Development of Novel Wound Dressings using Seaweed Derived Antimicrobial Compounds Characterised against Emergent Clinically Relevant Bacteria

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To my sister Valerie, whose journey ended the day this work began

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DECLARATION

No element of the research work described in this thesis, or in the thesis itself, except where otherwise acknowledged, has been previously submitted for a degree at this or any other institution. The work in this thesis has been performed entirely by the author.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistant
BHI	Brain heart infusion
CLSI	Clinical and laboratory standards institute
DCM	Dichloromethane
DPPH	1, 1-diphenyl-2-picryl-hydrazyl
DSC	Differential scanning calorimetry
DVS	Dynamic vapor sorbtion
EMEM	Eagals essential minimum media
EPA	Environmental Protection Agency
EtOAc	Ethyl acetate
EtOH	Ethanol
EUCAST	Committee on antibiotic susceptibility testing
FBS	Fetal bovine serum
FDA	Food and drug authority
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography mass spectroscopy
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HUFA	Highly unsaturated fatty acids
IDF	Insoluble dietary fibre
IR	Infrared spectroscopy
KOH	Potassium hydroxide

LC	Lethal concentration
LC-MS	Liquid chromatography mass spectroscopy
LCL	Lower confident level
LMW	Low molecular weight
MAE	Microwave-assisted extraction
MBEC	Minimum biofilm eradication concentration
MCT	Mercury cadmium telluride
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MMW	Medium molecular weight
MRD	Maximum recovery diluent
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	Thyazolyl blue tetrazolium bromide
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PLE	Pressurized liquid extraction
PPE	Personal protective equipment
R_f	Retention factor
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute media
SE	Standard error
SEM	Scanning electron microscope

SD	Standard deviation
SDF	Soluble dietary fibre
SFE	Supercritical fluid extraction
TCD	Total cell death
TDF	Total dietary fibre
TGA	Thermogravametric analysis
TLC	Thin Layer Chromatography
TPC	Total phenol content
TTC	2, 3, 5-triphenyl-tetrazolium chloride
UAE	Ultrasound-assisted extraction
UCL	Upper confident level
UV-Vis	Ultraviolet-visible
WHO	World Health Organisation

ABSTRACT

Seaweeds and their derivatives have recently been hailed for their impressive production of secondary metabolites for the purpose of self-preservation, and have been noted for their many bioactive activities. The emergence of antimicrobial-resistant (AMR) strains of pathogens has increasingly become an issue in the treatment of infections and has been reported to have been a driving force in the increasing cost of hospital care and higher risk of complications associated with the treatment of once trivial infections. The O'Neill report, commissioned by then UK Prime Minister David Cameron, in 2016 estimated that by 2050 more people will die of multi-drug resistant infections than of cancer. As a result, new sources of antimicrobials which could be developed into new antibiotics to combat AMR infections are being sought after.

An initial screen was carried out using solvent extracts from the seaweeds *Fucus serratus* and *Fucus vesiculosus* which were extracted using the solvents; water, methanol, acetone and ethyl acetate. These crude extracts were established to be antimicrobial against a range of bacterial wound pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA) clinically isolated from University Hospital Waterford. The most promising extract (water extracted *Fucus vesiculosus*) was analysed for anti-biofilm and cytotoxic activity. This extract demonstrated biofilm disruptive and biofilm preventative activities, but was, however, cytotoxic to all cell lines tested (HaCaT, HepG2 and THP-1). Once bioactivity was established, the method of extraction for the water extract from *F. vesiculosus* was developed using microwave assisted extraction techniques. This extraction method maintained antimicrobial activity of the extract and established an increase of 224 % in yield generated in 10 min as opposed to the 2 h standard using solvent extraction.

This extract was then soaked into inactive Aquacel® wound dressings and compared for antimicrobial activity over time to iodine, silver and honey commercially available antimicrobial wound dressings (Inadine, Aquacel® and Algivon respectively). The dressings soaked in seaweed extract maintained antimicrobial activity for a longer period of time than any of its commercially available counterparts. Additionally several wound dressing formulations were prepared, using naturally sourced polymers, incorporating the seaweed extract and the most structurally viable dressings were formulated for antimicrobial activity and flexibility.

The separation and identification of the antimicrobial in the extract was attempted using; TLC, bioautography, semi preparative HPLC, NMR, LC-MS, FTIR and UV-Vis analysis and the bioactive compound was determined to be fucoidan.

Chapter 1: Introduction

1.1 Introduction

Seaweeds are widely available primitive macroalgae which have been used as a food source for humans and animals for millennia (1). Their autotropic nature means they have the ability to produce a wide array of organic compounds from the simple resources found in the marine environment (2). The potential bioactivity of compounds produced by many species of seaweeds has been well documented over the years, endorsing the promise of this type of research (3–9). As such, seaweeds are a massively underexploited natural resource with potentially positive outcomes for the pharmaceutical industry.

1.1.1 Classification of Seaweeds

At their most basic, algae are chlorophyll containing organisms which can exist as microalgae or macroalgae in both aquatic and terrestrial environments (8). Seaweeds are benthic marine macroalgae which are abundantly present in the ocean. They are not categorised as either plant or animal and have a unicellular stage in their life cycle (10), as such they belong to the subkingdom Algae within the *Protista* kingdom.

The general structures of seaweeds can vary depending on the individual species of seaweed and whether or not the seaweed is unicellular or multicellular. In their simplest form, seaweeds can be unicellular or single celled organisms. These seaweeds can grow up to 1 inch in diameter (10). Multicellular seaweeds can be observed in a wider variety of forms. Uniseriate filaments (Figure 1.1), form when the cells in a seaweed form a chain and take a thread-like appearance (10). These chains can be microscopic, or macroscopic, depending on the species of seaweed.

Figure 1.1: Chaetomorpha, an example of uniseriate filaments (11).

Figure 1.2 depicts the possible structure of a seaweed including some of the typical appendages associated with seaweed. The frond refers to the whole length of the vegetation from the holdfast. The blade is the leaf-like component of the seaweed. They come in many various forms such as smooth, dented, perforated and segmented (10). The main purpose of the blade is to absorb nutrients from the water and to facilitate photosynthesis (11).

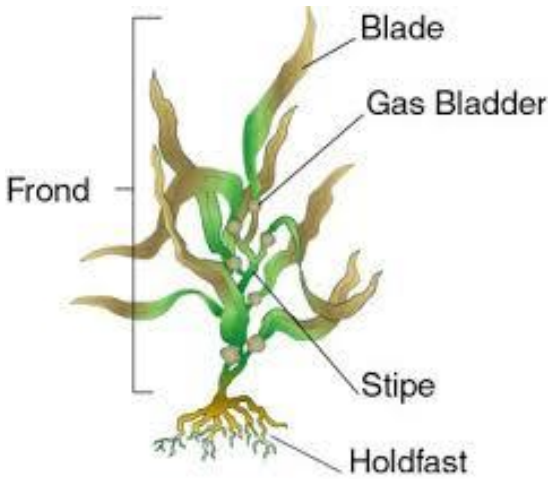

Figure 1.2: Structure of multicellular seaweed (12).

The gas bladder is not present on all seaweeds. It is an air-filled pocket at the base of the blade. These bladders serve the purpose of making the seaweed buoyant and seaweeds containing them are typically referred to as 'bladderwrack' (13). This allows the seaweed to be physically nearer to the water surface, where light can penetrate more easily; as such the bladders assist photosynthesis.

The stipe is similar to the stem in higher plants. It differs, however, as it is not a means of transporting nutrients around the organism (13). It serves the seaweed as a physical support and can also absorb nutrients from the environment (10).

The holdfast is the mechanism whereby benthic seaweeds can bind to the rock or surface on which they are based (13). Unlike the roots of a plant, the holdfasts main function is not the absorption of nutrients (10-11). The shape of the holdfast depends greatly on the structure to which it is binding. The autotropic nature of seaweeds leads to the production of various strong adhesives which adequately bind the relatively small holdfast to a structure for even large seaweeds (10).

As mentioned, seaweeds undergo photosynthesis and so contain photosynthetic protein pigments (11). They are differentiated based on the concentration of the protein pigments present in the seaweed and thus can be characterised by their colour. The categories of seaweeds are; Chlorophyta (green) (10), Rhodophyta (red) (11-12) and Phaeophyta (brown) (6). Irish examples of these are detailed in Table 1.1.

Table 1.1: Species of seaweeds found on the Irish coast (14,15).

Chlorophyta	Phaeophyta	Rhodophyta
Ulva lactuca	*Alaria esculenta*	*Palmaria palmata*
Enteromorpha intestinalis	*Fucus serratus*	*Porphyra*
Codium fragile	*Fucus spiralis*	*Chondrus crispus*
	Fucus vesiculosus	*Lithothamnion corallioide*
	Fucus serratus	*Polysiphonia lanosa*
	Himanthalia elongata	
	Laminaria digitata	
	Laminaria saccharina	
	Saccharina latissima	
	Ascophyllum nodosum	
	Alaria esculenta	

1.1.2 Uses of Seaweeds

Seaweeds are noted as an underutilised resource with many potential applications for various consumer industries (16). In 2018, the Irish Government issued the National Marine Planning Framework baseline report stating that seaweeds are increasingly being used as a source of high-value ingredients in bio-pharmaceuticals (17). It also noted that aquaculture in Ireland has increased by 44 % between 2014 and 2016 (17), which could potentially be due to consumers favouring natural products (18). In Ireland, seaweed is most commonly used as an animal feed and a fertiliser in rural communities (17).

1.1.2.1 Food

Marine macroalgae are used as a food source by coastal communities. The nutritional, bioactive and mineral content of the seaweed varies throughout the seasons and is very much dependent on environmental conditions and processing methods (7). Seaweeds are composed of approximately 80-90 % water, carbohydrates in the form of polysaccharides, protein, ash in the form of inorganic minerals, lipids, and dietary fibre (Table 1.2) (7,8). As mentioned the nutritional composition of these seaweeds are prone to variation between species (7).

From a dietary perspective, seaweeds are a rich food source owing to their high fibre content and protein. It has been established that increasing dietary fibre in the diet can significantly lower the risk of colon cancer (19). The protein within seaweed peptides are of high quality and can contain most of the eight essential amino acids, depending on the species and the season (20). Due to their low lipid content (1-6 % dry weight) (3), seaweeds are a negligible source of energy, however, they do contain a higher amount of saturated fatty acids compared to unsaturated fatty acids (8).

Seaweeds are also a source of vitamins: A, B_1, B_2, B_3, B_5, B_{12}, C, D and E, and folic acid (8,21). Minerals such as Ca, P, Na, K and iodine are also present, described as ash content (22). Vitamins and minerals are essential for general heath and maintenance of the human body (23).

Table 1.2: Approximate nutritional values of different species of seaweed compared to various edible foods (20-26). (Soluble dietary fibre (SDF), insoluble dietary fibre (IDF) and total dietary fibre (TDF))

NUTRITIONAL IMPORTANCE OF SEAWEED							
	Composition (9g/100 g of dry seaweed)						
	Carbohydrates	**SDF**	**IDF**	**TDF**	**Proteins**	**Lipids**	**Ash**
Chlorophyceae							
Ulva spp	42.1	NA	NA	NA	20.0-26.1	0.6-0.7	13.7-22.6
Enteromorpha spp	NA	21.3	16.8	38.1	10.0-21.0	NA	NA
Phaeophyceae							
Laminaia spp	49.1	9.2	26.9	36.1	6.7	1.6	19.2
Konbu	NA	NA	NA	NA	8.1-15.0	1.8	25.4
U.pinatifida	47.8	17.3-30.0	5.3-16.2	33.5-35.3	12.7-14.1	1.5-2.7	21.2-32.8
H.fusiforme	29.8	32.9	16.3	49.2	5.6-12.3	0.8-1.5	21.2-35.0
F.vesiculosus	34 - 66	9.80	40.29	50.1	1 - 11	1.2 - 4	23 - 36
F.serratus	26 - 62	NA	NA	16.0	10 - 17	0.4 - 3	19 - 22
Rhodophyceae							
P.tenera	40.5	14.6-17.9	16.8-19.2	33.7-34.7	33.0-47	0.7-1.6	8.5-8.7
Chondrus Crispus	54.8	22.3	12.0	34.3	11.2	2.6	14.2
Gracilarid	58.4	NA	NA	NA	7.9	0.1	17.8
Cyanophceae							
Nostoc	55.7	NA	NA	NA	20.9	1.2	7.5

In addition to raw consumption, seaweeds are also consumed through processed foods as agar\carrageenan, alginate and phytocolloids which are commonly used in food

products. These phytocolloids extracted from seaweeds are used as gelatine, suspension agents, emulsifying agents, stabilisers and to control the moisture present in food (7).

Similarly, seaweed can also play a role in the production of human food, as they are used as a fertiliser and as animal stock feed. Pigs fed on the seaweed extracts laminarin and fucoidan from *Laminaria digitata* for three weeks were found to contain bioactive antioxidant components into their muscles, without impacting pork flavour or quality. This resulted in an enhanced meat quality, as the meat was discovered to have an improved lipid stability through this process (24).

Fucoxanthin and one of its metabolites fucoxanthinol have been studied for their antioxidant, anti-tumour, anti-obesity, liver protective, anti-angiogenic and bone protective effects (25). Although these pigments can have a low bioavailability depending on other compounds consumed with them (24), they were shown to display no toxicity in mice and, therefore, have potential to be supplemented safely into the diet (26-28). It was found that by changing hens feed to include 15 % *Fucus serratus*, fucoxanthin was metabolised by the hens into fucoxanthinol and was transferred into the eggs of laying hens, presenting an inexpensive route to enhancing nutritional value of food (29)

The meat of rainbow trout was enriched by doubling of the iodine content within the fish through supplementing the trout's diet with 5 % *Gracilaria vermiculophylla.* This substitution was found to lower the lipid content within the flesh without affecting the protein content or threatening iodine overdose (28). Enhancing the nutritional content in meat has the potential to improve the human diet, as iodine can prevent the development of mental impairments and iodine deficiencies can cause goitre (30).

Seaweeds have been successfully used as fertilisers in coastal regions since the nineteenth century (17). However, seaweeds are becoming more relevant in this field as recently there is more emphasis placed on the environmental consequences of chemical fertilisers (31).

1.1.2.2 Cosmetics

Consumer trends are favouring natural products (18), and so seaweeds are becoming more popular as a natural alternative to typical synthetic cosmetics. Bioactive compounds isolated from seaweed have been found to have uses in the cosmetic industry (9). These properties coupled with the tightening of regulations surrounding the cosmetic industry makes seaweed extracts an attractive source of non toxic, bioactive ingredients.

Skin ages due to a number of internal and external factors. As well as exposure to UV light, pollution, nicotine and environmental wear and tear, skin can also appear aged due to the skins' tendency to lose some of its ability to cope with reactive oxygen species (ROS) produced during cellular respiration (9). Skin can come under oxygenative stress, hindering cell repair and giving an aged appearance (43). Antioxidants can neutralise these ROS and can act as free radical scavengers, hindering skin aging. Sulphated polysaccharides from *Ecklonia kurome* Okam (34), *Fucus vesiculosus* (35) and *Porphyra haitanensis* (36) all exhibited antioxidant properties and so can be used for the prevention of oxidation damage of the skin (43).

In addition, sulphated polysaccharides found in seaweed such as fucoidan from *Ascophyllum nodosum* seen in Figure 1.3 can act to regulate the degradation of proteins in the skin, further hindering the aging process by protecting the skins elasticity (16). This also doubles as a bioactive for the treatment of wrinkles.

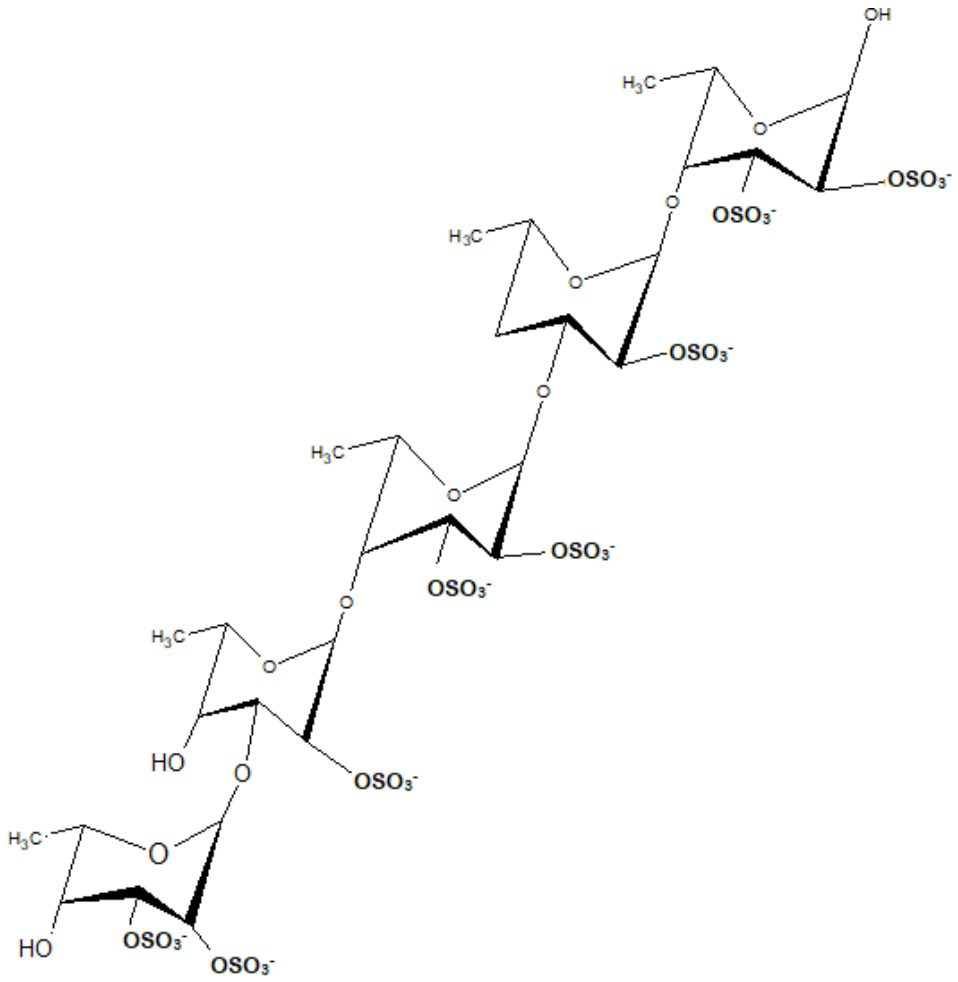

Figure 1.3: Possible structure of fucoidan from *A. nodosum* (37).

Protein peptides are used in the cosmetic industry to improve skin and make hair appear glossy. Seaweeds can be a cheap source of amino acids and so can be a valuable asset to this industry (20). Aosa biopeptide, a peptide isolated from *Ulva lactuca*, is currently used in skin creams (Biopeptide CL™, Ormedic balancing bio peptide créme, etc.) due to its ability to stimulate the production of collagen in human fibroblasts. Collagen is a structural protein found in the body, the breakdown of which can decrease skin elasticity and cause wrinkles (38).

1.1.2.3 Medicinal

Many seaweeds have been found to contain bioactive substances which have the potential to be a resource for novel natural pharmaceutically active molecules (8). As mentioned above in section 1.2.1, fucoxanthin and its relative metabolites have been found to be biologically active for a number of ailments. Fucoxanthin has been found to have anti-obesity properties; has lipolysis ability and acts as a lipogenesis inhibitor with the potential to alleviate diseases associated with excessive white adipose tissue in the body such as: type II diabetes, hyperlipidemia, hypertension, and cardiovascular disease (25). It was shown that supplementing the diet of obese mice with a 0.02 % dose of fucoxanthin significantly lowered body weight and fat accumulation (25).

Seaweed extracts have also been shown to contain anti-inflammatory compounds. A study isolating 3-O-b-glucopranosyl clerosterol from *Ulva lactuca* showed the steroid to inhibit oedema in mouse ears by 66 % when 1000 µg of extract was applied to the ear (40). Similarly, 5-hydroxypalisadin B, a secondary metabolite isolated from *Laurencia snackeyi* displayed anti-inflammatory activity in lipopolysaccharide-induced zebrafish embryos when compared *in-vivo* to the commercially available anti-inflammatory dexamethasone (41).

