual sample mass}} \quad \text{Equation 3}$$

Dried and re-weighed samples were stored under nitrogen at -20 °C for further analysis.

Preparation of discs and antibacterial screening of crude extract against pathogenic strain

Dried extracts from both seaweeds were aseptically dissolved in the solvents of their extraction at a concentration of 100 mg/ml. All antimicrobial screening discs were loaded with five consecutive aliquots of 10 µL. Negative control discs were loaded with 50 µL of each extraction solvent used for re-dissolving the sample. The final concentration of each disc was 0.1 mg/µL of crude extract. As a positive control, chloramphenicol discs (final concentration of 10 µg) were used. The bacterial pathogenic strain WIT 676 was aseptically inoculated from the glycerol stock (40 %v/v), stored at -20 °C at a concentration of 1:100 w/v and the antimicrobial activity was assessed using the Bauer-Kirby disk diffusion method.

Preparation of Mueller Hinton Agar Plates

Mueller-Hinton Agar (MHA) plates were prepared according to the standard procedure by dissolving 38 g of MHA powder in 1 l of deionised water. The quantity was scaled down and 11.4 g of MHA (LabaNEOGEN) was dissolved in 300 ml deionised water. The mixture was heated up slowly, with frequent agitation, and then boiled for 1 min to completely dissolve all solid components of powder. The conical flask, with dissolved MHA, was autoclaved at 121 °C, 1.5 bar, for 15 min followed by cooling down to 45 °C. The amount of MHA was measured using 20 ml sterile container to give each plate the same depth of 4mm. Plates were poured aseptically on a horizontal surface to give uniformity and were allowed to solidify at room temperature. Unused plates were stored in the fridge at -4 °C until the next day of analysis.

Antimicrobial Activity of Crude Extracts against MRSA Pathogen Strain

The antimicrobial activity of the crude extracts was tested against one clinical pathogenic strain, *WIT 676*, using the disc diffusion method. During analysis, standard size 6 mm paper discs were used throughout. All discs were dry and loaded with 5 mg/ml concentration, except the *Porphyra dioica* diethyl ether crude extract, which was loaded with 3 mg/ml due to insufficient yield obtained.

WIT-676 clinical pathogenic strain was aseptically inoculated from the glycerol stock (60% solution of sterile broth and 40% glycerol) at a concentration of 1:100 v/v in Brain Heart Infusion broth (BHI) and the inoculated broth was allowed to grow overnight at 37 °C. After overnight night incubation, 1 ml of broth with culture was centrifuged for 2 min at 4482 xg (13, 000 rpm) to produce a solid cell pellet. The supernatant was aseptically removed, and the cell pellet was carefully re-dissolved in 1 ml of sterile Maximum Recovery Diluent (MRD) to wash the cells and purify them without causing cell death. The washing procedure was repeated twice more to ensure that the cells were pure. Optical density (at 625nm) of MRSA bacteria was assessed using UV/VIS spectroscopy and was adjusted to between 0.10 - 0.12 OD by MRD dilution for all samples tested. Studies examining the bacterial standard OD and colony forming unit growth curves were previously carried out in WIT (Tan, 2013)

Sterile swabs were used to spread out evenly the pathogenic strain onto MHA plates pre-prepared, by swabbing gently the surface of the plates with culture in one direction following by rotation of the plate about 90 ° with even swabbing in this direction and rotating plate again about 45 °. Pre-prepared 5 mg/ml disks, as well as positive and negative controls were aseptically transferred to the swabbed plates and allowed to equilibrate at room temperature for 1 h to allow continuous diffusion of the crude extract from the discs to the agar. Antimicrobial activity was screened with all preloaded discs using the common chloramphenicol disc as the positive control. The negative control was a disc preloaded with the extraction solvent to show that solvent had no effect on antimicrobial activity. After 1 h of equilibration, plates were incubated at 37 °C in an inverted position for 18 h. After incubation the zone of inhibition was measured by ruler, with precision of millimetres. All sample extracts were tested in duplicate.