Fucoidan isolated from *Sargassum mcclurei, Sargassum polycystum* and *Turbinara ornata* were found to be anti-viral exhibiting a mean IC_{50} ranging from 0.33 to 0.7 g/ml (42). Fucoidans are sulphated polysaccharides; the structure and mode of action for the anti viral properties of these molecules remains unclear (42) Another study found that a methanol extract of *Symphyocladia latiuscula* worked as well as the commercially available anti-viral, acyclovir, against herpes simplex 1 in mice at a dose of 600 mg/kg/day, and also indicated that the extract could potentially be used as an alternative to acyclovir-resistant strains (44)

The development of high value products using seaweed is in agreement with the European Marine Strategy which aims to achieve and protect a high-quality environmental status for European waters (45).

The cytotoxic activity of seaweeds has been well established. Sulphated monoterpene extracts from the south African seaweeds *Plocamium suhrii* and *Plocamium cornutum* displayed greater cytotoxicity than the established chemotherapy drug cisplatin against

oesophageal cancer at IC_{50} 6.6–9.9 µM (46). A study found that a dietary polyphenol-rich extract from *Eucheuma cottonii* inhibited the growth of oestrogen-dependant MCF-7 (IC_{50} 20 µg/ml) and oestrogen independent MB-MDA-231 (IC_{50} 42 µg/ml) human breast cancer cells. However, this extract was found to be non- toxic to normal cells in rats (41).

Extracts from seaweeds have also been successfully shown to have antimicrobial properties (42,47) which could have implications for the Irish commercial market, as seaweeds have been identified as a potential high value addition to the International market (48), a break down for which can be observed in Figure 1.4

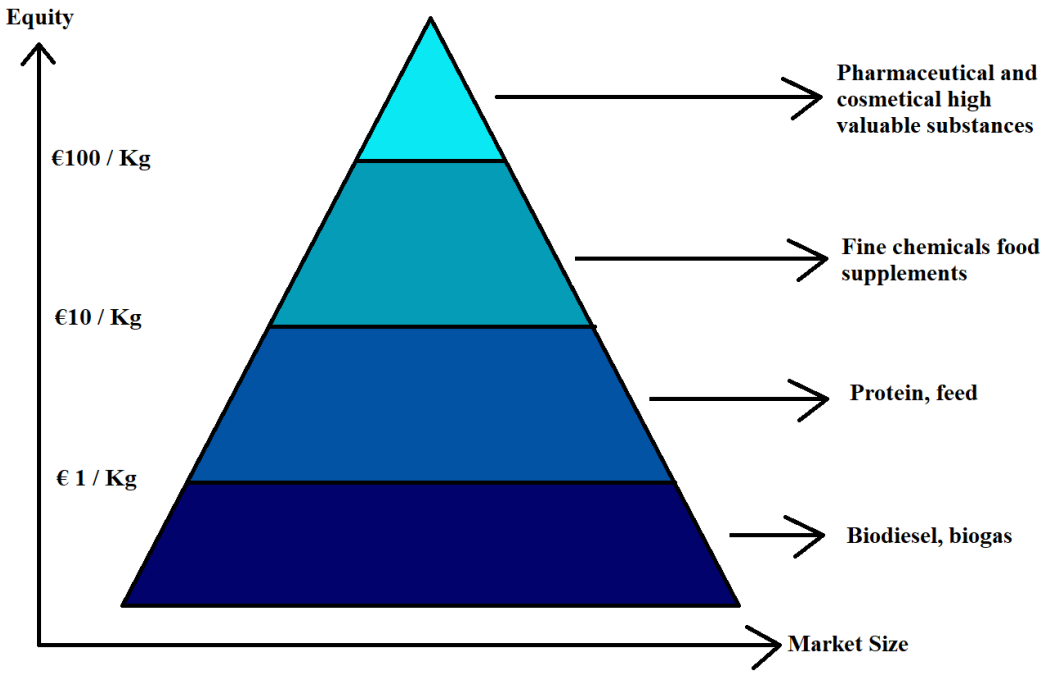

Figure 1.4: Algal value pyramid (49).

The development of high value products using seaweed is in agreement with the European Marine Strategy which aims to achieve and protect a high-quality environmental status for European waters (45).

1.2 The extraction of bioactive compounds from seaweed

1.2.1 Solvent Extraction

Conventional solvent extraction methods of bioactive compounds from seaweeds are often basic solvent extraction, of which there are many literature examples using different conditions and variations of this method, some of which are discussed below.

A study was conducted investigating the variations of bioactives throughout the year where *Ulva lactuca* was harvested off the coast of Wexford, Ireland and processed by washing in distilled deionised water, freeze drying, blending, sieving and storing the seaweed under nitrogen at -20 °C. The seaweed bioactives were extracted using a variety of solvents over a range of polarities at a ratio of 1:100 *w/v*. This study found that ethyl acetate consistently extracted an antimicrobial bioactive throughout the year (50).

Similarly, a study used the method of washing the seaweeds with fresh water, freeze drying, blending and storing the seaweeds at -20 °C. The bioactives were extracted from the seaweed powders by immersing the powder in acetone, chloroform, ethyl acetate, diethyl ether, methanol and water for various amounts of time (24 h, 48 h, 72 h). This study found that acetone extracts of nine different algae were active against *M. luteus* (inhibited by all 9 algae), *B. subtilis, S. aureus, Ps. fluorescens, B. cereus, Br. catarrhalis, P. vulgaris, E. coli, and S. marcescens*; all extracts inhibited five or more microbes with Gram positive strains being more vulnerable to the bioactives (3).

For the solvent extraction and assessment of antioxidant and antimicrobial activities in six edible Irish seaweeds, frozen seaweed samples were powdered using liquid nitrogen in a pestle and mortar. 5 g of the powdered seaweeds were extracted in 50 mL of solvent (acetone, ethanol, methanol or water) in a shaker incubator at 40 ° C for 2 h. The resulting mixtures were centrifuged at 10,000 rpm for 15 min and filtered to remove any remaining seaweed powder. Solvents were evaporated under reduced pressure and the methanol extract was found to display the largest range of antimicrobial activity (51).

For the extraction of potential antimicrobials from 54 different species of Indo-Pacific and Atlantic seaweeds; one study volumetrically measured 10 mL of seaweed tissue and soaked it in a solution of 1:1 dichloromethane and methanol. The resulting crude extract

was concentrated under vacuum and separated out using liquid-liquid extraction in ethyl acetate and water. It was found that 95 % of Indo-Pacific and 90 % of the Atlantic species of seaweeds used displayed selective antimicrobial activity against *Pseudoaltermonas bacteriolytica, Lindra thalassiae, Dendryphiella salina, Halophytophthora spinosa* and *Schizochytrium aggregatus* (6).

The seaweeds: *Valonia acgrophila, Halimeda opuntia, Lamouroux, Halimedatuna, Caulepa racemosa, Caulerpa mexicana* and *Ulva pertlusa* were harvested, cleaned of epiphytes and washed with fresh water in a study assessing their antimicrobial activities. The seaweeds were then dried with air at 25 °C, milled and left in diethyl ether solvent for 24 h at a ratio of 1:10 milled seaweed:solvent. The crude diethyl ether extracts were then tested for antimicrobial activity. All seaweed extracts, except that of *Caulerpa mexicana*, were found to be active against three or more of the four microbes tested. The extract from *V. acgrophila* exhibited the greatest antimicrobial activity and was determined to be a more effective antibiotic than the control, penicillin G (52).

F. vesiculosus was frozen at -20 °C, freeze dried and cut into small pieces before being extracted with 0.05 % trifluoroacetic acid (TFA) at a ratio of 1:10 seaweed:solvent at 4 °C for 24 h. The supernatant was decanted and the seaweed tissue was extracted again using TFA at 4 °C. Once the two extracted supernatants were added together, the solvent was rotary evaporated off from the sample until a final volume of 200 mL remained in the flask. The sample was separated into four different eluents by gradient reversed-phase chromatography. *E. coli, S. aureus, P. aeruginosa, S. epidermidis* and *P. mirabilis* were inhibited by at last one of the purified fractions (53). As can be seen from these studies, solvent extraction has been successfully used in the literature for the extraction of bioactives from seaweeds.

Most reviewed studies agree that processing the seaweed should include freeze drying and milling the samples into a powder (6,50–52). The solvent system required for the extraction appears to vary between bioactives from specific species of seaweed. Parameters such as the time and temperature of the extraction were different throughout the studies, with little work done in an attempt to developed the extraction. However, one study which examined *Nereocystis luetkeana* looked at extracting bioactives over different time periods at room temperature (3); the results indicated that there was no increased activity in the samples extracted beyond the 24 h mark.

While conventional solvent extractions are used often in the literature, they have the disadvantages of long extraction times and the requirement of copious amounts of solvents which require expensive disposal methods. As such, although this method is advantageous in its simplicity and low cost in terms of power requirements, other methods are often utilised with a view to increase extraction yields, lower extraction time, and to improve the environmental impact that this method can have.

1.2.2 Soxhlet extraction

Soxhlet extraction is a form of solvent extraction in which the solvent is recycled by reflux resulting in a concentrated extract. This techniques is also useful for the extraction of compounds with low solubility in the extraction solvent, however, it should be noted that it is not a suitable technique for thermolabile compounds (54). This technique requires a heat source to evaporate the extraction solvent present in the round bottomed flask. This solvent is then condensed into a liquid in the condenser where it will fall into a thimble where the sample is placed. Analytes will dissolve into the solvent in this section of the apparatus. Once the level of the solvent is high enough, the extract will be siphoned back into the round bottomed flask via gravity (55).

Natural products have been extracted in the past using Soxhlet extraction. Moshatine, a steroidal glycodside was isolated from *Centaurea moschata* seeds using sequential solvent Soxhlet extractions with n-hexane, chloroform and methanol. This extract went on to be purified and identified using HPLC analysis (56).

Vegetable oil was extracted from tobacco seeds by both Soxhlet and ultrasonic-assisted extraction (UAE) using hexane in a comparative study. Although Soxhlet was found to give a higher yield of vegetable oil with a 90 min extraction, UAE gave a relatively high yield in a 20 min extraction time at 25 ^{o}C. Similarly, vicine and convicine, pyrimidine glycosides, were extracted from faba beans using both Soxhlet and microwave assisted extraction (MAE) with a 1:1 MeOH:H_2O mix. This method noted that MAE obtained a 20 % higher yield than the traditional Soxhlet extraction using only two 30 s extractions in the microwave digester, this was speculated to be due to the degradation of thermolabile compounds during Soxhlet extraction (57).

Although Soxhlet extraction utilises less solvent and is less time consuming than traditional solvent extraction, it is being overshadowed by newer, 'greener' methods of extraction, such as MAE, which don't require large volumes of solvent to boil for long periods of time which can damage thermolabile compounds (58).

1.2.3 Microwave assisted extraction

Microwave assisted extraction (MAE) is considered an environmentally friendly and cost effective method of extraction as it reduces the need for the large volumes of solvents required by solvent and Soxhlet extraction (59). Microwaves are waves at radio frequency (0.3 - 300 GHz) which are accelerated at a sample in order to heat the sample. Typically, domestic microwaves run at a frequency of 2.54 GHz, with the intention of not interfering with radio waves. This frequency causes dielectric materials, such as water and other solvents, to undergo dipole rotation, which results in friction on a molecular level and results in the heating of the sample via ionic conduction (60).

Electromagnetic radiation can penetrate the solid sample resulting in changes in cell structure facilitating the extraction of compounds. This results in MAE being typically more efficient than solvent or Soxhlet extraction (61). Other modes of extraction of bioactives using MAE involve the pressure generated by the evaporating solvent in closed vessel systems resulting in stress on plant/seaweed cells which, in turn, results in the breaking of the cell and thus the release of its contents into the extraction solvent (62).

The terpenes limonene and carvone were successfully extracted from *Carum carvi L.* using MAE at 150 W with a maximum yield of 17.12 and 12.6 mg per gram of carvone respectively. This was as efficient as a conventional extraction with the lower extraction time of 10 min compared to 1 h for conventional extraction (63). Fucosterol, 24-methylenecholesterol and phyto were extracted from the seaweeds *Undaria pinnatifida* and *Sargassum fusiforme* in 220 min using MAE with over 97 % purity (64).

Phloroglucinol was extracted from the seaweed *Carpophyllum flexuosum* using MAE in a comparative study against solid-liquid extraction. A 70 % greater phloroglucinol yield was noted with the use of MAE in 3 min versus a 24 h solvent extraction (65). A

summary of conditions for the extraction of bioactive compounds from seaweed using MAE are detailed below in Table 1.3.

MAE does however have its shortcomings as there is a high initial cost associated with the purchase of the instrumentation, and also that microwaves can cause the degradation of thermolabile compounds (60). Regardless of this, organic compounds have been successfully extracted from natural products utilising this methodology.Methods such as MAE tend to require smaller volumes of solvents, shorter extraction periods and are capable of being streamlined into automation (66).

There are issues associated with the scale-up of MAE to an industrial level production, particularly due to problems relating to heat-transfer and the penetration of microwaves into the sample. These issues can often be ameliorated with the use of specific, commercially available microwaves for the purpose of microwave extraction (67).

Table 1.3: MAE extraction methodologies for natural product compounds from seaweeds.

Seaweed species	Compound	Bioactivity	Temp. (°C)	Power (W)	Solvent	Feed (g) : Flow (g/min) ratio	Pressure (psi)	Time (min)	Ref.
Dunaliella tetiolecta	Carotenoids	Antioxidant	56	-	H_2O	-	ATM	5	(68)
Fucus vesiculosus	Fucoidan	Antimicrobial Anticancer	70	-	H_2O	1-5/25g/mL	120	1	(69)
Palmaria, U. pinnatifida, H.elongata, L. ochroleuca, U. rigida	Iodine	Antimicrobial	200	1000	H_2O	0.025 g/mL	-	0-5	(70)
Ascophyllum nodosom	Fucoidan	Antimicrobial Anticancer	90	-	0.1M HCl	0.056 g/mL	-	15	(71)
Dunaliella tetiolecta	Fatty acids	Antimicrobial	-	490	CH_3Cl:MeOH 2:1	0.01 g/mL	-	2	(72)
Enteromorpha prolifra.	Polyphenols	Antioxidant	-	-	EtOH:H_2O 40:60	0.04 g/mL	-	25	(73)
Enteromorpha prolifra.	Polysaccharides	Antioxidant	70	700	H_2O	0.025 g/mL	-	25	(74)
Caulerpa racemosa	Phenolic compounds	Antioxidant	50	200	EtOH:H_2O 40:60	0.025 g/mL	-	40	(75)
Caulerpa racemosa	Phlorotannins	Antioxidant Antimicrobial	160	-	H_2O	0.033 g/mL	-	3	(76)
Carpophyllum felxuosum	Phloroglucinol	Anti-inflammatory	160	-	H_2O	0.033 g/mL	-	3	(65)
Ascophyllum nodosum,	Polyphenols	Antioxidant	75	1000	H_2O	-	-	20	(77)

1.2.4 Supercritical fluid & sub critical fluid extraction

Super critical and sub critical fluids are alternative substitutes for solvents that are typically used in the extraction of bioactives. They are greener than conventional organic solvents and often require less 'work-up' as the crude extract is deposited without solvent. Super critical fluids occur at a specific temperature and pressure above a substances critical point (78). When this point is reached, a substance loses the ability to be in a specific phase and begins to exist in a non-distinct hybrid phase of liquid and gas: as such the fluid retains properties from both liquid and gas phases, where it can diffuse through substances like a gas but also dissolve materials like a liquid. These hybrid phase properties can enhance the extraction process, by allowing a solvent to fully penetrate a bioactive containing product. The 'green' aspect of these fluids comes from the tendency of a solvents dielectric constant to change under conditions of high temperature and pressure; in this way compounds such as water and carbon dioxide can be manipulated to dissolve molecules that are soluble in environmentally unfriendly organic solvents (78,79).

While super critical fluid extraction is a greener method than that of solvent extraction, it does require a large initial investment. However, this may be countered with the reported increased extraction yields generated when compared to solvent extraction (80) and the reduced cost in solvent disposal. Some conditions for the extraction of compounds from natural products using super critical fluid extraction are detailed below in Table 1.4.

Table 1.4: Super and sub critical fluid extraction methodologies for compounds from various natural products.

Natural Product	Compound	Bioactivity	Temp. (°C)	Solvent	Feed (g) : Flow (g/min) ratio	Pressure (MPa)	Time	Ref.
Rhodiola rosea roots	Rosavin	Anti oxidant, anti stress	80	CO_2 + 10 % H_2O	1:324	20	3 h	(81)
Hyssop (Hyssopus ofcinalis L.)	Essential oil	Antipyretic	40	CO_2	-	9	-	(82)
Ginger	Essential oil	Antipyretic	30	CO_2 + MeOH	-	10	40 min	(83)
Garcinia mangostana	Xanthones	Antioxidant	40	CO_2 + 4 % EtOH	1:30	20	2 h	(84)
Lotus (Nelumbo nucifera)	Germ oil	Antioxidant	50	CO_2	200:0.5	32	2 h	(85)
Pinus Sp.	Flavonoids	Antioxidant	40	CO_2 + 3 % EtOH (w/v)	-	20	-	(86)
Artemisia annua L	Scopoletin, Artemisinin	Anti malarial	40	CO_2 + 16.25 % EtOH (w/v)	1:10	24.2	-	(87)
Green tea leaves	Caffeine	Stimulant	63	CO_2 + 95 % EtOH (w/v)	-	23	-	(88)
Hippophae rhamnoide	-	Antithrombotic, antiaterogenic	60	CO_2	1 Fruit : 60	45	-	(89)
Hibiscus cannabinus	Oil	Antioxidant	80	CO_2	100:25	20	150 min	(90)
Seaweeds								
Phaeodactylum tricornutum (microalgae)	Fucoxanthin	Antioxidant	100	EtOH	1:4.16	10.3	10 min	(91)

Table 1.4: Continued.

Natural Product	Compound	Bioactivity	Temp. (°C)	Solvent	Feed (g) : Flow (g/min) ratio	Pressure (MPa)	Time	Ref.
Eisenia bicyclis	Fucoxanthin	Antiooxidant	110	EtOH+H_2O 90:10	-	10.3	5 min	(92)
Chlorella ellipsoidea	Zeaxanthin	Anti macular degeneration	115.4	EtOH	-	10.3	23.3 min	(93)
Chlorella vulgaris (microalgae)	Carotenoids	Antioxidant	50	Acetone	0.8:-	10.3	20 min	(94)
Dunaliella salina (microalgae)	Carotenoids	Antioxidant	160	EtOH	6:-	10.3	17.5 min	(95)
Neochloris oleoabundans (microalgae)	Carotenoids	Antioxidant	112	EtOH	2:-	10.3	20 min	(96)
Haematococcus pluvialis (microalgae)	Carotenoids	Antioxidant	50	EtOH	-	10.3	20 min	(73)
Rhizoclonium hieroglyphicum (microalgae)	Omega 3 fatty acids	Lower cholesterol	120	CH_3Cl:MeOH	2.5:-	10.3	30 min	(97)
F.serratus, L.digitata, G. gracilis, C. fragile	Polyphenols	Antioxidant	100	EtOH+H_2O 80:20	2.5:-	6.9	25 min	(98)
Porphyra tenera, Undaria pinnatifida	Polyphenols	Antioxidant	130	Hexane: Acetone	0.2:-	13	20 min	(99)
Sargassum muticum	Polyphenols	Antioxidant	100	EtOH+H_2O 75:25	2.5:-	10.3	20 min	(100)
Ascophyllum nodosum, F.vesiculous, F. serratus	Polyphenols	Antioxidant	100	EtOH+H_2O 80:20	-	6.9	-	(101)
Haematococcus pluvialis	-	Antimicrobial	100	EtOH	-	10.3	20 min	(102)
Himanthalia elongata, *Synechocystis* sp.(microalgae)	-	Antioxidant, Antimicrobial	150	EtOH	-	10.3	20 min	(103)

This is by no means an exhaustive list of extraction techniques; other techniques include ultrasound assisted extraction (UAE) which involves the use of mechanical waves in a liquid based extraction which created areas of high pressure and low pressure in the medium as longitudinal waves collide, encouraging the extraction process. The resulting friction of the medium can cause a temperature increase, further assisting the extraction of compounds from natural products (104)

The antioxidant laminarin was extracted from *Ascophyllum nodosum*, in a study which found that that UAE using 60 % ultrasonic power for 15 min was more efficient than a 2.5 h solvent extraction at 70 ^{o} C (105). Another study into the UAE of antioxidant withanolides from *Witherania somnifera* using water and ethanol found that UAE improved the yield and antioxidant activity of the extracts, however this study noted that further work would be required to convert this method into an industrial technique (106). While UAE is a promising, environmentally friendly, extraction techniques, it has been noted as challenging to increase its scale to an industrial level (107).

1.3 Determination of Antimicrobial Activity

Natural products and their extracts have been used for millennia for flavour and to enhance food, as well as provide herbal remedies to everyday illnesses, such as the use of Chinese herbal medicine (108). In order to discover the bioactivity of these natural products and extracts, they must be screened for bioactivity. As the purpose of this work has a focus on the antimicrobial activity of seaweed extracts, some methods of screening products for antimicrobial activity are detailed below.

1.3.1 Disk diffusion assay

Antimicrobial diffusion methods such as disk diffusion and well diffusion are mainly used for qualitative purposes, however they do give an indication of the potency of the antimicrobial (109). Disk diffusion involves the bacterial inoculums being spread uniformly across a petri dish; samples for analysis are then loaded into solid disks and placed on the petri dish. Zones of inhibition can be visualised post incubation, the diameter of the zone can indicate the potency of the sample (109,110).

A disk diffusion assay using 25 µL of extract absorbed onto a sterile paper disk was used in conjunction with an antimicrobial assay in a study conducted by M.P. Puglisis *et al* (6). It was found that 95 % of Indo-Pacific and 90 % of the Atlantic species of seaweeds used displayed selective antimicrobial activity against *Pseudoaltermonas bacteriolytica, Lindra thalassiae, Dendryphiella salina , Halophytophthora spinosa* and *Schizochytrium aggregatu.* The inhibitory concentration was subsequently determined for active extracts as a more quantitative method (58).

Disk diffusion was also the method used for the antimicrobial assay of bioactive compounds from seaweeds in a study by Rosell *et al.*(3). 50 µL corresponding to 3.0 g of seaweed powder was absorbed onto a 8 mm sterile paper disk. After incubating the choice microorganism for 24 h at 37 °C, the zone of inhibition was calculated in mm (3). This study found that acetone extracts of nine different algae were active against *M. luteus* (inhibited by all 9 algae), *B. subtilis, S. aureus, P. fluorescens, B. cereus, B. catarrhalis, P .vulgaris, E. coli, and S. marcescens*; all extracts inhibited five or more microbes with Gram positive strains being more vulnerable to the bioactives (3).

1.3.2 Well diffusion assay

Well diffusion differs includes the use of a liquid sample, added into a hole cut into the agar inoculated with the bacteria (111). Upon incubation, zones of inhibition can be visualised and measured in diameter, similarly to the disk diffusion method. Well diffusion was determined to be more sensitive than disk diffusion (109). However, well diffusion is not compatible with non-aqueous samples as organic solvents are generally toxic to bacteria.

One study took standards of five previously identified the seaweed compounds responsible for antimicrobial activity; chondriol, cycloeudesmol, pre-pacifenol, laurinterol and debromolaurinterol. This particular study tested antimicrobial activity by the pour plate method which involved mixing the compound of interest with 10 mL of soy agar and streaking the bacteria of choice onto the agar plate; the plates were then incubated for 48 h at 36 °C (5). This method offers simplistic approach to antimicrobial

detection similar to the well diffusion assay, contrasting in that the other methods provide a quantifiable assay of the bioactive against a control.

Disk diffusion and well diffusion are suitable methods for testing antimicrobial activity as they are quick and display zones of inhibition which can quantify inhibition better than the pour plate method, which relies on controls tested on different petri dishes, introducing potential error between agar plates. Well diffusion appears to be superior to disk diffusion as it requires less sample as it removes the disk; the exact concentration of extract can be added directly to the well removing the load, dry, load steps associated with disk diffusion.

1.3.3 Broth dilution assay

The broth dilution method can determine the minimum inhibitory concentration of an antimicrobial compound, this is the minimum concentration required to inhibit the specified percentage of the bacterial inoculums. For example, the MIC_{50} is the concentration of the antimicrobial which will inhibit 50 % of bacteria in the sample (111). A sample is diluted in series at known concentrations, each of the samples are then added with bacterial inoculums and incubated accordingly. This antimicrobial concentration is then assessed spectrophotometrically comparing absorbance to the negative control containing bacteria and media only (109).

A minimum inhibitory concentration (MIC) method can be conducted on a 96 well microtiter plate (51). Extracts corresponding to 5 g of seaweed were dissolved in 2.5 mL of TSB broth; 200 µL of this enriched broth was transferred into the first column of the plate. 100 µL of TSB broth was added to the remaining wells. 100 µL of enriched broth from the first column was added to the second column, resulting in a 1:2 dilution of the extract; this trend was continued for all the columns except for the last which was to be used as a control. Finally 100 µL of $1x10^6$ CFU/mL suspension was added to each of the wells. The results were then obtained spectrophotometrically finding that *E. spirulina* was the most effective with a 97.58 % and 81.95 % inhibition against *E. faecalis* and *L. monocytogenes*, observed respectively (51).

The analysis of an antimicrobial extract against the bacteria *Pseudoaltermonas bacteriolytica* by placing 100 µL of a 1:160 dilution of *P. bacteriolytica* broth grown for 24 h into a 96 well sterile microtiter plate. 100 µL of extract was dissolved in methanol and broth and added to each well. The well volumes were then diluted 1:1 so that the MIC_{50} could be calculated. It was found that 95 % of Indo-Pacific and 90 % of the Atlantic species of seaweeds used displayed selective antimicrobial activity against *Pseudoaltermonas bacteriolytica, Lindra thalassiae, Dendryphiella salina, Halophytophthora spinosa* and *Schizochytrium aggregatus* (6).

Broth dilution method has advantages over the diffusion methods of antimicrobial assessment, as the determination of MIC is more sensitive and quantitative. However, as with the well diffusion method, the samples must have some solubility in aqueous solvents as organic solvents are typically toxic to microbes. The broth dilution method performed in a 96 well mircotiter plate is more expressive than the typical measurement of zones of inhibition in terms of area, however this method is not as rapid.

1.3.4 Bioautography

Bioautography is a simple and effective technique for the separation and subsequent screening of antimicrobial activity from an extract. It involves the initial separation of the extract via thin layer chromatography (TLC) which is then aseptically placed in a sterile petri dish and layered with inoculated media. Any antimicrobial fractions will appear as clear, inhibited zones which can be measured on their own, or visualised more clearly using a stain such as 2,3,5-triphenyltetrazolium chloride (TTC) which converts to the pink coloured formazan on reaction with living microbes (112). Bioautography has the advantage of being able to separate antagonistic or bioactive compounds from one another, potentially uncovering further activity (113). Some examples of this technique are:

Six antimicrobial compounds were extracted from the plant *Piper betle L.* and found to be antimicrobial in nature via bioautography against MRSA and vancomycin resistant *Enterococcus* (VRE). Ethanol extracts of this leaf revealed two antimicrobial fractions via bioautography. These fractions were then identified using gas chromatography-mass spectroscopy (GC-MS) analysis (114).

A study investigating the seasonal variations of bioactives from seaweeds compared the typical disk diffusion antimicrobial assay and bioautography found that bioautography was a more sensitive method (50). This method included spotting 30 µL of extracts from *U. lactuca* onto a silica TLC plate. Post separation and bioautography, the plates were treated with TTC resulting in active 'bands' which can be viewed in Figure 1.5.

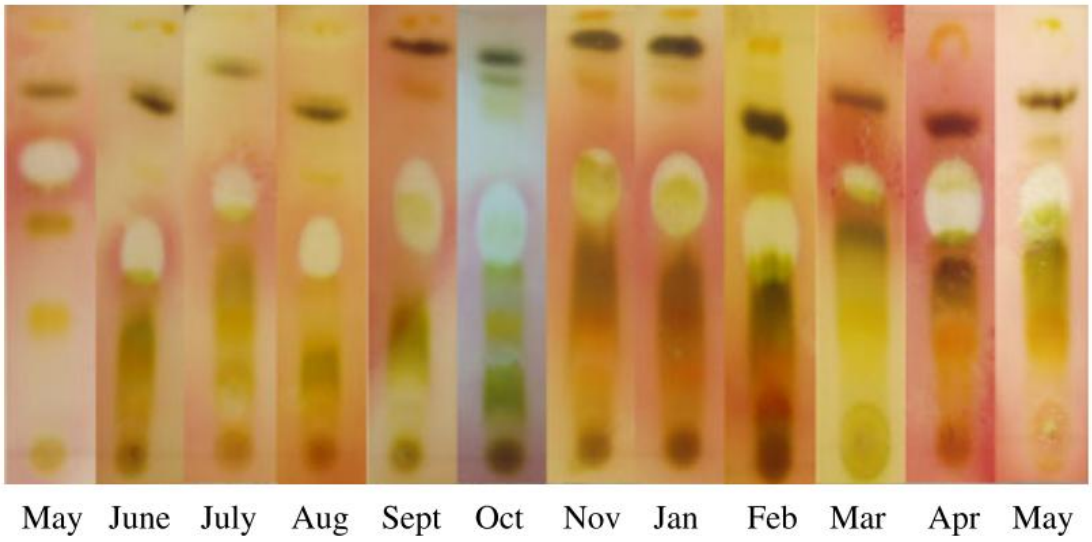

Figure 1.5: Seasonal study using bioautographic separation of EtOAc extracts from *U. lactuca* (50).

Bioautography, a more descriptive and sensitive method compared to disk diffusion (50), can be a helpful method in separating bioactives from any potential bioactive antagonists in the extract. It also gives a means by which the R_f value of the bioactive in a particular solvent system can be calculated giving an easy scale up to preparative TLC (57). This information can be obtained to indicate the polarity of the compound giving a hint as to the class of compound displaying bioactivity.

1.4 Structural elucidation of bioactive natural compounds

The nature of the pharmaceutical and medical device industries are highly regulated and as such, do not leave room for any unpredictable variations in composition that natural

products can have. Considering this, it is generally thought that bioactive compounds found in extracts of natural products should be purified and identified. Regulating bodies such as the FDA have highly regulated standards of approval which make it difficult to approve pharmaceuticals with natural variation. As such, bioactive compounds discovered in natural products have been synthesised to remove some of this natural variation (115).

Many spectral techniques can be used for the structural elucidation of compounds, such techniques can analyse data generated against a 'bank' of data for known compounds. For example the global natural products social networking database (GNPS) (116) and the National Institute of Standards and Technology (NIST) (117) are online databases with spectral libraries against which natural product data can be compared.

Spectroscopic techniques such as Fourier transfer infrared (FTIR), ultraviolet-visible (UV-Vis), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy can be used to aid the structural elucidation of compounds. UV-Vis and FTIR analysis rely on the principal that specific bonds absorb light at specific wavelengths, as such; the wavelengths at which bioactive compounds absorb light can give information on the nature of the bonds present in the compound and, thereby, indicates the functionality present in the molecule.

UV-Vis spectroscopy is based on the principle that electrons in molecules absorb energy and become excited by photons into higher energy states. The amount of energy absorbed is directly proportional to the wavelength or frequency of the light transmitted through the sample (118). This information can then be used to determine the nature of the bond present in the sample. The Woodward-Hoffmann rules were devised based on this where the maximum wavelength absorbed by a molecule can be predicted based on the bonds present in said molecule (119). For example electron n-π^* transitions that typically occur in carbonyls under the influence of UV-Vis light generally occur at wavelength of 270 - 300 nm for saturated carbonyls and 300 - 350 nm in conjugated systems (120).

FTIR spectroscopy works on much the same principle as UV-Vis spectroscopy, albeit with a longer wavelength of light. This lower energy light does not have the energy to excite electrons, however, at specific frequencies, bonds can absorb energy causing them to vibrate and/or rotate. As specific bonds and functionality absorb specific

frequencies of light, this gives information about the functionality present in the molecule.

Mass spectroscopy is frequently used in the structural elucidation of organic compounds, and in particular, to find the molecular weight of compounds. It is based on the principle that positive ions separate based on mass/charge ratio in a magnetic field. Positive ions of the compound are produced via hard ionisation (electron impact ionisation (EI)) or soft ionisation techniques (electrospray ionisation (ESI)). Soft techniques typically result in the addition of a proton to the molecule, and can be detected as the molecular mass of the compound plus one. Hard ionisation techniques result in the generation of a positive ion by the removal of an electron, this results in a much more unstable species which often fragments, and can be characterised based on the fragmentation pattern (121). Examples of FTIR, UV-Vis and LC-MS spectra can be visualised in section 6.3.5. of this work.

^{1}HNMR and ^{13}CNMR can be used for the structural elucidation of organic compounds; this technique requires atoms with an uneven number of protons such as hydrogen or radioactive carbon (C^{13}). This technique provides information on the relationship between carbon and hydrogen atoms in a molecule (36). With a combination of data including the chemical shift at which a peak occurs and the splitting pattern of the peak, functional groups in the structure can be determined and pieced together. NMR is a useful technique, and is used in conjunction with other techniques to determine the structure of novel compounds (113). While all of the mentioned techniques are useful for the structural elucidation of compounds, they must be used in tandem to correctly determine molecular structures.

Phlorotannins from *Sargassum fusiforme* were structurally elucidated using ultra high performance liquid chromatography, diode array detector, triple quadrupole mass spectroscopy (UHPLC-DAD-QQQ-MS) and Ms/Ms profiling (122). These techniques were used to obtain the molecular weight and MS/MS fragments of several phlorotannins separated by UHPLC. The fragmentation patterns were then used to identify known phlorotannins present in *S. fusiforme*, including fuhalols, fucophlorethols, eckols and phlorethols. A novel isoflavone 3-(4-(glucopyranosyloxy)-5-hydroxy-2-methoxyphenyl)-7-hydroxy-4H-chromen-4-one 1 was isolated and

structurally elucidated from *Ononis angustissima L* using several NMR techniques (123).

An antimicrobial polysaccharide (Figure 1.6) was extracted from the fungus *Hericium erinaceus* and structurally elucidated using FTIR, GC-MS, ^{13}CNMR and ^{1}HNMR spectroscopy (124). FTIR was used to identify functionality and to compare the spectra of the extract to the known monosaccharides, fucose, galactose and glucose. Once the identities of the monosaccharides were confirmed, their position and branching were established using NMR analysis.

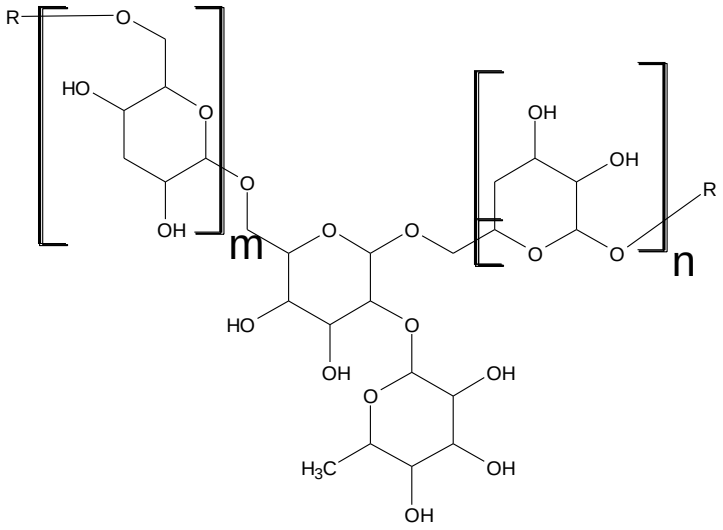

Figure 1.6: Proposed polysaccharide structure for antimicrobial compound from the fungus *Hericium erinaceus* (124).

1.5 Wounds

The skin serves as a barrier to protect the interior of the body from environmental hazards such as infection and debris. A rupture in the skin which disrupts its structure and function due to illness or injury can be described as a wound (125,126). Wounds can have a detrimental effect on an individual causing disability and mortality.

Wounds are classified as acute or chronic depending on the length of time in which it takes them to heal. Acute wounds typically heal in 8-12 weeks (127) and are caused by the tearing of skin via mechanical action which includes penetrative traumas such as

knife, gunshot and surgical wounds. Acute wounds can also include burns of skin caused by fire or chemicals. Thermal burns can vary in their degree and treatment depending on the temperature, extent of the burn and how many layers of the skin are damaged. Acute wounds follow the normal stages of healing (127).

Wounds are categorised as chronic when they require greater than 12 weeks to heal (127). Chronic wounds do not advance through the typical stages of healing in a timely manner. This can be due to an impediment in the progression from one stage to another or due to various external factors that can arise such as repeated injuries, necrosis, uncontrolled inflammation, underlying physiological conditions such as diabetes, infection and other conditions which compromise the immune system of the patient (125,128). Up to 60 % of chronic wounds are possibly kept in the inflammation stage of healing due to the formation of a bacterial biofilm (129).

1.5.1 Wound Healing

1.5.1.1 Haemostasis

Ruptures in the skin often cause bleeding from damaged blood vessels and platelets in the blood coagulate to form a plug to the new wound. This formation is the body's first line of healing as it stops the loss of blood through the wound site and protects the site from further intrusion of debris or microorganisms. As such haemostasis is successful when the wound is plugged and a matrix, whereby cells can travel during the healing process, is provided by a fibrin clot (128,130).

Clots form via the crosslinking of fibrin fibres with thrombospondin, fibronectin and vitronectin present in blood plasma (130). Fibrin fibres are produced by the enzymatic cleavage of the protein fibrinogen by the enzyme thrombin which is produced by surface activated platelets (131). Platelets present in circulating blood are activated by environmental factors and are embedded in the fibrin clot matrix to release cytokines and growth factors on degranulation (132).

1.5.1.2 Inflammation

Platelet degranulation along with the introduction of bacterial proteins at the wound site signals for white blood cells and neutrophils, which induces monocytes, to migrate to the wound from circulating blood. Exudate, a liquid containing nutrients, electrolytes, inflammatory mediators, proteins, enzymes and growth factors are produced at this stage in the healing process. Exudate provides a moist environment for optimum healing, however too much or too little exudate affects the appropriate healing of wounds. Therefore, exudate must be observed and managed as a part of wound treatment (133).

The established role of neutrophils is thought to clear the wound site of bacterial invasion and dead tissue via the generation of reactive oxygen and nitrogen species (134), while encouraging the migration and activation of monocytes, cytokines and chemokins to the wound (130,135,136). Both neutrophils and monocytes are large phagocytes which are some of the first white blood cells to respond to injury and are responsible for a cascade of inflammatory responses. Inflammation is an important factor in wound healing, as it facilitates the synthesis of a new extracellular matrix (137).

Neutrophils are the most abundant white blood cell, as such they are present immediately after skin trauma occurs causing bleeding (138). They themselves produce low levels of pro-inflammatory mediators. However, their true inflammatory response relies on their ability to summon monocytes from circulating blood (136). As mentioned, research has shown that neutrophils can themselves be a source of pro-inflammatory cytokines which activate local keratinocytes and fibroblasts early for cell repair (130). Neutrophils are the main leukocyte involved in the early stages of the healing process. Unless the wound is infected, they stop accumulating in the wound after a few days, after which exhausted neutrophils are phagocytosed by tissue macrophages (130).

Monocytes are white blood cells which originate in bone marrow. These cells patrol the blood and can differentiate into macrophages according to environmental stimulus. Monocytes instigate inflammation by secreting cytokines. Once this vital part of the wound healing process has started, monocytes can change their phenotype to form microorganism hunting macrophages which clear wound debris. These macrophages for

the phagocytosis of bacteria continue to gather monocytes from the blood (130). Monocytes can also differentiate into anti-inflammatory macrophages for aiding tissue repair and controlling inflammation (139,140). Activated macrophages can release cytokines and growth factors, thus continuing the work of earlier neutrophils (130). A schematic diagram of this process is presented in Figure 1.7.