3. Results

Water content analysis

Each seaweed analysis was carried out in triplicate to obtain the mean standard deviation of water content and confirm the consistency of the seaweed sample analysed. Results are shown in Table 1. Both seaweeds exhibited high water content around 80 %; but samples of *Porphyra dioica* exhibited a higher water content compare to *Fucus Vesiculosus*, due uniformity of the seaweed.

Table 1. Water content analysis results of *Porphyra dioica* and *Fucus vesiculosus* (n = 3)

Name / sample no.	Wet sample mass (g)	Dry sample mass (g)	Water content (%)	Mean water content (%), \pm SD (%)
<i>Porphyra dioica</i>				
Sample 1	5.15	1.18	77.08	80.05 \pm 2.88
Sample 2	5.36	1.06	80.22	
Sample 3	5.36	0.92	82.84	
<i>Fucus vesiculosus</i>				
Sample 1	5.19	0.97	81.31	77.66 \pm 4.07
Sample 2	5.28	1.14	78.41	
Sample 3	5.20	1.39	73.27	

Extraction Yields

Extraction yields from both seaweeds, *Porphyra dioica* and *Fucus vesiculosus*, were obtained from dry blended seaweeds using a variety of organic solvents. The presence of unknown diverse metabolites in both types of seaweeds with different polarities raised the opportunity to explore the effect of the solvent on extraction yield (Cunha & Grenha, 2015), (Kasanah, et al., 2015). The final extraction yields were expressed in milligrams and as a percentage (see **Error! Reference source not found.**). The yields obtained were relevant to discs preparation for the antimicrobial screening. Only *Porphyra dioica* was extracted in ethyl acetate (99.9% Macron, log P=0.711) at room temperature. The same seaweed was also extracted in methanol:water (1:1) by solvent extraction at room temperature at a concentration of 1:100 w/v and a 2 h Soxhlet extraction at a concentration of approximately 1:30 w/v due to the promising antimicrobial results of this type of seaweed in this solvent and solvent mixture (see Table 3).

The highest yield from both seaweeds was obtained from the water-based extracts. The difference between water and methanol extracts from *Porphyra dioica* was not significant (water extract yield = 428.20 mg \pm 79.13 mg, methanolic extract yield = 383.30mg \pm 16.5 mg compared to *Fucus vesiculosus*, where the water extract was hugely predominant compared to other extraction yields of the other solvents used (water extract yield = 838.00 mg \pm 62.80 mg, methanolic extract yield = 351.00 mg \pm 35.25 mg). Solvents with higher log P values and lower polarity had a negative effect on extraction yields obtained. The lowest yield of crude extract was acquired from the *Porphyra dioica* diethyl ether extract. The lowest yield obtained from *Fucus vesiculosus* was also from the same solvent, but compared to *Porphyra dioica* the yield was 20 times higher (104.70 mg \pm 17.60 mg compare 4.09 mg \pm 0.36 mg; log P=0.870).

Yields obtained from the *Porphyra dioica* water:methanol (1:1) extract was very difficult to compare, because different concentrations were used in both extractions. The average percentage yield from the Soxhlet extraction obtained was actually lower (572.4 mg \pm 97.62 mg) than the room temperature extraction (303.60 mg \pm 29.00 mg) compared to the amount of sample used.

Table 2. Extraction yields from *Porphyra dioica* and *Fucus vesiculosus* obtained from room temperature extraction by various solvents of different polarities.