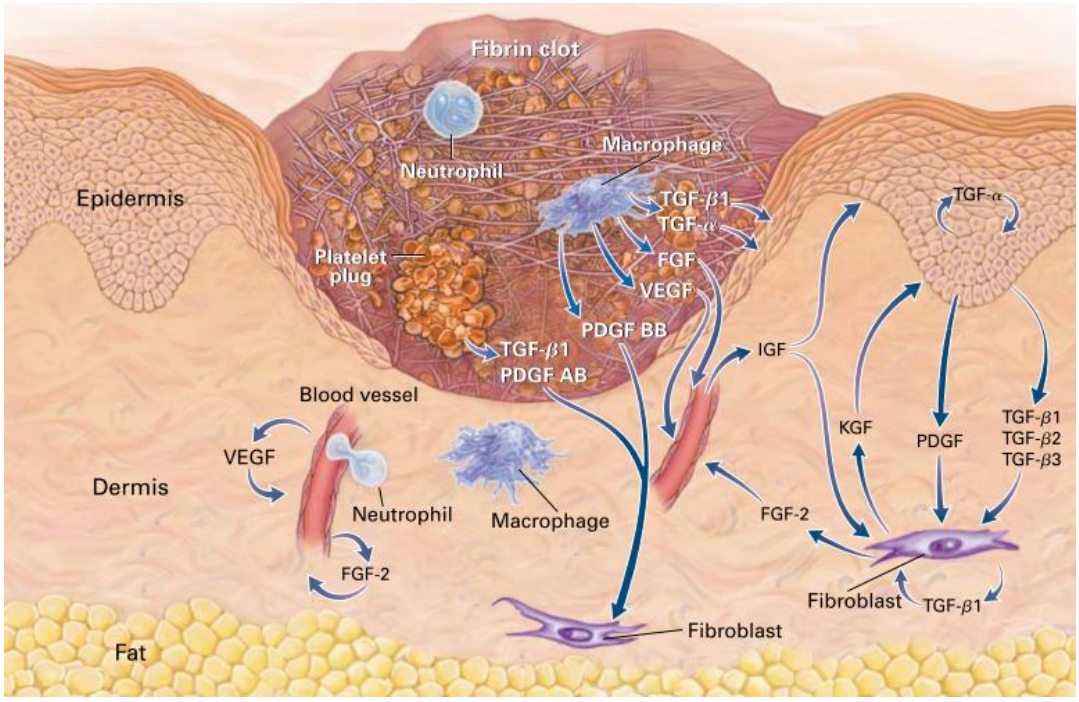

Figure 1.7: Schematic diagram of the inflammation phase in day three of wound healing (126).

However, neutrophils and monocytes are also capable of generating oxygen radicals to clear the wound of debris. These radicals are highly reactive and can compromise the integrity of healing tissues (135). In addition, neutrophils and monocytes can and do encourage inflammation by signalling for inflammatory cytokines. While inflammation is an important step in wound repair, it can be harmful in the later stages of healing where uncontrolled inflammation can be a feature of chronic diseases (141) and be responsible for scarring (136).

1.5.1.3 Reepithelialization

Subsequent to the formation of the clot and the recruitment of neutrophils/monocytes to the wound site for inflammation, reepithelialization begins just hours after injury (126). The fibrin clot allows epithelial cells to migrate through the matrix. Epidermal cells and skin appendages such as hair follicles quickly work to remove clotted blood and damaged tissues from the site. An eschar (scab) forms on the surface of the wound site for the continued protection of the wound (142). Meanwhile a dissolution phase begins in the wound whereby hemidesmosomal bonds are broken between the epidermal layer and its supporting membrane. This allows for lateral movement of epidermal cells in the fibrin clot and facilitates interactions between epidermal cells, proteins and stroma type 1 collagen (126,130). The transfer of epidermal cells acts to physically dissect viable tissue from the non-viable tissue. During this stage, the epidermal cells also produce collagenase and plasminogen activator, both of which act to degrade collagen and other proteins within the extracellular matrix facilitating the migration of epithelial cells.

Fibroblasts, endothelial cells and keratinocytes are meanwhile proliferating within the extracellular matrix. These cells secrete and synthesise a number of mediators responsible for the stimulation and control of the extra cellular matrix synthesis, reepithelialization and angiogenesis.

Granulation tissues invade the wound site to form a new stroma for the healing of the dermal layer. This new tissue has a granular appearance due to the many capillaries which invade the area forming a vascular network. Numerous growth factors are released at this stage promoting angiogenesis (130). The stroma itself replaces the fibrin clot over time and is fabricated mainly from fibroblasts. Fibroblasts are present in the surrounding tissues and actively divide at the edge of the exposed dermis. They are also recruited from fibrocytes circulating in the blood stream and from bone marrow progenitor cells (138)(128).

Epidermal cells proliferate at the edge of the wound site to cover the denuded area with a monolayer of keratinocyte's (epidermal cells) thus ending the reepithelialization stage of wound healing (126,130,142). As well as the proliferation of epidermal cells, bulge stem cells present in the outer root of hair follicles commit to form epidermal phenotypes in the event of injury, aiding the reepithelialzation process (138).

By the end of the reepithelialzation stage, the site is slightly raised at the surface and lacks any skin appendages such as hair (142). Underneath the skins surface, collagen degradation and synthesis is ongoing as the wound enters the remodelling phase.

1.5.1.4 Remodelling

The remodelling of a wound takes place subsequent to the injury and can take years to occur. Disorganised collagen in the extra cellular matrix is continually being degraded and synthesised to increase the tensile strength of the wound site (142). Macrophages still present in the wound continue to secrete growth factors such as TFG-β1 which encourage fibroblasts to produce and remodel the collagen-rich extra cellular matrix forming a more robust site (130). The mechanical forces involved in the contraction of the wound alongside the activation of fibroblasts by TFG-β1, transforms fibroblasts into myofibroblasts. Myofibroblasts express α-smooth muscle actin, which are capable of further contracting the wound site (130). This contraction of the wound site serves to bring the margins of the wound closer together, resulting in a smaller scar area (143). Collagen deposits increase in density over time as macrophage and fibroblasts reduce in concentration at the wound site and capillaries stop growing in the site (143). Remodelling often results in fibrosis (a scar) which contains no skin appendages and little circulation.

1.5.2 Wound Infections

Wounds make people more vulnerable to infection as pathogens can penetrate the body far more easily when there is an impairment in the skin. Understanding the nature of the pathogens that infect wounds is key to developing a treatment that will improve patient healing. The types of pathogens that infect an open wound can depend on the types and acquisition of the wound. Often, bacteria that are naturally found on the skin such as *S. aureus* can infect a wound. Typically these infections can be dealt with by the body's immune system or easily with the aid of a healthcare professional. They can, however, become an issue if the wound is infected with antibiotic-resistant bacteria or in the case of immuno-compromised patients.

As mentioned, white blood cells present in the circulatory system are capable of phagocytosis. Low levels of bacteria in wounds has the benefit of encouraging the bodies inflammatory response and kick starting the healing process (130). However, the body's immune system can be overwhelmed by infection, which may require topical or systemic antimicrobials to treat.

The complexity of a wound infection is directly associated with the number of cells that have inoculated on the wound site and the virulence of the invading microbial species (144). The ability of the host to defend itself against infection is also a factor in wound infection acquisition (144).

There are five progressing stages to an infection: contamination, colonisation, local infection, spreading infection and systemic infection. Contamination and colonisation require vigilance but can often be overcome by the patient's immune response and without treatment (129,145). Biofilms may form on advancement of a local infection, resulting in more cumbersome treatment for the wound (129). Local infections are generally identified according to; changes in pain and exudate oedema and the presence of friable bright red granulation tissue. The presence of lesions around the wound, lymphangitis, redness which reaches greater than 2 cm from the wound edge, and general malaise are indicators that the infection has spread (129,145). Severe wound infections are generally treated with antibiotics (146).

A 2015 survey of 1759 patients presenting with infected wounds in the UK found that 72.4 % of the pathogens collected were *S. aureus*, 15.8 % of which were MRSA. Other common wound pathogens include *b-Streptococci*, *Streptococcus pyogenes*, *Enterococci*, *Enterobacteriaceae* and *Pseudomonas aeruginosa* (147). Two separate studies, one in 2001 (148), and one in 2010 (146), support these species as typical bacteria found in wounds. It was also highlighted that wounds should be treated for a broad spectrum of microorganisms as wounds are often infected with a multitude of both Gram positive and Gram negative bacteria (146).

1.5.2.1 Wound Pathogens

The types of pathogens that infect an open wound can depend on the types and acquisition of the wound. Surveys carried out on wound pathogens have revealed pathogens that are typically associated with infected wounds (148–152)

1.5.2.2 Staphylococcus aureus

Staphylococcus aureus is a bacteria commonly associated with wounds. A report commissioned by the European Centre for Disease Control and Prevention (ECDC) identified *S. aureus* as one of the ten most common microbes to infect European wounds, with it being present in 10.2 % of wound infections in 2013 (152). Another Ethiopian study found that *S. aureus* made up 41.6 % of pathogens in the wounds of patients from 2003 to 2010 (49). *S. aureus* can live on the skin and in the mucous membranes of healthy individuals. It is estimated that 20 - 30 % of the population are carriers of this organism and that 80 % will be infected in their lifetime (52).

S. aureus is a Gram positive bacteria that has a diameter between 0.7 - 1.2 μm. It develops circular golden cultures that form in grape like clusters shown in Figure 1.8 (53). The golden pigment associated with *S. aureus* is caused by antioxidant carotenoids which protect the cell from oxidants produced by the immune system (54). The ability of *S. aureus* to excrete cytolytic toxins that form β-barrel pores causing the lysis and death of host cells is the cause of disease. *S. aureus* also secretes an antigen toxin, which can cause gastroenteritis and toxic shock syndrome upon ingestion (54). In addition, they also produce coagulase and haemolysins which convert fibrinogen to fibrin, clotting the hosts blood and thus protecting the bacteria from the host immune system which travels via the circulatory system (55).

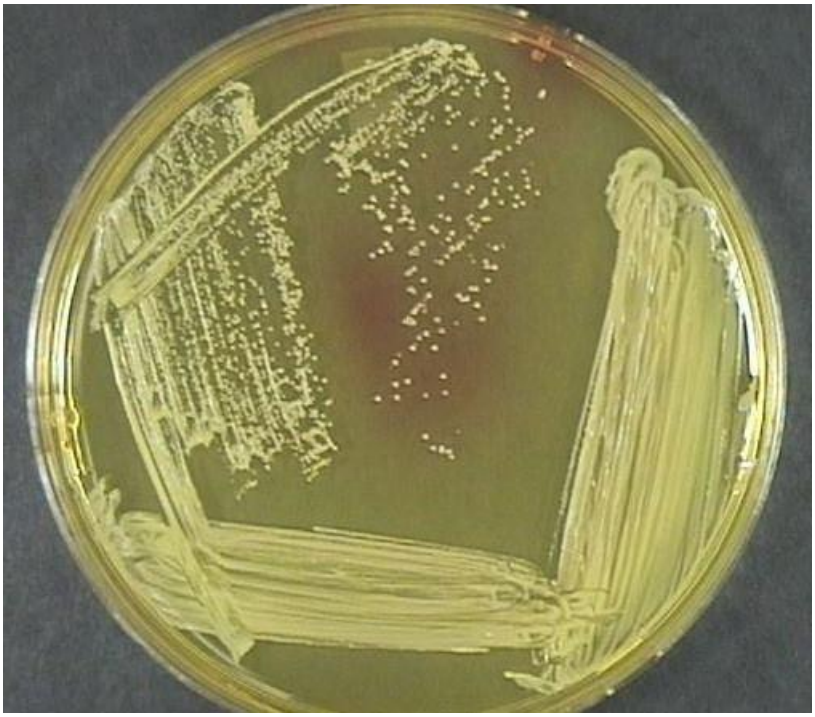

Figure 1.8: *S. aureus* grown on mannitol agar (157).

Antibiotics based on the β- lactam ring have been used to treat *S. aureus* infections. As with all antibiotics based on penicillin, the β - lactam ring binds with transpeptidase, the enzyme responsible for peptidoglycan synthesis. As peptidoglycan is a major component of the cell wall, the cell undergoes lysis and dies (54). This will be explained in greater detail in section 1.5.3.

Resistance to these antibiotics began when *S. aureus* developed the enzyme, group β-lactamase. These enzymes bind to the β - lactam ring so that it no longer resembles the natural substrate of transpeptidase, d-alanyl-d-alanine of the peptidoglycan stem peptide. The second mechanism for resistance is where the penicillin binding proteins (PBP) in the cell are mutated and thus altered slightly. In this way the β - lactam ring has little to no affinity for PBP (56). The evolution of *S. aureus* has caused the development of methicillin-resistant *S. aureus* (MRSA) (54) and vancomycin resistant *S. aureus* (VRSA) (159).

1.5.2.3 Pseudomonas spp

Pseudomonas spp are a highly diverse Gram negative group of bacteria which are found in most terrestrial and aquatic environments on earth. They are opportunistic microbes and can act as pathogens commonly infecting open wounds, eyes and lungs. *Pseudomonas spp* commonly infect wounds. It was found in a survey that *Pseudomonas spp* accounted for 6.8 % of wound microorganisms according to the ECDC in Europe during 2013 and 18.4 % of pathogenic isolates in wounds from 2003 to 2010 in Dessie regional laboratory, Ethiopia (49). Another survey found that *Pseudomonas spp* accounted for 21.21 % of pathogens found in the wounds of 2458 patients swabbed in the University of Port Harcourt Hospital in Port Harcourt, Nigeria (149).

Pseudomonas spp are 2-4 microns in length and are rod shaped bacteria possessing polar flagella, which are responsible for the motility of the bacteria. The cultures form in green clusters as can be seen below in Figure 1.9, due to the presence of the pigments: pyocyanine (green-blue) and pyorubrin (yellow-green) in the media (*Pseudamonas* selective agar) (160).

Figure 1.9: *Pseudomonas aeruginosa* grown on *Pseudomonas* selective agar (161).

Pseudomonas aeruginosa is a strain of *Pseudomonas spp* which commonly infects wounds. Its pathogenic properties are due to its secretion of exoenzymes, which cause the rearrangement of the host cells cytoskeleton protein actin, leading to host cell death (162). Exotoxins are also secreted by *Pseudomonas*, the toxins that penetrate the cell are phospholipases which cause cell death (163).

Antibiotic resistant *Pseudomonas spp* have been in circulation for years, indicating that there is need for a new antibiotic that has the ability to stop this type of infection (164). As one 2015 study found that 90 % of the *P. aeruginosa* tested were either multidrug resistant or extremely multidrug resistant (165), in addition to *Pseudonmonal* infections being observed in >35 % of the fatalities corresponding to infections in one review (166), this is becoming a serious issue.

1.5.2.4 Escherichia coli

E. coli typically inhabits the intestines of humans and animals without harm to the host. However, *E. coli* can become pathogenic on ingestion, or on contamination of a wound. *E. coli* is a Gram negative bacteria with the shape of a short rod. They form circular clusters as shown in Figure 1.10. It was discovered that *E. coli* made up 34.4 % of the microbes in infected wounds in Europe in 2013 (152) and 16.4 % of pathogens in the wounds of patients from 2003 to 2010 in Dessie regional laboratory, Ethiopia (49)

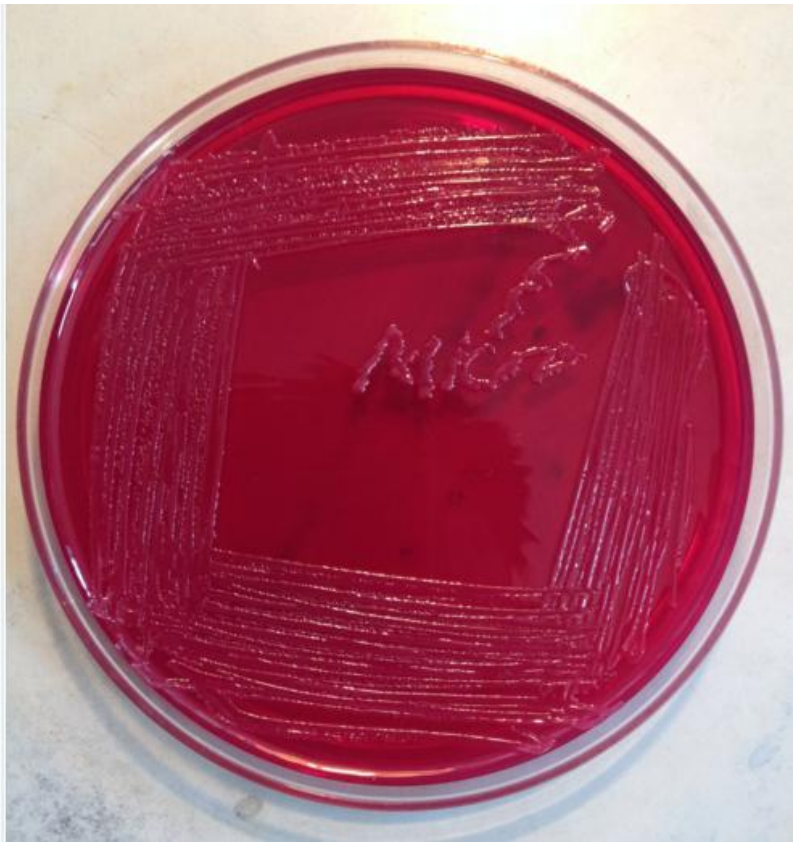

Figure 1.10: *Escherichia coli* on MacConkey Agar (167).

Pathogenic strains of *E.coli* can form a biofilm over the site of infection, ensuring colony adhesion to the site. The secretion of cytotoxins and enterotoxins such as the Shiga toxin which slices RNA in host cells, thereby, stopping protein synthesis, leads to cell death. Toxins stopping host cell divisions such as cytolethal distending toxin and cycle-inhibiting factor toxin also give *E.coli* its pathogenic status (168).

Pathogenic *E.coli* infections are typically treated with cephalosporin antibiotics (169). Cephalosporin antibiotics work in much the same way as β-lactam antibiotics. *E.coli* is also acquiring antimicrobial resistance. A study of 767 strains of *E.coli* isolated from sewage treated with 24 different antibiotics, found that 40 % of the overall samples were resistant to one or more of the antibiotics used (169).

1.5.2.5 Biofilms

Biofilms are the production of a polymeric matrix by bacteria that aggregate for their inhabitation and their improved adherence to surfaces. Bacteria produce biofilms for a myriad of reasons, however, this optimisation of their environment can make bacterial infections difficult to treat (170). Materialisation of these three dimensional bacterial structured communities with natural antimicrobial resistance certainly promotes the survival of bacterial cultures, including those with pathogenic tendencies (171,172). Biofilms were first noted on the teeth in the form of a sticky plaque, which develops on the aggregation of several species of oral flora.

Biofilms result in clusters of sessile microbial cells surrounded by a matrix becoming affixed to a surface to the extent where they cannot be removed via gentle rinsing. The matrix differs depending on the specific organism involved but typically is composed of a polysaccharide material (173). Microorganisms account for only 10 % of the dry mass of the Biofilm, with the remainder being the surrounding matrix. There can be mineral crystals, debris and blood components incorporated into the biofilm depending on the nature and environment of the microorganisms in question. Biofilms facilitate gene transfer between bacterial cells, promoting the evolution of antimicrobial resistance (173).

Biofilms can form in wounds at the local infection stage of a wound infection. At this stage they interrupt the inflammation stage of the healing process resulting in a delayed or halted repair of the wound site (129). The presence of a Biofilm results in an infection which the immune system is rarely able to resolve (174).

Biofilms are often treated with antibiotics, however they can respond inconsistently to treatment due to the protective effects of the biofilm (174). Bacteria protected by the biofilm have a higher antibiotic tolerance than their planktonic counterparts (170). This is due to a number of possible reasons such as: antibiotic permeation, eflux pump systems, varying environments within biofilm and the presence of free radical scavengers within the biofilm.

Antibiotics do not operate well in the biofilm environment. Different physical environments can exist within a biofilm to cater for a diverse ecosystem of bacteria (172).

Some antibiotics cannot tolerate the variation in environmental factors such as pH, viscosity, and anaerobic pockets. For example, macrolids and tetracycline antibiotics are inactive at low pH, while aminoglycosides are inactive in areas of low O_2 for biofilms formed by anaerobic bacteria (170,174). Bacteria in biofilms often have efflux pump systems which actively remove antibiotics from the cell, making them harder to eliminate and contributing to antimicrobial resistance (170,175,176).

Nutrients are not distributed evenly in the biofilm leaving pockets of malnourished bacteria. These cells slow down and enter a starvation response leaving them dormant and less susceptible to antibiotics. This results in layers of cells in the three dimensional biofilm matrix that can survive antibiotic activity (170,175,176). The bacteria in biofilms do not need to divide as rapidly as bacteria in their planktonic state. Many antibiotics act on bacteria during cell division lessening the potency of the antibiotic in biofilms (177).

Some bacteria produce enzymes which can be present in the biofilm and deactivate antibiotic compounds. For example, *P. aeruginosa* produces β-lactamase enzyme that lyses the β-lactum ring in penicillin-based antibiotics (170). Due to the above issues, bacteria in biofilms can be 10-1000 times more resistant to treatment by antibiotics (177).

As mentioned, the presence of a biofilm can prolong the inflammation stage of the wound healing process. This can be due to rhamnolipid-producing bacteria, such as *P. aeruginosa*, which are protected from phagocytosis by macrophages (170). As the bacteria are still present regardless of the immune response, more leucocytes are recruited to the site exacerbating local inflammation (170,174). The biofilm can also act as a scavenger for free radical oxygen species, adding to the protection of bacteria from the immune response (170).

1.5.2.6 Biofilm Disruption

As mentioned, the formation of oral biofilms in the form of plaque is the most noted and earliest recorded management of biofilm formation. Oral biofilms can form in 48 h, thus it is recommended that people engage in oral debridement twice a day (brushing, flossing etc.) (178). Biofilms require more than a gentle rinsing for removal as such,

biofilm debridement is a poor method for wound management as it further disrupts the wound site.

There is, however, research into anti-biofilm agents for the management of biofilms. Many of these agents can be impregnated into medical devices such as catheters to stop the formation of a biofilm (179). Agents such as lactoferin and EDTA, hinder the adhesion of biofilms onto a surface (178). Other agents such as gallium disrupt iron metabolism (178). Xylitol and dispersin B impair biofilm matrix development (178,180). Silver nanoparticles which are commonly used in wound dressings have the ability to detach biofilms from surfaces by destroying the polymeric matrix cell wall (181). It was speculated in one study that this could be utilised in the contact lens industry (182). A 2014 review also stated that biosurfactants can also be potent anti-biofilm agents and can be used for the treatment and management of biofilms formed by a broad spectrum of bacteria (183).

As plants and algae have to similarly contend with pathogenic biofilms, studies looked to natural sources for evidence of biofilm disruption. Extracts from marine sources have since demonstrated antibiofilm activity. For example, microscope slides treated with 100 mg/mL water extracts from *Ulva lactuca*, fabricated into zinc oxide nanoparticles, were proven to disrupt biofilms formed by *Bacillus licheniformis* (85 %), *Bacillus pumilus* (79 %) *E. coli* (82 %) and *Proteus vulgaris* (80 %) (184). Methanol extracts from *S. wightii* were found to reduce biofilms produced by; *V. parahaemolyticus* (80 %), *A. baumannii* (71 %), *S. aureus* (80 %) and *V. anguillarum* (76 %), better than ascorbic acid, the positive control in the study (68 %, 35 %, 46 % and 36 % respectively) (185).

1.5.3 Wound Infection Treatments

Contaminated and infected wounds are currently treated throughout the world with a number of various antimicrobial agents in different drug forms, such as systemic antibiotics or wound dressing formulations (186). Antimicrobials may be delivered via topical preparations or as part of a systemic antibiotic regime.

1.5.3.1 β-Lactam Antibiotics

β-lactam antibiotics are oral antibiotics that have been relied on heavily since the discovery of penicillin, the first antibiotic in the 1920's (187). Penicillin, and its derivative drugs, disrupts the synthesis of peptidogylcan (188). As Gram positive bacteria have higher levels of peptidoglycan in their cell wall than that of Gram negative bacteria, penicillin's are commonly used in the treatment of Gram positive infections (189).

The β-lactam ring in these antibiotics resembles the d-alanyl-d-alanine end of the peptidoglycan stem peptide, the natural substrate for the bacterial enzyme transpeptidase responsible for the biosynthesis of peptidoglycan in the bacterial cell wall. The inhibition of transpeptidase prevents the bacterial cell from re-establishing a cell wall after cell division, causing lysis of the cell and consequently cell death (189,190). The structural similarity between penicillin and the d-alanyl-d-alanine end of the peptidoglycan stem peptide can be seen below in Figure 1.11.

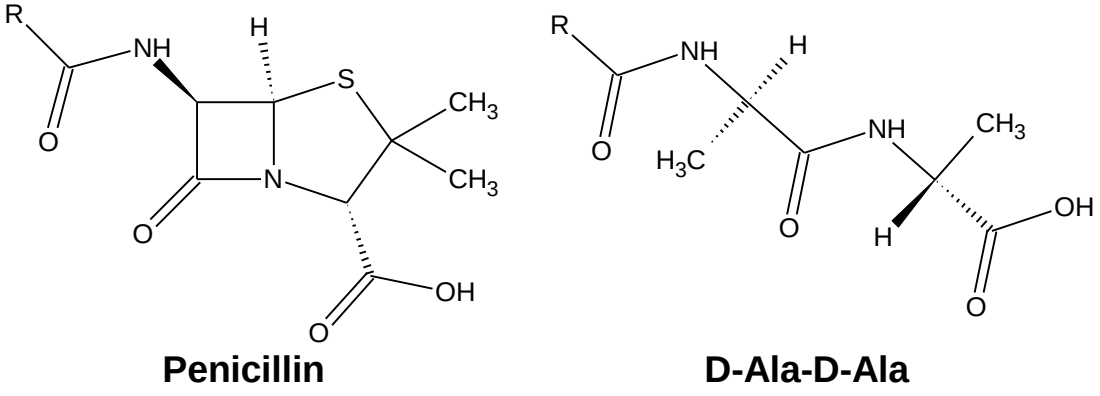

Figure 1.11: The structural similarity between penicillin and the d-alanyl-d-alanine end of the peptidoglycan stem peptide.

The structure of penicillin has been varied throughout the years in an attempt to stay ahead of the ever evolving antimicrobial resistance that many strains of pathogens are exhibiting (188). In these drug variations, the substituent's of the four membered β-lactam ring are varied to disguise the molecule from β-lactamase enzymes and penicillin

binding proteins (PBP) produced by the resistant strain. β-lactamase enzymes hydrolyse the sigma bond between the carbonyl carbon of the β-lactam ring and the α nitrogen (as can be seen in Figure 1.12) before the target enzymes for the drug are reached (190–192).

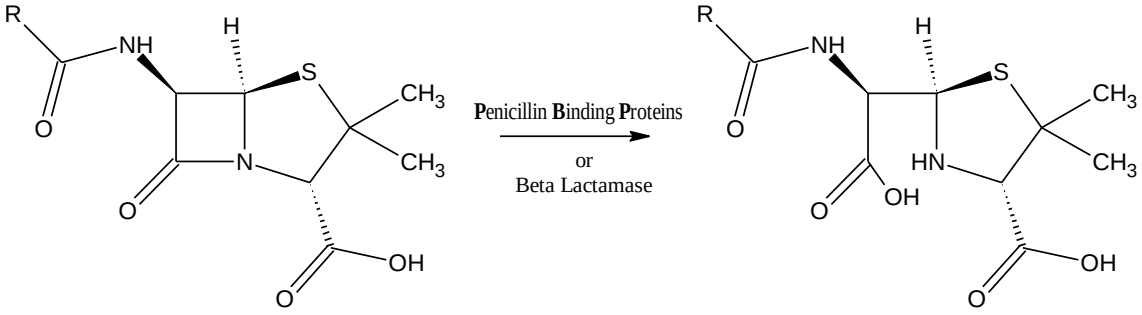

Figure 1.12: Mode of action for the inhibition of penicillin by β-lactamase (193).

β-lactamase inhibiting drugs such as clavulanic acid are included in the formulation with β-lactam drugs. The enzyme inhibitors attempt to bind to β-lactamase or the PBP, inhibiting the enzymes ability to render the antibiotic inactive (188). However, with the emergence of new forms of β-lactam resistance in strains such as MRSA, it has become apparent that new antimicrobials with different modes of action will be required for future management of wounds.

1.5.3.2 Vancomycin

Vancomycin is a potent antibiotic available since the 1950's that is active against Gram positive bacteria. Vancomycin is a large glycopeptide that is structurally dissimilar to other commercially available antibiotics as can be seen below in Figure 1.13. Its usage has increased in recent years due to the prevalence of MRSA, however vancomycin resistance has been noted in some strains of *S. aureus* as early as the 1960's (194).

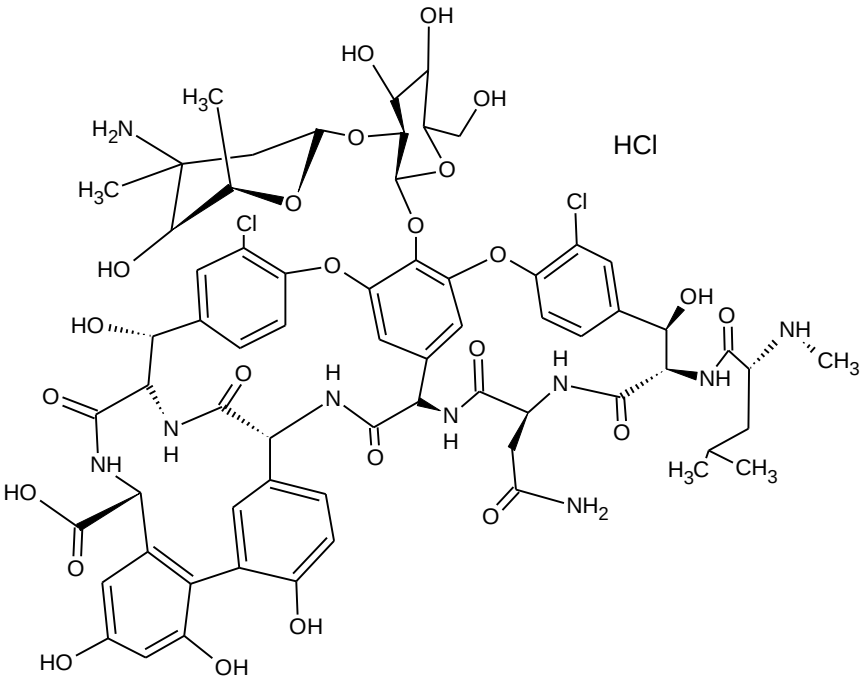

Figure 1.13: Chemical structure of vancomycin hydrochloride.

The mechanism of action for vancomycin is like that of penicillin as it relies on the prevention of peptidoglycan formation in the cell wall of Gram positive bacteria. Its mode of action differs, however, as vancomycin obstructs the second stage of peptidoglycan synthesis. Vancomycin has affinity for the d-alanyl-d-alanine at the free carboxyl end of the peptidoglycan stem peptide. Once bound to this peptide, the enzyme peptidoglycan synthase cannot catalyse the synthesis of peptidoglycan in the cell wall due to the steric hindrance that vancomycin presents. In this way, the building blocks for peptidoglycan are rendered inactive (194,195).

Without a means of peptidoglycan synthesis in Gram positive bacteria, cell lysis will occur causing death. However, bacteria are also evolving resistance to vancomycin. As vancomycin was relied on as a treatment for MRSA, many wound pathogens have developed resistance to this antibiotic. The microbial mechanism for resistance in *S. aureus* appears to be the bacterial alteration of the d-alanyl-d-alanine residue to d-alanyl-d-lactate. Vancomycin does not display high affinity for the d-alanyl-d-lactate residue rendering the antibiotic harmless to the cell (159).

1.5.3.3 Sulphonamide Antibiotics

Sulphonamide antibiotics are bacteriostatic in nature as they stop the reproduction of bacteria. Often, these drugs are more relied on in veterinary care as a proportion of the human population is allergic to the drug (196). The structure of sulphonamide antibiotics can be seen below in Figure 1.14. Drug derivatives are synthesised by varying substituent's from the thioamide nitrogen.

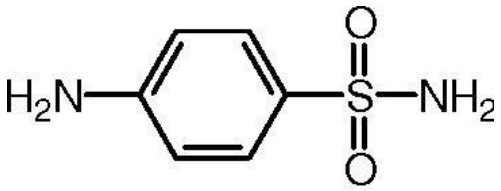

Figure 1.14: The chemical structure of sulphonamide (197).

Sulphonamides typically inhibit the synthesis of fatty acid hydroxylase 2 (FAH_2 or folate) in bacteria. FAH_2 is important in the biosynthesis of nucleic acid for DNA and RNA production in the cell. Without the ability to produce DNA, the cell can neither repair nor reproduce; this stops the spread of the infection and leads to eventual colony death. As folate is not synthesised in mammals but taken in through the diet, this mechanism does not harm the host (198,199).

The mode of action works in two ways; the drug inhibits the enzyme dihydropterorate synthase by acting as a pseudometabolite. This enzyme catalyses a reaction between p-amino benzoic acid (PABA) and 2-amino-4-hydroxyl-6-hydroxymethyl dihydropteridine diphosphate (AHHDD), the outcome of this reaction is a precursor to folonic acid. Sulphonamides also condense with AHHDD, removing AHHDD as a reactant in the synthesis of folate thus stopping the cell from synthesising folate (198,199).

Sulphonamide resistance has been demonstrated by bacterial strains which display the ability to reduce drug permeability into the cell. These strains also increase PABA production resulting in the enzymatic competitive inhibition favouring PABA as a substrate. In addition, the modification of the active site in the enzyme dihydropterorate

synthase so that it no longer recognises sulphonamide drugs as a substrate, can occur (199).

1.5.3.4 Silver

The antimicrobial properties of silver have been recognised since ancient times; in 400 B.C.E. (before common era) Hippocrates theorised that silver controlled disease (200). However the use of silver as a treatment in wound infections became more common in the 18th century when silver nitrate was used to treat wound ulcers (201). Studies since have recognised silver as an antimicrobial agent leading to it being accepted by the FDA in the 1920's (201).

Silver-based wound dressings are a common measure for wound management, particularly in the treatment of multi-drug resistant wound infections such as MRSA (202). Silver is used topically for infections due to its tendency to cause mortality, weight loss, immunological effects and the alteration of liver enzymes when taken in quantities greater than the daily tolerable intake of 2.5 µg/kg of body weight per day (203). The topical use of silver is less likely to result in a systemic overdose. However, many adverse side effects of the topical use of silver have been noted such as argyria, a blue discoloration of the skin (203). The cytotoxic and genotoxic side effects of silver nano particles were investigated in a study which suggested that the generation of reactive oxygen species damages DNA via mitochondrial dysfunction, causing this toxicity (204).

Topically, silver is used in either antimicrobial ointments such as silver nitrate ointment and silver sulphadiazine cream or it is incorporated into wound dressings which release silver ions to the wound over time (125). Silver nitrate ointment initially delivers a higher concentration of silver ions compared to silver sulphadiazine based creams, however as silver sulphadiazine releases the silver ions more slowly with a sustained release profile, it is the better option for preventing and managing wounds (205). Silver-based wound dressings also incorporate a sustained release profile, indicating that they are effective for wound management. An evaluation of several silver-based wound dressings against typical wound pathogens: *S. aureus, P. aeruginosa* and *C. albicans* determined that silver can be used as an effective treatment for wound infections (206).

A study evaluating the efficiency of silver-lined washing machines in controlling bacteria typically associated with contaminated fabric (*S. aureus and E. coli)* found that while the silver ions released during the wash cycle were effective against both bacteria, *S. aureus* was inhibited to a greater degree. It was speculated that this could have been due to the greater quantity of peptidoglycan in the cell wall of the Gram positive *S. aureus* compared to the Gram negative *E. coli*. This suggests that the mode of action of the silver ions is by compromising the cell structure of the bacteria causing cell death (207).

Silver, a known antimicrobial has been incorporated into many wound dressings on the market including Acticoat (206,208). It has been experimented with in wound dressing in nanocoatings (209), incorporation into gelling fibres (210) as well as the general incorporation of silver into nylon (205). Silver has the advantage of also having anti-biofilm properties (180,182). Still, silver has been proven to be toxic (203,204) and is expensive, resulting in the suitability of these dressings for short term use only.

Many bacterial strains are developing resistance to silver as an antimicrobial agent, indicating that although silver is effective, continued reliance on silver as a wound treatment is unsustainable (211). This in addition to the side effects of silver ions and the high cost of producing disposable wound dressings incorporated with the precious metal, indicate that there is a need for a new antimicrobial wound dressing in the market (212).

The advantages of using silver wound dressings is their broad spectrum antibiofilm (180,182), and antimicrobial activity against wound pathogens with minimal bacterial resistance (213). However silver dressings have the disadvantage of being expensive (213), and adverse effects such as liver toxicity (214).

1.5.3.5 Honey

Honey has been used as an alternative antimicrobial throughout the world. Due to the emergence of antibiotic resistant bacterial strains, the use of honey has been revisited by the medical community (215). In 2007 the FDA approved the use of honey in medical devices such as MediHoney^{TM} via a 501(k) (216), however this approval was specific for the dressing and not honey as a drug in its own right.

Honey is thought to inhibit bacterial growth due to its high osmolarity and its ability to restrict bacterial access to water. Some honeys produce hydrogen peroxide, broadening its ability to kill bacteria (148). A study conducted on honey showed that honey stimulates the production of pro healing inflammatory cytokines from monocytic cells. Monocytic cells are known for their importance in cell repair, indicating that honey improves the body healing response (215).

Honey has proven to be effective at killing many strains of different types of pathogens such as: *S. aureus*, MRSA, *Acinetobacter baumannii*, *Enterococcus faecalis*, Vancomycin resistant *Enterococcus* (VRE) [VR *E. faecalis* and VR *E. faecium*], *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae and P aeruginosa* (217). In particular, honeys' power to kill multi-drug resistant bacteria and its ability to aid the healing of chronic wounds make it a good candidate for hydrogel and wound dressings for the treatment of wounds (217–220) (221).

The use of honey as an antimicrobial is exclusively on the treatment of wounds due to its mechanism of action, requiring the infected area to be in contact with a high dose of honey (148). There is a spectrum of literature detailing products from neat honey, to ointment and dressing formulations such as MediHoney^{TM} (222). The natural origins of honey make it an attractive product for consumers, particularly as the consumer market is looking for more natural bioactives (223).

The antimicrobial efficiency of honey coupled with the fact that no toxic side effects of the use of honey as an antimicrobial agent have been reported, makes honey an excellent natural product-based antimicrobial (224,225). However, the demand for honey as a wound dressing is decreasing due to the availability of dressings with better market uptake such as alginate and foam based dressings (226).

Honey is inexpensive and rarely results in adverse effects however while effective against acute wounds, honey does not significantly improve healing time in chronic wounds (144).

1.5.3.6 Iodine

Iodine has been commonly used for over 150 years as a antiseptic (227), and has been noted for its rapid bactericidal, fungicidal, virucidal, tuberculocidal, and sporicidal activities (228). Considering this and its proven efficiency against wound pathogens

such as; *S. aureus*, *E. coli* and *P. aeruginosa*, it has been formulated into antimicrobial wound dressing preparations (229).

The mechanism of action for iodine as an antimicrobial comes from the liberation of molecular iodine (I_2) which rapidly penetrates microorganisms, damaging proteins, nucleotides, and fatty acids via oxidative stress leading to cell death (227,229). The binding of molecular iodine to thiol and sulphydryl groups present in proteins and enzymes can denature them, rendering them disfunctional (229). Phospholipid membranes are also altered by iodine blocking hydrogen bonding within amino acids in the membrane; this is devastating for the cell and rapidly results in cell death (228,230,231)

Iodine has been associated with irritation and staining in aqueous and alcohol preparations (227) over the years which has resulted in concerns over the potential toxicity of iodine as an antimicrobial for wounds, particularly with usage over longer periods of time (229). Additionally, iodine has been known to interact with medications such as lithium, increasing the risk of hypothyroidism (229).

Considering its broad range of activity and that there has been no reported bacterial resistance to iodine as an antimicrobial agent (232), it is an attractive active ingredient for antimicrobial wound dressings. Additionally iodine is less expensive than silver as an antimicrobial and more effective at treating wound infections than honey, however it does have the disadvantage of adverse effects (233).

1.5.3.7 Seaweeds as Antimicrobials

Most modern day antibiotics are based on the penicillin and sulphanilamide antibiotics invented in the 1920's. Pathogenic microbes are evolving immunity to these drugs, as such new drugs with a new mode of action need to be made available to the population. Natural products such as seaweeds are a rich source of bioactive compounds with the potential to provide for this gap in novel antimicrobial compounds (202,234,235).

An ethyl acetate extract of *Ulva lactuca* was found to be potently active against *Staphylococcus aureus, Bacillus subtilis* and MRSA in a study investigating the seasonal changes in seaweed bioactivity. Bioautography was determined to be a more

sensitive antimicrobial assay than disk diffusion for this investigation. The maximum inhibition of MRSA, *S.aureus* and *B. subtilis* was determined with the limitation of being season dependant (57).

Five antimicrobial compounds: chondriol, cycloeudesmol, pre-pacifenol, laurinterol, and debromolaurinterol were extracted from marine algae and tested against the microbes: *S. aureus, Salmonella choleraesuis, Mycobacterium smegmatis, C. albicans* and *E. coli*. All compounds successfully inhibited the growth of the Gram positive bacteria, suggesting that the mode of action is selective for Gram positive bacterial cell walls. Only debromolaurinterol and chondriol inhibited the growth of Gram negative strains of *S. aureus* and *C. albicans,* implying that these antimicrobials have a broader spectrum of activity (48).