<i>Porphyra dioica</i> – extractions (room temp.)				<i>Fucus vesiculosus</i> – extractions (room temp.)								
Solvent	Sample no.	Sample mass (g)	Yield (mg)	Yield mean (mg) \pm SD (mg)	Yield (%)	Mean (%)	Sample no.	Sample mass (g)	Yield (mg)	Yield mean (mg) \pm SD (mg)	Yield (%)	Mean (%)
Diethyl ether	Sample 1	2.5158	4.50		1.79		Sample 1	2.5100	124.30		4.95	
	Sample 2	2.5656	3.82	4.09 \pm 0.36	1.49	1.61	Sample 2	2.5609	99.70	104.70 \pm 17.60	3.89	4.14
	Sample 3	2.5700	3.95		1.54		Sample 3	2.5318	90.20		3.56	
Ethyl acetate	Sample 1	2.5077	256.80		10.20		Sample 1	-				
	Sample 2	2.5304	259.10	252.46 \pm 9.56	10.20	10.01	Sample 2	-				
	Sample 3	2.5070	241.50		9.63		Sample 3	-				
MeOH	Sample 1	2.5900	382.80		14.78		Sample 1	2.5708	312.80		12.17	
	Sample 2	2.5288	399.80	383.30 \pm 16.50	15.81	15.08	Sample 2	2.5488	357.80	351.00 \pm 35.25	14.06	15.08
	Sample 3	2.5051	366.80		14.64		Sample 3	2.5113	382.30		15.22	
Water	Sample 1	2.5040	347.10		13.86		Sample 1	2.5366	859.20		33.87	
	Sample 2	2.5551	505.20	428.20 \pm 79.13	19.77	16.82	Sample 2	2.5187	767.30	838.00 \pm 62.80	30.46	32.97
	Sample 3	2.5260	432.30		16.82		Sample 3	2.5659	887.40		34.58	

Legend - Only *Porphyra dioica* was extracted in ethyl acetate solvent.

Table 3. Methanol / Water extractions at room temperature and Soxhlet extractions carried out on *Porphyra dioica* (n=3).

<i>Porphyra dioica</i> - methanol/water extraction						
Solvent	Sample no.	Sample mass (g)	Yield (mg)	Yield mean (mg) ± SD (mg)	Yield (%)	Mean (%)
Room temp. extraction	<i>Sample 1</i>	2.5183	308.00	303.60 ± 29.00	12.23	11.90
	<i>Sample 2</i>	2.5873	330.10		12.76	
	<i>Sample 3</i>	2.5430	272.60		10.72	
Soxhlet extraction	<i>Sample 1</i>	5.0253	475.90	572.40 ± 97.62	9.47	11.35
	<i>Sample 2</i>	5.0047	570.02		11.39	
	<i>Sample 3</i>	5.0866	671.10		13.19	

Antimicrobial Activity of Crude Extracts against MRSA Clinical Pathogen Strain

Antimicrobial activity was tested against one clinical pathogenic strain, WIT 676, using the disk diffusion method. During analysis standard size 6 mm paper discs were used throughout. All discs were dry and loaded with 5 mg/ml concentration, except the *Porphyra dioica* diethyl ether crude extract, which was loaded with 3 mg/ml due to insufficient yield obtained. Bacterial cell concentration was estimated by measuring cell turbidity at OD₆₂₅, previous studies for bacterial standard OD and colony forming unit growth curves were previously carried out in WIT (Tan, 2013). Antimicrobial activity was screened with all preloaded discs using the common chloramphenicol disc as the positive control. The negative control was a disc preloaded with the extraction solvent to show that solvent had no effect on antimicrobial activity.

The results obtained from antimicrobial screening against the clinical pathogen strain WIT 676 displayed various results from the crude extracts (see Table 4). *Porphyra dioica* ethyl acetate crude extracts was the only solvent extract that exhibited antimicrobial activity at room temperature. The mean zone of inhibition measured was 8 mm. The solvent mixture water:methanol (1:1) crude extracts exhibited a zone of inhibition of 9 mm at room temperature. The same mixture of solvents used in Soxhlet extraction showed a higher zone of inhibition of 13 mm. It needs to be stated that concentration during the extraction techniques used was much higher, approximately 1:30 w/v, compared to the room temperature extraction concentration of 1:100 w/v. The Soxhlet extraction was carried out according to the method of Mendes *et al.* (Mendes, et al., 2013) with modifications due to the different capacity of glassware for Soxhlet apparatus. The yield of crude extract obtained from the Soxhlet extraction (1:25 w/v) was actually lower than the yield obtained from the room temperature extraction (1:100 w/v) followed by the method developed in WIT by (Tan, et al., 2012), but antimicrobial activity of the crude extract from Soxhlet extraction was higher when the discs from both crude extracts were loaded with the same concentration of 5 mg/ml of crude extract per disc.