S. aureus, B. subtilis, E. coli, and a yeast, *Candida albicans* were successfully inhibited in an investigation of extracts from the following seaweeds: *Valonia acgrophila, Halimeda Opuntia, Lamouroux, Halimedatuna, Caulepa racemosa, CauIerpa mexicana* and *Ulva pertlusa.* All seaweed extracts, barring that of *Caulerpa mexicana,* were found to be active against three or more of the four microbes tested. The extract from *V. acgrophila* exhibited the greatest antimicrobial activity and was determined to be a more effective antibiotic than the control, Penicillin G (52).

Acetone extracts of nine different algae were tested against *Micrococcus luteus* (inhibited by all nine algae), *B. subtilis, S. aureus, Ps. fluorescens, B. cereus, Br. catarrhalis, P. vulgaris, E. coli, and S. marcescens*; all extracts inhibited five or more microbes with Gram positive strains being more vulnerable to the bioactives in the seaweed extracts. This study speculated that bioactivity could be due to the high quantity of fatty acids present in the seaweeds, due to the extracts exhibiting similar inhibition to commercially available fatty acids (3).

Six edible Irish seaweeds: *Laminaria digitata, Laminaria saccharina, Himanthalia elongata, Palmaria palmata, Chondrus crispus* and *Enteromorpha spirulina* were solvent extracted and tested against the common food spoilage bacteria: *Listeria monocytogenes, Salmonella abony, Enterococcus faecalis* and *Pseudomonas aeruginosa.* All methanol extracts from the seaweeds were active against all four of the bacteria to various degrees. *Himanthalia elongata* was found to display the greatest

antimicrobial activity, as 6.40 mg/mL displayed similar activity to the controls: sodium benzoate and sodium nitrate (51).

1.6 Potential application of seaweed derived antimicrobials incorporated into wound dressings

1.6.1 Introduction to wound dressings

Wound dressings serve the purpose of protecting open wounds from external debris and pathogens, thereby giving the wound the opportunity to heal. The rate at which the wound heals depends on a number of local and systemic factors such as; presence of infection, presence of debris, poor blood supply, advanced age and vitamin deficiency (125).

The fundamental purpose of a wound dressing is to optimise healing by providing the appropriate conditions whereby the body can repair itself, and by managing exudate from exuding wounds. Traditionally, wound dressings comprised of a simple, clean absorbent material, with the sole purpose of being a barrier to protect the wound from the environment. However, as understanding of the nature of different wounds is increasing, so too is the development of specialised wound dressing materials (236). More recently dressings have been used to actively facilitate wound healing by acting as a platform for drug delivery (202,237). Although these more advanced wound dressings are by comparison more expensive than the traditional cotton or gauze dressings, they are thought to save money in terms of labour and wound management (237). As advanced dressings are engineered to facilitate changing once every few days rather than several times a day, this reduces disturbance of the wound healing process and there is less wound management required for a shorter period of time (237). As such, there is a greater variety of active wound dressing materials available on today's market, as indicated in Figure 1.15. This industry is thriving to the point where it is estimated that the bioactive wound dressing market will reach 13.5 billion USD by 2026 (238).

Conditions for the ideal wound healing environment have been extensively researched and it has been established that a moist healing environment provides optimum

therapeutic conditions for cell reepithelialisation (239). This is thought to be due to the free movement of epithelial cells in moist conditions (240). Modern dressings aim to facilitate this fact by providing moist conditions, and other pro-wound healing features include thermal insulation (241), pain reducing wound dressings (242), vapour exchange (243) and the incorporation of antimicrobial agents to prevent and treat wound infections (144,205,244,245). The conditions provided by the wound dressing for optimal healing is individual to the wound. There is no 'one size fits all' as dry wounds would require a more moist dressing, while exudate needs to be absorbed and managed in exuding wounds (239). Owing to the various needs of different types of wounds, patient care guidelines on selecting the appropriate wound dressing can vary according to Figure 1.15.

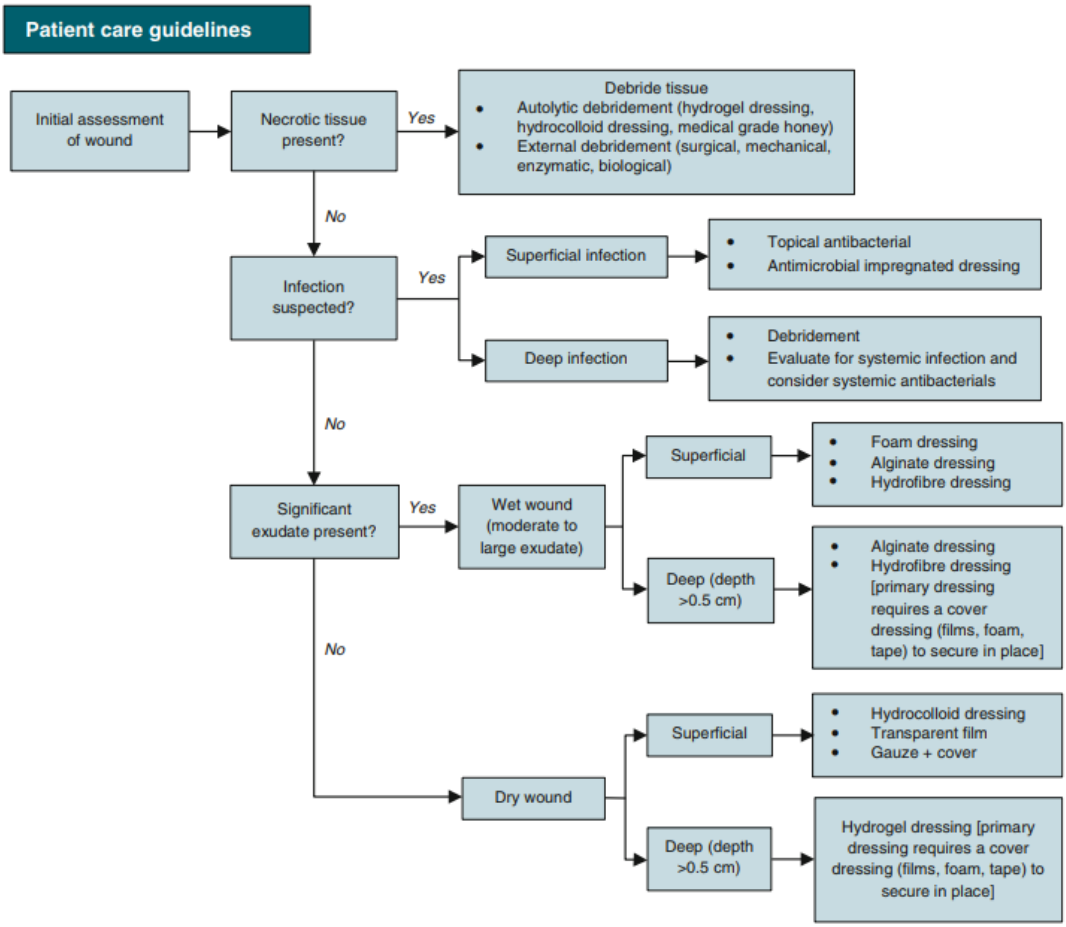

Figure 1.15: Wound dressing selection post assessment (246,247).

Wound dressing polymers from natural sources such as cellulose and pectin are valued due to their biocompatibility with topical application, mechanical tensile strength and biodegradability (237). They may also have therapeutic properties such as antimicrobial, anti-inflammatory and analgesic properties in their own right, making them attractive components for wound dressings (248). However, there are advantages to using synthetic components in these applications as synthetic polymers can be designed to have tight specifications. For example, pore size can be calculated and synthesised to a high degree of accuracy to promote the desired release profile of a drug (249).

1.6.2 Antimicrobial wound dressings

Antimicrobial wound dressings have obvious benefits as topical forms of drug delivery, particularly considering the issues around the systemic antibiotic contraindications on patients (250,251), and the increasing risk of antimicrobial resistance from antibiotic usage (252). Local drug delivery can be used to target higher concentrations of antimicrobials at the site of infection, negating systemic toxicity (253). There are two motives to include a bioactive, antimicrobial dressing for wound management; for the treatment of an active infection, or for prophylactic therapy.

Antimicrobial dressings for the treatment of wound infections habitually require a burst release of the antimicrobial to eradicate the initial infection, followed by a sustained release to protect against secondary infection (254). Conversely, prophylactic therapy is typical for the prevention of infection, particularly in wounds that get infected easily such as; chronic wounds for instance, diabetic foot ulcers (180), and burns in which the immune response is impaired and, therefore, carry a higher chance of infection (255). Prophylactic wound dressings require the antimicrobial to be released slowly over time to prevent the need for frequent dressing changes (254), and therefore, dressings made with prophylaxis in mind should feature a sustained release profile.

Considering this information, a 'smart' wound dressing with a burst release of the antimicrobial followed by a sustained release would be the most versatile and, therefore, desirable formulation for the treatment of, and protection from, wound infections (254).

1.6.2.1 Antimicrobial solid dressings

Solid wound dressings such as hydrocolliod, hydrofiber, film and gauze dressings are often used for the treatment of wet and dry wounds of varying severity (247). Inert gauzes are commonly used as primary wound dressings. However, many studies have been conducted on incorporation of bioactives in to these dressings to promote wound healing and control infections (220,236,245,256–258).

Bacterial cellulose is biosynthesised cheaply in high volumes by *Acetobacter xylinum.* Cellulose is an effective natural polymer used in wound dressings due to its mechanical strength (259) and its ability to encourage wounds to undergo granulation and epithelialization aiding in the natural healing process. Its biocompatibility and strength makes cellulose a suitable bioscaffold for severe wound types (260) such as, chronic wounds (261). Cellulose also has the ability to be incorporated with therapeutic drugs, demonstrated in a study by M. Ul-Islam *et al.* which demonstrated the fortification of bacterial cellulose composite films with antimicrobial montmorillonite that resulted in a wound dressing effective against *E. coli* and *S. aureus* (262).

One such study evaluated a hybrid of synthetic and natural wound dressings was synthesised and evaluated in a study concerning the healing of burn wounds on guinea pigs. In the study, a 50:50 poly[DL-lactic-co-glycolic acid] polymer was formed with homogenous pore size of 1.0 - 1.5 microns. This polymer was then backed with a collagen dressing and loaded with the antibiotic gentamicin. The collagen section of the dressing was intended to promote wound healing after the release of the drug. A burst release and a sustained drug release profile were generated by varying the pore size in the synthetic polymer. The guinea pigs were observed to heal faster with a slow drug release profile compared to commercial Melolin®, Aquacel®, and Ag controls (249).

Acticoat (Figure 1.16) is one of many commercially available silver based, three-ply gauze dressing, produced for the antimicrobial treatment of wounds (263). It has been shown to reduce the bio-burden of wounds from pathogens such as; MRSA, *S. aureus, E. coli, E. cloacae, P. vulgaris, P. aeruginosa* and *A. baumanii* (180,219,263)

Figure 1.16: Acticoat, commercially available silver antimicrobial wound dressings layered with cotton gauze and woven silver sheets (264).

1.6.2.2 Antimicrobial foams and gels

Foams, gels and ointments are sometimes recommended for the treatment of superficial wounds (247). Foams, gels and ointments can be used where frequent dressing changes and sometimes require a secondary dressing to fix them in place. They can be associated with unpleasant, malodorous discharge of the wound (186). Foams have been used to pack severe wounds, however they are not recommended due to complications with fragments of the foams being a source of toxic shock syndrome (265).

Plyhexamethylene biguanide antimicrobial foam dressings were found to significantly reduce the bacterial burden in chronic superficial wounds (266), however, this study recommended frequent dressing changes of as many as three times a week.

Ip *et al*. compared different commercially available wound preparations containing silver as an antimicrobial. Contreet, the only foam formulation tested proved better in the rapid reduction of MRSA than any solid formulations tested (263). However, this study was only performed over a 24 h period and, therefore, there was no indication of how this dressing would perform over a longer time.

Chitin is a natural amino-polysaccharide found in the hard shell of crustaceans and insects as well as in the cell-wall of fungi. Chitosan is a natural derivative of chitin, whereby chitin is dealkylated using the base sodium hydroxide. Chitin is a highly ordered crystalline polymer with little hydrophilic properties, however on dealkylation

to chitosan it gains the ability to swell. The formulation of wound dressings with different ratios of chitin and chitosan cause different favourable properties to be exposed (260). For example, one study found that 50 % dealkylation of chitin resulted in a water soluble substance similar to an ointment. Upon application of this ointment on wounds on mice ears, it was found to stimulate granulation and epithelialization in the mice in addition to reducing inflammation, making it an excellent wound dressing (267).

Chitosan has been reported to have antimicrobial activity making it an attractive material for use in wound dressing (237). Gel film wound dressings were formulated using chitosan and a variety of organic acid solutions with antimicrobial activity for a low-cost dissolvable wound dressing. It was noted that the chitosan solutions made in lactic acid showed the greatest antimicrobial activity against *S. aureus* and *P, aeruginosa* (268).

1.6.2.3 Antimicrobial hydrogels

A hydrogel is a three dimensional polymer with hydrophilic properties. As hydrogels appear as a viscous liquid, they are ideal for unusually-shaped wounds. They do, however, require a secondary solid dressing to maintain the hydrogel in contact with the wound bed. Hydrogels are recommended for the treatment of deep dry wounds (247), as the liquid nature of the hydrogel ensures that the wound is kept moist for optimal healing, while its hydrophilic properties mean that many hydrogels can swell and absorb excess exudate, reducing the risk of complications in chronic wounds (237).

Polyvinylalcohol [PVA], polyvinylpyrrolidone [PVP] and polyvinyl oxide are often used as materials in hydrogel formulations. These polymers are attractive as they are cheap, often adhesive, non-toxic and hydrophilic and can be impregnated with drugs for topical treatment of wound infections (260). The hydrophilicity of the dressing is important in wound healing as hydrophilic dressings can maintain moist conditions which optimise healing while discouraging the build-up of exudate, ideal for hydrogel formulations (237).

A synthetic hydrogel made from PVA and PVP was successfully impregnated with a natural antimicrobial extract from the red seaweed *Polysiphonia lanosa.* The extract was

tested against several common wound pathogens including MRSA. The drug release profile of this hydrogel showed an initial burst release of the antimicrobial, reducing the bacteria by 70-90 % within 30 min followed by a slow release over 97 h. This action was found to be similar to that of the commercially available wound dressing Acticoat® (202). Burst release followed by a sustained release profile is an advantage in wound dressings as they raise the likelihood that the entire microbial population in the wound is exterminated, minimising the risks of antimicrobial resistance developing (202).

Similarly, a PVA-derivative was used to synthesise a hydrogel for targeted gentamicin delivery. This hydrogel released the antibiotic in the presence of the enzyme thrombin, which is present in the wound upon infection. As the concentration of thrombin in the wound is directly proportional to the level of infection, so was the antibiotic dose from the hydrogel. This mechanism served to deliver an appropriate dose of antibiotic with the aim to alleviate the complications and ramifications associated with antibiotic overdose (269).

Chitin has been successfully incorporated with several therapeutics for the bioactive treatment of wounds including fucoidan (260). The fucoidan-enriched hydrogel dressing was found to significantly improve healing of dermal burn wounds in rabbits in two complimentary studies (243,270)

1.6.2.4 Antimicrobial alginate dressings

Alginates have many great applications in the bioengineering of wound dressings due to their biocompatibility, biodegradability and ability to absorb water up to twenty times their own weight (260). As such, alginate dressings are often in their own class of wound dressing, which can include hydrogels made with alginate, for wet and heavily exuding wounds (247).

Alginates are polysaccharides extracted from seaweeds. They consist of two mannuronic acid residues bonded with a 1-4 glycosidic bond along with two guluronic acid residues as can be seen in Figure 1.17 (260,271). Seaweed can produce compounds of a bioactive nature (9,41,51,272–274). Alginate is one such bioactive as it exhibits mild antimicrobial properties and effectively manages exudate (275), making it an attractive addition to a wound dressing formulation (260). In addition, alginates are

often used in the formation of hydrogel wound dressings aiding in providing a moist environment for wound healing. However, they often require a secondary bandage-type dressing to physically hold them in place (260).

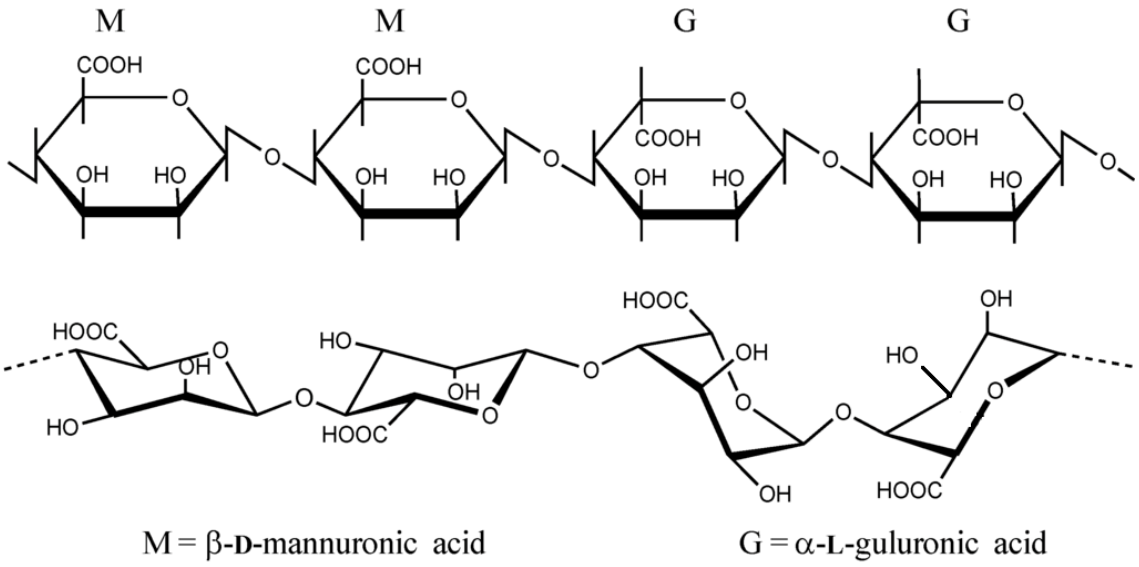

Figure 1.17: Alginate structure showing mannuronic acid and guluronic acid residues (205).

Alginate based wound dressings can be impregnated with more potent therapeutics as demonstrated by E. Kamoun *et. al* in a study which synthesised a PVA and alginate hydrogel with antibiotic ampicillin incorporated into its membrane. This resulted in a dressing with antimicrobial activity against *S. aureus, P. aeruginosa* and *Proteus vulgaris, in vitro* (276).

Another study that utilised alginate, gelatin and borax to form a hydrogel showed that the dressing provided excellent conditions for wound healing in rats. The dressing demonstrated 90 % water absorption while maintaining a moist wound bed for optimal healing. The dressing could be a good base for incorporation of therapeutic drugs or topical drug delivery as a new wound dressing (277).

Two commercially available collagen/alginate matrix dressings were imbibed with ionic silver as an antimicrobial agent successfully inhibited the growth of *S. aureus* after 30

min of application. These dressings were determined to have a sustained release and could be worn for up to five days effectively and were recommended or use on chronic wounds (206).

1.7 Potential of seaweed extracts as bioactive agents in wound dressings

Bioactivity including antimicrobial activity from seaweed extracts has been noted extensively in the literature which has been mentioned already in section 1.3.3.7. and 1.6.1. This activity has included the antimicrobial and antibiofilm activity of wound pathogens such as MRSA, *S. aureus, B. subtilis* and *P. aeruginosa.* This extensive bioactivity could be exploited and has the potential to be incorporated into medical devices such as wound dressings. As discussed in section 1.4, antimicrobial wound dressings are often used in the treatment of wounds both for the management and prevention of wound infections. In particular, the emergence of multi-drug resistant strains of infections have increased the pressure and cost of treating these infections, which are often transmitted via wounds (252). New treatments for these infections are being sought, driving the bioactive wound care market, which is expected to reach $13.5 billion by 2026 (238). In 2018, it was noted in over 65 % of the wound dressings used were required for moist wounds (238).