Table 4: Antimicrobial activity of crude seaweeds extracts of different organic solvents against WIT 676 MRSA pathogen strain (n=6). Inhibition zones are reported as a clear zone (including 6 mm in diameter discs). (a) Positive control Chloramphenicol antibiotic discs 10 µg and (b) negative control was 50 µg/ml of appropriate solvent loaded onto disc.

Solvent	Yield mean (mg)	Test 1 – zone of inhibition (mm)/ ± SD	Test 2– zone of inhibition (mm) /± SD (mm)	Positive Control ^a (mm)	Negative Control ^b (mm)
<i>Porphyra dioica</i>					
Diethyl ether	4.09	-	-	-	-
Ethyl acetate	252.46	7.67 ± 0.58	8.33 ± 0.58	29	-
Methanol	383.30	-	-	-	-
Water	428.20	-	-	-	-
<i>Fucus vesiculosus</i>					
Diethyl ether	104.70	-	-	-	-
Methanol	351.00	8.33 ± 0.58	7.67 ± 0.58	30	-
Water	838.00	8.00 ± 1.00	8.67 ± 0.58	33	-
<i>Porphyra dioica</i>					
Water/MeOH Room temp.	303.60	9.33 ± 0.58	-	30	-
Water/MeOH Soxhlet	572.40	13.00 ± 1.00	12.33 ± 2.52	30	-

The *Fucus vesiculosus* methanol and water crude extracts exhibited the highest antimicrobial activity against the MRSA pathogen strain tested. Both extracts showed activity with mean zones of inhibition of 8 mm, including the standard 6 mm disc. The water extract yield was much higher compare to the methanol yield, which could support the hypothesis that more unwanted materials were present in the water extracts compared to the methanol extracts.

Effect of Solvent during Extraction on Antimicrobial Activity

A variety of extraction methods were carried at room temperature with various volumes of solvent present during extraction at concentrations of 1:40, 1:60, 1:80 and 1: 100 w/v. The experiments were performed using the seaweed *Fucus vesiculosus* and the extraction solvent was methanol. All methods and techniques, including antimicrobial screening, were performed using the same disc concentrations. The lowest yield of extraction was obtained at a concentration of 1:40 w/v and the highest yield of extraction was obtained at a concentration of 1:100 w/v. This was not an unexpected result, previous literature indicated the lack of inhibitory activity against some pathogenic strains (Tan, et al., 2012). For antimicrobial screening, the highest zone of inhibition was shown by extracts with the lowest amount of solvent present during extraction. Increasing the amount of solvent during extraction had a negative effect on antimicrobial activity and the zone of inhibition became smaller (see Table).

Table 5. Antimicrobial activity of *Fucus vesiculosus* after extraction of seaweed in different amounts of solvent, but discs loaded with same concentration of 5 mg per disc of extracts (n=6). Inhibition zones are reported as a clear zone (including 6 mm in diameter discs). (a) Positive control Chloramphenicol antibiotic discs 10 µg/ml and (b) Negative control was 50 µg/ml of appropriate solvent loaded on the plane disc.

<i>Fucus Vesiculosus</i>					
Concentr. (w/v) - methanol	Mean Yield (mg)	Test 1 – zone of inhibition (mm) / ± SD (mm)	Test 2– zone of inhibition (mm)/ ± SD (mm)	Positive Control ^a (mm)	Negative Control ^b (mm)
1/40	227.06	12.00 ± 0.00	12.33 ± 0.58	27.5	-
1/60	228.13	10.00 ± 0.00	9.00 ± 1.00	27	-
1/80	312.60	9.70 ± 0.58	10.00 ± 0.00	28	-
1/100	383.30	8.33 ± 0.58	7.67 ± 0.58	30	-

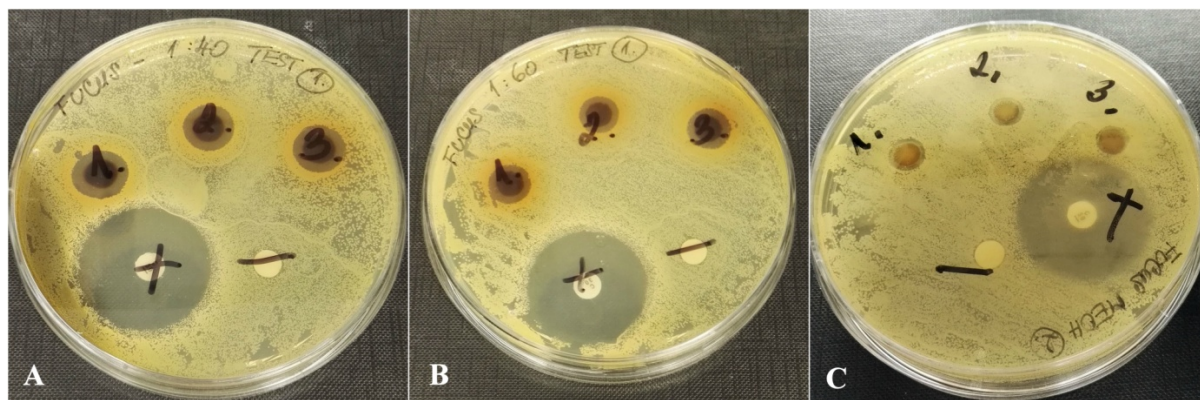


Figure 1. (A) *Fucus vesiculosus* showing highest zone of inhibition 12 mm in concentrated extracts 1:40 (w/v), (B) *Fucus vesiculosus* zone of inhibition 10 mm at a concentration of extract 1:60 (w/w) and (C) *Fucus vesiculosus* zone of inhibition 8 mm at a concentration 1:100 (w/v). All discs were loaded with 5 mg/ml of each extract after re-dissolution at a concentration of 100mg/ ml. Positive control was Chloramphenicol antibiotic discs 10 µg/ml and negative control was 50 µg/ml of the appropriate solvent loaded on the disc.

4. Discussion

The main objective of this study was to evaluate the antimicrobial activity of two different seaweeds *Porphyra dioica* and *Fucus vesiculosus* collected on the Irish coast against the pathogen strain WIT 676. Water content analysis was performed on both seaweeds to determine the percentage of water present in both seaweeds. Both seaweeds exhibited high water content around 80 %, with results for *Porphyra dioica* 80.05 ± 2.88 % and *Fucus vesiculosus* 77.66 ± 4.07 %. *Porphyra dioica* exhibited higher consistency after drying compared to *Fucus vesiculosus*. It was due to higher variability of seaweed structure at *Fucus vesiculosus*, which contains bladders and harder stipes compared to *Porphyra dioica*. Water content analyses were carried out to determine the actual % of water, which could influence further antimicrobial analysis.

Extraction, selection of solvent and extraction technique played crucial role for further antimicrobial analysis. The lowest yield of crude extract was acquired from the *Porphyra dioica* diethyl ether extract. The lowest yield obtained from *Fucus vesiculosus* was also from the same solvent, but compared to *Porphyra dioica* the yield was 20 times higher ($104.70 \text{ mg} \pm 17.60 \text{ mg}$ compared to $4.09 \text{ mg} \pm 0.36 \text{ mg}$; $\log P=0.870$), which contradicts previous reports (Tuney, et al., 2006). The highest yield from both seaweeds was obtained from the water-based extractions. The difference between the water and methanol extracts from *Porphyra dioica* was not significant (water extract yield = $428.20 \text{ mg} \pm 79.13 \text{ mg}$, methanolic extract yield = $383.30 \text{ mg} \pm 16.5 \text{ mg}$) compared to *Fucus vesiculosus*, where the water extract was hugely predominant compared to other extraction yields of the other solvents used (water extract yield = $838.00 \text{ mg} \pm 62.80 \text{ mg}$, methanolic extract yield = $351.00 \text{ mg} \pm 35.25 \text{ mg}$). Solvents with higher p log values and lower polarity had a negative effect on extraction yields obtained. Only *Porphyra dioica* water:methanol (1:1) extraction, was performed by two different types of extractions. The average percentage yield from the Soxhlet extraction obtained was lower (572.4 mg or 11.39%) than the room temperature extraction (303.60 mg or 11.90%) compared to the amount of initial sample. Usually increased temperature and pressure should have an increasing impact on the yield obtained (Foon, et al., 2013), but it could also have a negative impact on compounds with antimicrobial activity, which may not be thermally stable and increase the quantity of unwanted components extracted.