Bioactive compounds derived from seaweed sources could potentially be developed into wound dressings, alleviating this issue. Alginate from seaweed is noted for its therapeutic abilities and moisture management in wound environments. Natural products such as alginate would be appropriate for incorporation with seaweed based bioactives, making for a biodegradable, natural product with the capacity to manage moist wounds.

To date, while many studies have demonstrated the bioactivity of compounds and extracts derived from seaweeds, only one study incorporating an antimicrobial seaweed extract into a wound dressing has been noted in the literature (202), and this has not yet, entered the commercial market.

The ability of natural products to be naturally biodegradable (278) is particularly attractive to a market which is increasingly aware of the issues with single use items facing today's world. Considering that environmental concern is in vogue, it is not surprising that consumer trends are vying for natural solutions, such as seaweeds, to everyday problems such as treating wounds (18).

1.8 Overview of Thesis

Treating wounds can be complicated as the desirable properties of the dressing material are completely dependent on the type of wound. This review has alluded to one threat that all wounds need protection from and treatment for; infections. It is with this in mind that the capacity of wound dressings to imbibe or be themselves antimicrobial substances is an attractive and versatile feature.

There is a need for new, antimicrobial management systems for the treatment of infections. The rise of antimicrobial resistance has led to a requirement to discover novel antibiotics with new germicidal mechanisms that bacterial strains currently resistant to broad spectrum antibiotics have no defence against. Wounds are commonly infected in everyday life and as part of health care associated infections. As wounds provide an entry point for contamination and infections, antimicrobial wound dressings are a logical investment for the protection against infection.

There is much literature surrounding the notion of natural based wound dressings (237,244,260,279,280), of which seaweeds are a major contributor in terms of providing sodium alginate as a material (256). Yet, the application of imbibing wound dressings with bioactive, antimicrobial seaweed extracts is a rarer find. There is very limited literature on the use of seaweed based extracts for use in a wound dressing, an exception to this is a study carried out in W.I.T. published in 2013. This involved the synthesis of a novel hydrogel incorporated with water extracts from *Polysiphonia lanosa* which retained antimicrobial activity subsequent to incorporation of the extract into a wound dressing (202).

Still, the need for new antimicrobials is vast and the requirement for all wounds to be protected or treated against infections offers potential for seaweed derived antimicrobial wound dressings to be a novel addition to the commercial market. The scope of this thesis is to produce a novel antimicrobial wound dressing. The seaweeds *Fucus vesiculous* and *Fucus serratus* will be screened for antimicrobial activity against typical wound pathogens including MRSA isolated from a clinical setting by our collaborators, University Hospital Waterford. The bioactivity of these extracts can then be further assessed by performing antibiofilm and cytotoxicity assays. For the conservation of resources, a method of extraction of antimicrobial extracts can be developed.

Subsequent to this, the extract(s) can be incorporated into novel wound dressing formulations, and characterised for their respective antimicrobial activity. Purification and identification of the most potent antimicrobial extract(s), will then be focused on, as the synthesis of antimicrobial agents would make up-scaling their production straightforward.

Chapter 2: Antimicrobial screening of seaweed extracts

2.1 Introduction

Seaweeds have evolved to produce an assortment of bioactive compounds for the purpose of self-preservation (7,10,281). These bioactives can be extracted from seaweeds and utilised by people for a range of applications; from supplements to pharmaceuticals (7,8,28,42,47,50,235,282). The industrial potential for bioactives of natural origin is vast. Antimicrobial natural products could have the ability to improve the treatment of modern multi-resistant infections. Bacteria have evolved to reject various established antibiotics. As such, infections are becoming more serious leading to increased treatment cost and a higher risk of complications associated with the treatment of once trivial infections (158,176,252). Many organisms produce secondary metabolites of a bioactive nature. These compounds may be novel and as such have a greater chance of activity against the more dangerous strains of infections which are already resistant to commonly used antibiotics due to their previous lack of use (283).

Wound treatment and management comprises a sizable cost both in financial and societal terms (284). There is, therefore, a constant drive for better forms of treatment within the healthcare system. Wound management is a significant burden to the taxpayer in terms of treatment materials, hospitalisation and nurse expertise in after care. This cost is on the increase with the appearance of antimicrobial resistant bacterial strains that commonly infect wounds (285). This can be due to not only the increased cost of treatments, but also the prolonged stay of AMR patients in hospital care, increasing the cost. These longer treatments can amplify the burden with the risk of healthcare associated infections (HCAI), European estimates of which indicate that annually 4.1 million patients will acquire a HCAI which costs approximately €7 billion and results in 37,000 deaths (284). Irelands National Action Plan 2017-2020 stated that it is not currently possible to estimate the cost of AMR in Ireland since the emergence of Carbapenemase Producing *Enterobacteriaceae* (CPE) in 2012 (285).

A 2016 UK review has predicted that multi resistant bacterial strains such as MRSA, which is a known wound pathogen, will be responsible for 10 million deaths per annum by 2050 (252). It was stated that antimicrobial resistance costs the USA 20 billion dollars in annual costs and that the projected costs globally for AMR by 2050 is $100 trillion (252). As such, there is scope to improve the management of wounds by enhancing wound dressings in areas such as; tackling antimicrobial resistance and

biofilm formation, managing exudates, designing active dressings to aid wound healing and the topical delivery of drugs. Currently there are expensive and cumbersome treatments for the management of wound infections (145,164,252). As such, applying seaweed-derived antimicrobials for the treatment of wound infections would be a constructive application with potential commercial feasibility.

Seaweeds have previously demonstrated antimicrobial activity in several studies. There are many example of studies showing activity including; extracts from *Polysiphonia lanosa* against *S. pyongenes, S. aureus, B. subtilis* and *E.coli* (286), *Himanthalia elongata* against *Salmonella abony* and *Listeria monocytogenes* (287) and solvent extracts of *Sargassum oligocystum* and *Sargassum crassifolium* which were antimicrobial against 8 different bacterial pathogens including Gram negative *E. coli* (288). Methanol extracts from five species of Irish seaweeds *L. digitata, L. saccharina, H. elongata, P. palmata,* and *E. spirulina* were shown to inhibit the growth of *Listeria monocytogenes, Salmonella abony, Enterococcus faecalis* and *Pseudomonas aeruginosa* in a study carried out in 2010, indicating the potential of Irish seaweeds (51).

Species of *Fucus* have been shown to have antimicrobial activity (289) and antimicrobial protection (290) which indicates that there is potential in testing solvent extracts from this bladder wrack for their antimicrobial activity. The water soluble carotenoid fucoxanthin from *Fucus vesiculosus,* which is also present in *Fucus serratus* (29), was determined to inhibit *Cytophaga* KT0804, *Bacillus aquimaris* and *Ulvibacter littoralis* (291). Ethanol extracts of *Fucus vesiculosus* were reported to have antimicrobial activity against *S. aureus* and *B. subtilis* (292). Many phlorotannins such as eckol, dieckol and phloroglucinol, from *Fucus sp.* have been identified for their antimicrobial activity (235). For example, polyhydroxylated fucophlorethol extracted, purified and identified from *F. vesiculosus* was found to be antimicrobial against *E. coli, S. aureus, P. aeruginosa, S. epidermidis* and *P. mirabilis* (53).

MecC MRSA was discovered by the misdiagnosis of bovine mastitis in 2007 where MRSA was incorrectly typed as MSSA due to the lack of the mecA gene which typically confirms the presence of MRSA (293). As such, mecC strains of MRSA are considered more cumbersome in terms of diagnosis which can result in a slower onset of treatment for the resistant strain. GISA strains of MRSA are resistant to vancomycin and its derivative antibiotics, meaning that other 'last stand' antibiotics such as linezolid

are required to treat these infections (294). Linezolid is an oxazolidinone antibiotic which was approved by the FDA in 2000 for the treatment of Gram positive infections, particularly those exhibiting drug-resistance (295,296). Oxazolidinone antibiotics are bacteriostatic and, therefore, work by inhibiting the reproduction of bacteria cells rather than killing them. This is achieved by binding to a ribosomal subunit and inhibiting the initiation complex necessary for the synthesis of proteins (297,298). Without the ability to make proteins, the cell cannot reproduce, stopping the infection. Mutation of the sites on the ribosomal and other subunits that linezolid binds to is the main mode of resistance that some strains have undergone (297).

With this in mind, extracts from the seaweeds *Fucus vesiculosus* and *Fucus serratus* were tested against various wound pathogens with the aim of finding the extract with the broadest spectrum of antimicrobial activity against wound pathogens. The seaweeds *Fucus vesiculosus* and *Fucus serratus* have exhibited antimicrobial activity in a study previously carried out in W.I.T. (273), indicating their potential as a starting point for this project. In addition, a collaboration with University Hospital Waterford resulting in access to clinically relevant and isolated strains of pathogenic bacteria that were currently being treated in wound infections at the time of their donation in 2016. Among these isolates were two mecC positive strains of MRSA, a glycopeptide intermediate *S.* aureus (GISA) strain of MRSA and linezolid resistant *E. faecium* and *S. epidermidis.*

2.2 Experimental Procedure

2.2.1 Harvesting and preparation of seaweeds

The two seaweed species, *Fucus vesiculosus* and *Fucus serratus*, were harvested at Fethard-on-Sea, Co. Wexford, Ireland (52°11'53,68"N, 6°49'34,64"W, Figure 2.1 and Figure 2.2) on the 25th September 2015. The seaweeds were cut at the stipe of the algae and placed in a cooler box for transportation. Visually, not more that 10 % of the seaweed population of the beach was harvested for each species to enable the seaweed species to repopulate the beach. The seaweeds were taken from a variety of rocks to ensure that the complete harvest was a representative sample of the seaweed from that beach.

Figure 2.1: Fethard-on-Sea, Co. Wexford, Ireland (52°11'53,68"N, 6°49'34,64"W).

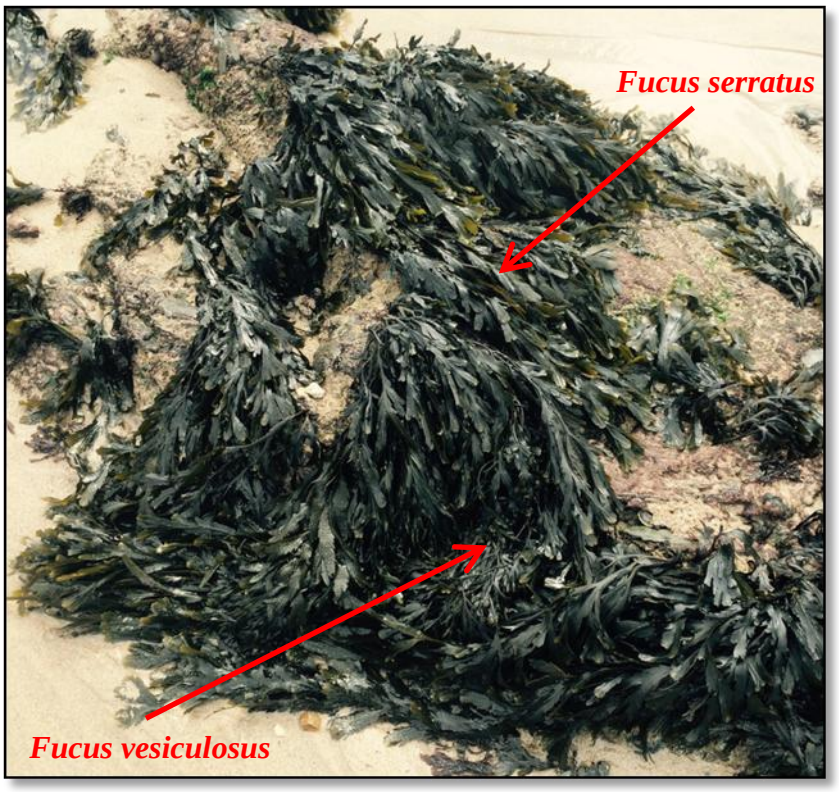

Figure 2.2: Seaweeds *Fucus serratus* and *Fucus vesiculosus* growing on beach rock.

The seaweeds were transferred to a sink of tap water where they were rinsed and manually cleaned of any epiphytes and beach debris. The cleaned seaweeds were then rinsed in distilled deionised water (SG Water Germany) (Figure 2.3) before blot drying excess water from the surface of the seaweeds and storing the seaweed in paper envelopes. They were then frozen at - 20 ^{o}C. This process was carried out on the day of harvesting. Once frozen, the seaweed was placed in a freeze drier (VirTis, SP Scientific, PA, USA) to ensure the complete dehydration of the seaweed.

Figure 2.3: *Fucus vesiculosus* post epiphyte debridement and rinsing.

Once freeze dried, the seaweed was weighed and stored in a sample bag under nitrogen gas until needed for processing.

The seaweed was processed by blending and sieving until the particle size was less than 850 µm as can be seen in Figure 2.4. The blended seaweed powder was placed in a sample bag under nitrogen and kept at -20 ^{o}C for storage until required for further analysis.

Figure 2.4: Dried seaweed blended to a particle size < 850 nm.

2.2.2 Water content analysis

Water content analysis was performed on both *Fucus vesiculosus* and *Fucus serratus* to be able to relate generated extract yields to wet mass of seaweed. A 5 g sample of each seaweed was allowed to dry in the base of a glass petri dish in an oven at 100 °C for three days. These samples were weighed every 24 h until the same weight was achieved two days in a row. Upon removal, the samples were allowed to cool in a desiccator containing sodium sulphate as a drying agent for a further day. This process was repeated in triplicate on two separate days to ensure the validity of the results.

Water content of each seaweed was calculated by taking the percentage of dried seaweed weight compared to the wet seaweed weight. This was repeated six times for each of the replicates.

2.2.3 Production of crude seaweed extracts

Seaweed powder was extracted by various solvents by shaking the powder in a volume of solvent on a shaker incubator at room temperature (19 °C) for 2 h at a speed of 200 rpm at a ratio of 1:100 *w/v*. A total of three extractions of 2.5 g seaweed powder in 250 mL of solvent were carried out for each seaweed in each solvent during this investigation. The solvents distilled deionised water, methanol (99.5 % HPLC grade), ethyl acetate (99.5 % HPLC grade) and acetone (99.8 % HPLC grade) were used in this experiment. All solvents used were purchased from Fisher Scientific, Dublin except distilled deionised water (SG Water, Germany). These solvents were chosen based on promising results from a previous study carried out at W.I.T (273).

Post extraction, the acetone, ethyl acetate and methanol extracts were filtered by vacuum through a Buchner funnel using Whatman No. 1 filter paper (Whatman, Kent, UK.). The filtrate was then washed into a 500 mL round bottom flask and rotary evaporated to dryness at 25 °C (Bibby heated water bath, Heidolph Laborota 4000 motor unit condenser, Vacuubrand vacuum pump, Heidolph, Nurenberg, Germany). The dried extract was then re-solvated in less than 8 mL of the extraction solvent, transferred to pre-weighed 8 mL glass amber vials and dried under nitrogen to dryness. Dried extracts were stored under nitrogen at -20 °C.

Water extracts were separated from their respective seaweed powders by centrifugation at 4500 rpm for 4 min. The supernatant was then decanted into a glass beaker, frozen at -20 °C and freeze dried. The solid water extracts were then transferred to pre-weighed 8 mL amber vials and stored under nitrogen at -20 °C as before.

A time study was carried out in water by extracting the seaweed powder using solvent extraction for 1 h, 2 h, 12 h, and 24 h to investigate the effect of time on the solvent extraction method. The 2 h crude water extract of the seaweed was also autoclaved and tested for antimicrobial activity using the disk diffusion method detailed in section 2.2.5 as an initial investigation of the effects of heat on the antimicrobial activity of the sample. Generated yields were calculated as % yield by Equation 2.1.

Equation 2.1: $\% \text{ yield} = \frac{\text{Mass of dry extract (g)}}{\text{Mass of seaweed powder (g)}} \times 100$

All organic solvents were disposed of in correct 'flammable waste' or chlorinated waste' containers for safe disposal.

2.2.4 Determination of crude extract dose

Prior to the assessment of antimicrobial activity via the disk diffusion method (described below), a small concentration study was carried out to determine how much extract was to be used per disk in this screen. Dried crude acetone and water extracts were aseptically dissolved in the solvent of their extraction at a concentration of 100 mg/mL. These solutions were then spotted in 10 µL aliquots until the desired concentration of 1 mg, 3 mg and 5 mg per disk were achieved. These disks of increasing concentration were then tested against a clinical strain of MRSA (MRSA 618) and MRSA NTCC using the disk diffusion method described below.

2.2.5 Antimicrobial activity of crude seaweed extracts against wound pathogens

The antimicrobial activity of the extracts of the seaweeds *Fucus vesiculosus* and *Fucus serratus* was assessed using the disk diffusion assay (299).

Dried extracts were aseptically dissolved in the solvent of their extraction at a concentration of 100 mg/mL. Disks were loaded with five 10 µL aliquots of this solution, allowing the disks to dry fully between loads until there was a concentration of 5 mg of crude extract per disk, as can be seen in Figure 2.5. Negative control disks were loaded in a similar manner with 50 µL of the extraction solvent in five 10 µL aliquots. Chloramphenicol disks (10 µg, Oxoid, Basingstoke, UK) were used as a positive control throughout this assay. A preliminary investigation comparing the dose to be used was undertaken by loading 3 mg and 5 mg on disks and comparing the data.

Figure 2.5: Disks loaded with 5 mg of crude seaweed extract.

Bacterial strains detailed with their point of origins in Table 2.1 and their antimicrobial susceptibilities in Table 2.2, were used as part of this screen. These strains were stocked

in a 60:40 *v/v* solution of sterile broth : glycerol and stored at - 20 ^{o}C. The bacterial strains were inoculated aseptically from their glycerine stocks at a concentration of 1:100 *v/v* in Brain Heat Infusion broth (BHI, Oxoid Basingstoke, UK). The inoculated broth was allowed to grow overnight at 37 ^{o}C.

Table 2.1: Origin of bacterial strains screened, incubated at 37 °C on Mueller Hinton agar plates in aerobic conditions. All strains barring 'type strains' were obtained from University Hospital Waterford.

Bacterial Strain	Site of Patient Infection	Bacterial Strain	Site of Patient Infection
MRSA 618	Blood	**MRSA 683**	Leg ulcer
MRSA 619	Blood	**MRSA 684**	Foot
MRSA 620	Nose	**MRSA 685**	Leg ulcer
MRSA 621	Mouth	**MRSA 686**	Breast wound
MRSA 666	Perineum	**MRSA 687**	Shoulder ulcer
MRSA 667	Groin	**MRSA 688**	Nasal swab
MRSA 668	Nose	**MRSA 689**	Blood culture
MRSA 669	Abdominal Wound	***E.coli* 625**	Type strain
MRSA 670	Nose	***E.coli* 626**	Blood
MRSA 671	Sputum	***A.baumani* 629**	Blood
MRSA 672	Sputum	***E.cloacae* 630**	Blood
MRSA 673	Catheter specimen	***P.microbilis* 635**	Type strain
MRSA 674	Penis	***S.aureus* 631**	Blood
MRSA 675	Penis	***S.aureus* 632**	Blood
MRSA 676	Eye	***K.pneumoniae* 638**	Type strain
MRSA 677	Ear	***K.pneumoniae* 639**	Blood
MRSA 678	Foot	***K.pneumoniae* 640**	Peritoneal fluid
MRSA 679	Breast wound	***K.pneumoniae* (CRE)(NDM) 662**	Faecal swab
MRSA 680	Skin around catheter	***K.pneumoniae* (CRE)OXA-48 663**	Peritoneal fluid
MRSA 681	Buttock pressure sore	***E.faecium* VRE (LZD-R) 664**	Rectal swab
MRSA 682	Wound	***S.epidermidis* (LZD-R) 665**	Axilla swab

Table 2.2: Details of MRSA types isolated from clinical setting detailing their antibiotic susceptibility and sensitivity obtained from University Hospital Waterford, all of which were utilised as part of this study.