It must be stated that the amount of extracted material has no direct correlation to the antimicrobial activity of certain extracts to the MRSA pathogenic strain. Crude extracts contain a variety of unknown compounds and the lowest yield obtained could contain an unknown compound or a mixture of compounds responsible for antimicrobial activity against the MRSA clinical pathogen strain WIT-676. In previous studies, antimicrobial testing from various seaweeds was performed to find the highest zone of inhibition for dissimilar pathogens including MRSA by various research groups (Dae-Sung Lee, Min-Seung Kang, Hye-Jin Hwang, 2008) (Moubayed, et al., 2017) (Tan, 2013).

Extraction concentrations and methods used vary also. The initial extraction technique and concentration used in this study were solvent based extraction at 1:100 w/v developed by (Tan, et al., 2012), which was a high dilution compared to the 1:2 w/v (Mendes, et al., 2013), 1:20 w/v (Sameeh, et al., 2016) or 1:40 w/v (Ibtissam, et al., 2009). *Fucus vesiculosus* demonstrated a relatively high antimicrobial activity in water and methanol extracts against the pathogenic strain WIT-676, with a mean inhibition zone of 8 mm for both extracts. Both high polar solvents with low log p values gives a brief indication that the compounds responsible for antimicrobial activity would be polar compounds.

Previously the antimicrobial activity of *Fucus vesiculosus* was attributed to polyhydroxylated fucophlorethol, which possesses antimicrobial activity against Gram-positive and Gram-negative bacteria (Sansdalen, et al., 2003). Dae-Sung and co-workers (Dae-Sung Lee, Min-Seung Kang, Hye-Jin Hwang, 2008) identified a compound from *Ecklonia stolonifera* responsible for antimicrobial activity as dieckol, which is a highly polar molecule also. Further research demonstrated that phlorotannins are the only group of tannins present in the brown algae (*Phaeophyta*), of which *Fucus vesiculosus* is a presentative seaweed. Phlorotannins are polymers of phloroglucinols (1,3,5 – trihydroxybenzene), which vary in their molecular masses and may generate up to 15 % of dry mass of brown algae (Ragan & Glambitza, 1986).

Porphyra dioica extracts demonstrated the highest antimicrobial activity in ethyl acetate and water:methanol (1:1) extracts at room temperature with mean zones of inhibition of 8 mm and 9 mm, respectively, against the same pathogenic strain WIT 676. Soxhlet extraction with the same mixture of solvents (water:methanol, 1:1), but with a higher extraction concentration of approximately 1:30 w/v possessed the highest antimicrobial activity with a zone of inhibition of 13 mm against WIT-676 pathogenic strain. Antimicrobial activity detected from ethyl

acetate and water:methanol extractions, which are different solvents with divergent polarities, suggested another hypothesis that *Fucus vesiculosus* and *Porphyra dioica* seaweeds will probably contain more than one compound responsible for antimicrobial activity. However, this was not in accordance previous research that observed that the most successful solvent for extractions used was diethyl ether compared to acetone and methanol (Tuney, et al., 2006). Neither *Fucus vesiculosus*, nor *Porphyra dioica*, did not support this statement, because both extraction yields were lowest in this solvent (see Table 2) and did not exhibit any activity against MRSA in those solvents (see Table 4). Further analysis would be required to confirm any synergism of compounds present (Tallarida, 2011) by further separation analysis, such as thin layer chromatography, bioautography, column chromatography and antimicrobial testing of individual bands by bioautography technique (Dewanjee, et al., 2015).

Porphyra dioica was only seaweed which was extracted by Soxhlet extraction. This was done based on previous reports of antimicrobial activity of this seaweed (Mendes, et al., 2013). The method used in this experiment was modified, to include the use of lyophilised seaweed in the soxhlet extraction (Salvador, et al., 2007). Previous research compared fresh and dry seaweed material and they observed lower antimicrobial activity in fresh extraction samples compared pre-treated ones by freeze-drying (de Campos-Takaki, et al., 1988). It was hypothesised that the water present in fresh sample could have negatively influenced the extraction concentrations of compounds responsible for antimicrobial activity.

The diversity of previously used extraction concentrations (Tan, et al., 2012) (Mendes, et al., 2013) (Sameeh, et al., 2016) (Ibtissam, et al., 2009) led to the experiment in this study only being performed with *Fucus Vesiculosus* in methanol, which resulted in an influenced zone of inhibition in antimicrobial activity. The experiment was performed at concentrations of 1:40, 1:60, 1:80 and 1: 100 w/v (see Figure 1). The highest antimicrobial activity against the MRSA pathogenic strain WIT 676 was revealed at a concentration of 1:40 w/v with lowest extraction yields (see, Table 5). Increasing yield resulted in decreased antimicrobial activity for *Fucus vesiculosus*, which suggests that more unwanted material was extracted, which possibly lead to a negative synergistic effect on the antimicrobial compound(s) present. Additionally, unwanted material extracted could have a masking effect on the compound or mixture of the compounds responsible for antimicrobial activity. All these hypothesis would have to be confirmed experimentally; however, large inhibition zone from more diluted extracts have previously been reported (Ibtissam, et al., 2009), (Kolanjinathan & Stella, 2009).

The limitations of this study include the effect of the extract concentration on antimicrobial activity was performed only on one type of solvent and seaweed and further studies would be required. The experiments performed could be repeated with any type of seaweed and solvent to modify extraction yields or to estimate what concentration of solvent could be used in extraction of each specific seaweed. This estimation would modify the concentration of solvent used, which could lead to decreasing the ecological and financial burden in future extractions, with the potential to develop an optimised extraction method.

5. Conclusions

Antimicrobial activity of *Fucus vesiculosus* and *Porphyra dioica* extracts in various solvents were successfully screened against the MRSA pathogen strain WIT 676. Crude seaweed extracts from the polar solvent water:methanol (1:1) and the intermediate polarity solvent ethyl acetate possessed highest antimicrobial activity against the pathogenic strain WIT 676 for

Porphyra dioica with zones of inhibition measured as 13 and 9 mm, respectively, from extracts concentrations of 1:30 w/v and 1:100 w/v. Extracts from polar solvents water and methanol (separately) possessed the highest antimicrobial activity for *Fucus vesiculosus* with equal zones of inhibition of 8 mm measured, at a concentration of 1:100 w/v. The effect of the solvent used during extraction on the antimicrobial activity showed the highest zone of inhibition at a concentration of 1/40 w/v, and resulted in a 12 mm zone of inhibition for the methanol-based extraction of *Fucus vesiculosus*. The red seaweed *Porphyra dioica* demonstrated higher antimicrobial activity compared to the brown seaweed *Fucus vesiculosus*, but both seaweeds revealed promising results for their metabolite extracts for further use.

6. Future Work

Further analysis would require modification of the method, which could be designed specifically for a certain type of seaweed to obtain a maximum yield of compound, or mixture of compounds, responsible for antimicrobial activity. Fractioning and purification of the crude extract by thin layer chromatography could lead to the identification of components leading to chemical elucidation of compounds responsible for antimicrobial activity (Shanmughapriya, et al., 2008). Metabolites responsible for antimicrobial activity and susceptibility towards MRSA WIT676 strain could be further tested by bioautography technique (Dewanjee, et al., 2015). Additional analysis would also be required to analyse the different compounds for chemical and physical properties (Tan, et al., 2013). Antimicrobial screening would need to be enlarged to other MRSA pathogenic strains and other bacterial strains also. Incorporation of selected purified components responsible for antimicrobial activity into polymers, such as chitosan and alginate with potential development of a novel wound dressing based on natural products is a future application of this work.

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8. References

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