	MRSA												
	618	**619**	**620**	**621**	**666**	**667**	**668**	**669**	**670**	**671**	**672**	**673**	**674**
Chloramphenicol	S	S	S	S	S	S	S	S	S	N/A	N/A	S	S
Ciprofloxacin	N/A	N/A	N/A	N/A	R	R	R	R	R	R	R	R	R
Flucloxacillin	R	N/A	R	R	R	R	R	R	R	R	R	R	R
Gentamicin	S	S	R	S	S	S	S	S	S	S	S	S	S
Clindamycin	N/A	N/A	N/A	N/A	R	S	R	R	R	R	R	S	R
Daptomycin	S	S	N/A	N/A	N/A	N/A	N/A	S	N/A	N/A	N/A	N/A	N/A
Erythromycin	R	R	R	R	R	S	R	R	R	R	R	S	R
Fusidic acid	S	S	S	S	R	R	S	R	S	S	R	S	R
Cefoxitin	R	R	R	R	R	R	R	R	R	R	R	R	R
Linezolid	S	S	S	S	S	S	S	S	S	S	S	S	S
Mupirocin	S	S	R	S	S	S	S	S	S	S	S	S	S
Rifampicin	S	S	S	S	S	S	S	S	S	S	S	S	S
Cotrimoxazole	N/A	N/A	N/A	N/A	S	S	S	S	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	S	S	S	S
Teicoplanin	S	S	S	S	N/A	N/A	N/A	S	N/A	N/A	N/A	N/A	N/A
Vancomycin	S	S	S	S	N/A	N/A	N/A	S	N/A	N/A	N/A	N/A	N/A
Trimethoprim	S	S	R	S	N/A	N/A	N/A	N/A	N/A	N/A	N/A	R	N/A
Co-amoxiclav	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	R	N/A
Cephalexin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	R	N/A
Nitrofurantoin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	S	N/A
Novobiocin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	S	N/A

'S' indicates susceptible and 'R' indicates resistance. N/A indicates non-applicable.

Table 2.2: Continued.

	MRSA											
	675	**676**	**677**	**678**	**679**	**680**	**681**	**682**	**683**	**684**	**685**	**686**
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin	R	R	R	R	R	R	R	R	R	S	S	R
Flucloxacillin	R	R	R	R	R	R	R	R	R	R	R	R
Gentamicin	S	S	S	S	S	S	S	S	S	R	S	S
Clindamycin	R	R	R	R	S	S	R	R	R	R	R	R
Daptomycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Erythromycin	R	R	R	R	S	R	R	R	R	S	R	R
Fusidic acid	S	S	S	S	S	S	R	S	S	R	S	R
Cefoxitin	R	R	R	R	R	R	R	R	R	R	R	R
Linezolid	S	S	S	S	S	S	S	S	S	S	S	S
Mupirocin	S	S	S	S	S	S	S	S	S	S	S	S
Rifampicin	S	S	S	S	S	S	S	S	S	S	S	S
Cotrimoxazole	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	S	S	S
Teicoplanin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	S	N/A	S
Vancomycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	S	N/A	S
Trimethoprim	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Co-amoxiclav	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cephalexin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nitrofurantoin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Novobiocin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

'S' indicates susceptible and 'R' indicates resistance. N/A indicates non-applicable. NOTE: strains 687 and 688 are mecC, strain 689 is GISA

After incubation, 1 mL of cultured broth was centrifuged at 13,000 rpm for 2 min to generate a cell pellet. The supernatant was removed aseptically and the pellet re-suspended in 1 mL of sterile maximum recovery diluent (MRD) with the intention of washing the cells without shocking the cells to death. This was repeated a further two times to ensure that the cells were clean of metabolites before the adjustment of the bacteria to an optical density (OD_{625}) of 0.10 - 0.12 (equivalent to 0.5 McFarland Standard or 10^7 - 10^8 colony forming units, cfu/mL).

The bacterial standard optical density and colony forming unit growth curves were carried out in a previous study in WIT* (273). This data was used in order to reach the OD_{625} of 0.10 - 0.12 so that the cells remained in the log phase, as mentioned above (273).

A sterile swab was used to spread adjusted bacteria onto Mueller Hinton agar (MHA, Oxoid Basingstoke, UK) plates by swabbing the surface of the plates with culture within 15 min of adjustment, then rotating the plate 60 °C and spreading the bacteria before rotating a further 60 °C and spreading the bacteria again. The 5 mg disks prepared as per section 2.2.2, including the positive and negative controls, were transferred aseptically to the swabbed plates and allowed to chill in a refrigerator at 4 °C for 5 h to allow for uninterrupted diffusion of the extract on the disks into the agar. The plates were then incubated overnight in the inverted position at 37 °C.

*Note: Log growth curves were attempted and failed, hence use of literature data carried out on the same strains of pathogens.

2.2.6 Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were tested using the CLSI standard broth dilution method (300). Dried extracts were dissolved to a starting concentration of 50 mg/mL in Mueller Hinton Broth (MHB). Of this solution, 100 µL was then added to each of four wells (3 x extract + bacteria, 1 x extract) of a 96 well microtitre plate, serial twofold dilutions were then carried out on these samples using MHB as diluent so that the concentration ranged from 25 mg/mL to 0.1563 mg/mL. Control samples included three wells of 100 µL of

10 µg/mL chloramphenicol as a positive control, six wells of 100 µL MHB, three wells as a media only control and three wells as negative controls (bacteria).

Separately, a 1 % inoculation of MRSA (676) was prepared in BHI and allowed to incubate overnight at 37 ºC. The subsequent cells were then washed in triplicate with MRD and adjusted to a McFarland standard of 10^7-10^8 colony forming units per mL as described in section 2.2.4. The adjusted solution was diluted 1:100 *v/v* in MHB resulting in a final solution concentration of 10^5-10^6 cfu/mL. Of this solution, 100 µL was loaded into each of the three rows of sample wells, the three positive control wells and the three negative control wells. A volume of 100 µL of sterile MHB was then added into the remaining wells (extract and media only controls).

The plate was incubated overnight at 37 ºC and subsequently read at 620 nm on a plate reader. MIC was subsequently calculated according to Equation 2.2:

Equation 2.2: $\text{Absorbance}_{(\text{sample})} = \text{Abs}_{(\text{extract + bacteria})} - \text{Abs}_{(\text{extract})}$

$$\% \text{ Inhibition} = \frac{\text{Abs}_{(\text{bacteria})} + \text{Abs}_{(\text{sample})}}{\text{Abs}_{(\text{bacteria})}} \times 100$$

This method was repeated in triplicate on different days. Minimum bactericidal concentration (MBC) was performed by spreading 50 µL from each well with the MIC reading onto MHA plates. The MBC was determined to be the lowest concentration which resulted in no growth after overnight incubation at 37 º C.

2.2.7 Safety procedures for microbiological work

All microbiological work was carried out in a Class II pathogen lab with an emphasis on safety and containment of pathogenic substances. The Standard Operating Procedure (SOP) for the pathogen lab was followed, including: all items brought into the lab were autoclaved on exit. Only pathogen lab coats must be worn in the pathogen lab, gloves must be used at all times and hands washed at exit. All waste in pathogen lab must be

autoclaved subsequent to disposal and benches washed down with 70 % *v/v* ethanol in water after use. All contaminated organic solvents were evaporated to dryness and diluted with virkon, before disposal.

2.2.8 Statistical analysis

Results are reported as the mean ± standard deviation. Comparisons between yields were made using one-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test. All statistical analysis was performed using Minitab 17 software for Windows 10 operating system with a 5 % statistical significance level ($p<0.05$).

2.3 Results and Discussion

2.3.1 Water content in seaweeds

The absolute removal of water in the powdered seaweed is important for the preservation of the seaweed, as traces of solvent molecules can aid the degradation of antimicrobial compounds. Dehydration is one of the world's oldest methods of preservation and works by removing water which acts as an activator for enzymes responsible for the decomposition of seaweed and aids microbial spoilage (301).

From an analytical perspective, the presence of water in the seaweed powder could skew results for yields and activity for the extraction and assessment of solvents of various polarities. Water molecules present in the seaweed could potentially act to alter the polarity of the extracting solvent system, thus changing the types of compounds being extracted.

As can be seen in Table 2.3, the water content in the seaweeds *Fucus serratus* and *Fucus vesiculosus* was 75.0 % and 71.9 % respectively. This is comparable to the 76.3 % and 68.0 % found by Tan *et. al.* (273) in the same season of 2009 in the same location. Similarly, a 2018 review stated that the moisture content in literature and found that *F. vesiculosus* and *F. serratus* is 74 -84 % and 80 - 81 % respectively (302). This information is useful in a practical sense as it provides a reference point as to what to expect for the yield of dry mass obtained from the harvested seaweed. For techniques such as freeze drying, it can be unclear as to when the sample is completely dry and oven drying could potentially degrade compounds responsible for antimicrobial activity. Using this data, an expected mass can be calculated over three days, saving time by making the drying/weighing/drying cycle redundant.

Table 2.3 : Water content in the brown seaweeds *Fucus serratus* and *Fucus vesiculosus* (n=6).

Seaweed Species	**Water Content (%)**
Fucus serratus	75.02 ± 2.2^{a}
Fucus vesiculosus	71.90 ± 1.1^{b}

Data (n=6) are presented as the mean ± SD; Data that do not share a common superscript are statistically different ($p<0.05$; One-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test).

2.3.2 Crude solvent extraction yields from the seaweeds F. serratus and F. vesiculosus

The yield of crude extract generated from the seaweeds *F. serratus* and *F. vesiculosus* harvested on the 25th of September 2015 for different solvents at a ratio of 1:100 *w/v* are displayed in Table 2.4. A seasonal study carried out previously on the extraction yields of seaweeds indicated that the time of year that the seaweed was harvested has an impact on the antimicrobial activity and yield of crude compounds that will extract into the solvent (273). As such, these yields could vary if this study is repeated at a different time of the year.

A 1:100 *w/v* ratio of seaweed powder to solvent was used so that at no point during the extraction was the solvent fully saturated. This was repeated three times ensuring that solvent saturation was not a factor in the extraction, indicating that the yields generated were representative of the extraction process for the specific solvent.

This study found that the quantity of crude extract from the seaweed increased with an increase in solvent polarity. This correlation between increasing solvent polarity and yield generated was also found in a study assessing the antioxidant capabilities of phlorotannins extracted from *F. vesiculosus* (303). This is not an unexpected result as carbohydrates, which are polar, account for approximately 20 - 50 % of dried seaweeds (304). Carbohydrates are polyhydroxy ketones or aldehydes which are polar due to the

large number of hydroxyl groups on the molecule. Proteins (3-11 % dry mass (20)) and lipids (1-6 % dry mass (281)) can also have varying degrees of polarity, as such, some can be solubilised by polar solvents while the remainder will be either solubilised by non-polar solvents or be completely insoluble.

The polarity of the solvent used to generate crude extracts from *F. vesiculosus* and *serratus* appears to have an influence on the specific antimicrobial activity of the extract in this study, which can be visualised in Table 2.8 and 2.10. It was noted that an increase in the range and extent of antimicrobial activity occurred with an increase of the polarity of the solvent used in the extraction. These results will be discussed in detail below in section 2.3.6.

Table 2.4: Percentage crude extract yields for solvents of decreasing polarity (n=3).

	% of Extract			
	Water	**Methanol**	**Acetone**	**Ethyl Acetate**
Polarity Index (305)	10.2	5.1	5.1	4.4
Fucus serratus	32.51 ± 1.57^{a}	13.15 ± 1.70^{b}	5.59 ± 0.74^{c}	3.96 ± 0.28^{d}
Fucus Vesiculous	28.31 ± 1.54^{e}	12.93 ± 1.21^{b}	5.81 ± 0.55^{c}	3.96 ± 0.39^{d}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different ($p<0.05$; One-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test).

A study conducted on solvent extracted seaweeds found that water extracts for *Fucus serratus* and *Fucus vesiculosus* yielded 39.6 % and 23.9 % of the dry mass respectively (273). This is in contrast with the results from this study which was 32.51 % and 28.31 %. However, this is not a surprising result, as seasonal variation is to be expected.

Similarly, the extracts for *Fucus serratus* and *Fucus vesiculosus* with acetone (5.59 % and 5.81 %) are in contrast to the 4.04 % and 4.47 % of dry mass extracted as part of the

same study previously carried out on *Fucus* seaweeds (273). Ethyl acetate extracts generated 3.96 % for both *Fucus* species used in this study, which is in contrast to the published data of 2.73 % for *Fucus serratus* and 2.05 % for *Fucus vesiculosus* (273).

The crude extract generated from methanol was 20.2 % and 8.43 % for *Fucus serratus* and *Fucus vesiculosus,* respectively, which differs significantly from the 13.15 % and 12.93 % generated as part of this study, however, as stated, this study used seaweeds harvested in March of 2010 (273). Differences in yields could be due inter-year or seasonal variation that occurs as changes in environmental factors, such as the weather and sea conditions, have an effect on the different metabolites produced by the seaweed and thus on their composition (50).

2.3.3 Determination of crude extract dose.

Crude acetone and water seaweed extracts were tested for antimicrobial activity against MRSA NCTC (National Collection of Type Cultures) and the clinically isolated strain of MRSA 618 using the disk diffusion method outlined in section 2.2.4. MRSA NCTC and 618 were chosen as strain 618 is an example of a clinical strain and MRSA NCTC is a standard, commercially available strains of MRSA. This investigation was carried out to determine the optimum dose of crude seaweed extract to use in the disk diffusion assay for screening the seaweed extracts for antimicrobial activity against MRSAbv618 and MRSA NCTC.

Anti-MRSA activity was discovered in a previous study carried out in WIT on *Fucus vesiculosus* extracts generated by solvent extraction in acetone and water (273). The study, carried out by Tan *et. al* demonstrated the antimicrobial effects of water, methanol, ethanol, acetone and ethyl acetate extracts of *Fucus vesiculosus* against MRSA NCTC (273). This study was used as the basis for choosing acetone and water as solvents for the determination of the extract dose, 1 mg, 3 mg and 5 mg were chosen following on from an initial screen carried out in WIT by Tan *et. al* (273). A concentration of 1 mg/disk was used as in this study and exhibited some activity against MRSA from the acetone and water extracts. However, as higher concentrations than 1 mg may be required to identify activity that is missed due to low concentrations, 3 mg and 5 mg were also tested to ensure activity could be observed.

Table 2.5 summarises the results of this analysis. It was found that 5 mg loaded disks gave higher antimicrobial activity than 3 mg or 1 mg, as might be expected. As both the 1 mg and 3 mg water extracts did not show any activity against MRSA NCTC and MRSA 618, 5 mg was chosen as the dose to be used for the screen of crude seaweed extracts against all MRSA strains. The emphasis of the study was to establish the presence or absence of activity and not dose-dependent activity. Once activity was observed, further development of the dose could always be carried out in future studies. The use of a 5mg dose reduced the probability of missing activity and gave a very clear picture of the range of activities of the extract.

A study comparing the well diffusion to the disk diffusion method over a range of concentrations of natural product antimicrobials against *S. aureus* found that 1 mg/disk was enough to expose the majority of the antimicrobial activity, while 5 mg was the lowest dose where all the extracts were capable of inhibiting the bacteria to varying degrees (111). More specifically for seaweed extracts, biooassays of methanol:acetone crude extracts of the seaweeds, *Dictyota flabellata, Padina concrescens, Laurencia johnstonii, Gymnogongrus martinensis, Ulva lactuca* and *Codium fragile* against pathogenic bacteria such as *S. aureus* and *B. subtilis* used 1.95 mg per disc with the disc diffusion method (306). Conversely, only 10 µg of sulphated polysacharides extracted from *Sagassum swartzii* was used against *E. coli* (307).

While the range of 10 µg (307), to 5 mg (111), were shown to successfully assess antimicrobial activity, it was thought that at lower doses, the disk diffusion method was not sensitive enough to detect sought after antimicrobial activity.

Table 2.5: Results of antimicrobial assay of 2 different solvent extracts against MRSA NCTC and MRSA 618 at different concentrations.

	Water						Acetone					
	MRSA 618			MRSA NCTC			MRSA 618			MRSA NCTC		
	1mg	3mg	5mg	1mg	3mg	5mg	1mg	3mg	5mg	1mg	3mg	5mg
Fucus serratus	-	+	+	-	-	++	-	-	+	-	+	+
Fucus vesiculosus	-	+	+++	-	+++	++++	-	+	+++	-	+++	++++

[a]Chloramphenicol antibiotic disk - 10 µg/disk. [b]Negative control - 50 µL of specific solvent. Inhibition zone reported as diameter of clear inhibition (including 6 mm disk) in mm; - indicates no inhibition, + indicates inhibition zone of 6 mm - 7 mm, ++ indicates inhibition zone of 7.1 mm - 8 mm, +++ indicates inhibition zone of 8.1 mm-10 mm, ++++ indicates inhibition zone of > 10 mm. (n=3)

The differences in activity exhibited between *Fucus vesiculosus* and *Fucus serratus* were notable in this study. As can be seen in Table 2.4, *F. vesiculosus* is far more active than *F. serratus* against MRSA for both water and acetone extracts. This is supported by work carried out by Tan *et al.* (273) where the extracts from *F. vesiculosus* produced more potent antimicrobials against MRSA than their *F. serratus* counterparts. These differences in antimicrobial activity between the two seaweeds could be due to genetic differences present between the seaweeds and therefore differences in the secondary metabolites they produce. However, compositional variations between *F. vesiculosus* and *F. serratus* will be reviewed.

A 2018 study comparing the two seaweeds noted that there is a difference in composition between the two which can be seen in Table 2.6 (308).

Table 2.6: Composition differences in % dry mass between *F. serratus* and *F. vesiculosus* (302).

	Seaweed species	
% Composition	***F. vesiculosus***	***F. serratus***
Moisture	71 - 84	80 - 81
Carbohydrates	34 - 66	26 - 62
Fibre	4 - 59	16.0
Protein	1 - 11	10 - 17
Lipid	1.2 - 4	0.4 - 3
Ash	23 - 36	19 - 22
Fucoidan	3.4 - 25.7	13 - 24.4
Alginic acid	8.4 - 58.8	10.5 - 22.2
Laminaran	0.6 - 7	1.0 - 19.0

These compositional differences could possibly be the reason why there is a difference in activity between *F. serratus* and *F. vesiculosus.* However, as laminarin has shown to be antimicrobial (105), it would be expected that *F. serratus* would have a higher activity than that of *F. vesiculosus*. This discrepancy has a number of possible explanations, such as; different saline conditions can change the composition of seaweed (309), similarly, the year and seasons that the seaweeds are harvested also as an effect on the antimicrobial compounds in seaweed (50). The antimicrobial may not be in any way related to the laminarin content and *F. serratus* could contain an antagonist to any antimicrobial compounds that may be present. Similarly, *F. vesiculosus* may contain synergistic compounds, which could be enhancing the antimicrobial activity of these extracts.

F. serratus and *F. vesiculosus* are noted to both contain fucoidan which has been noted to be antimicrobial in the literature (302,310,311). However, the structure of the fucoidan varies between these two species (302), which may account for the variation in

antimicrobial activity shown. Interestingly, a search of the literature for the antimicrobial activity of fucoidan notes the fucoidan to be extracted from brown seaweeds such as; *F. vesiculosus* (312), *S. wightii* (313) and *A. nodosum* (314) and not *F. serratus.* The antimicrobial activity noted here may be due to a different compound however, as it was noted that the antimicrobial compound(s) present in *F. serratus* were less stable than those in *F. vesiculosus* as evidenced by the both the time study (Table 2.7) and the autoclave study (Table 2.8) discussed in later sections of this work.

F. vesiculosus was determined to have 4.7 % and 33.5 % phospholipids and glycolipids respectively. These represent a higher percentage composition of phospholipids and glycolipids than those of *F. serratus* which were determined at 2.7 % and 30.1 % respectively (315). This could potentially be the source of the difference between the antimicrobial activities of *F. vesiculosus* and *F. serratus* noted as part of this study due to the reported antimicrobial activity of phospholipids from both *F. vesiculosus* and *F. serratus* (316). Phenolic content in *F. vesiculosus* was determined to be greater than that of *F. serratus* in Danish waters (0.608 % vs. 0.412 % respectively) (317), which may be a factor in the differences displayed in this study between the antimicrobial activity of *F. serratus* and *F. vesiculosus* as phenolic content has also been associated with antimicrobial activity (318).

F. vesiculosus containing superior antimicrobial ability than that of *F. serratus* in this study may be due to any one, or all of these differences in composition indicated in the literature to date.

2.3.4 Antimicrobial activity of crude seaweed extract

Disk diffusion is recommended by the Clinical and Laboratory Standard Institute (CLSI) as a standard method for the assessment of antimicrobials (319). As such, disk diffusion was the antimicrobial assay chosen for the evaluation of the antimicrobial activity of *Fucus vesiculosus* and *Fucus serratus.*

The eight crude seaweed extracts were tested for antimicrobial activity against a range of clinical isolates donated by University Hospital Waterford in order to establish the extract with the largest zone of inhibition against the pathogens typically found in

wounds. The donated isolates are listed in Tables 2.7 and 2.8 with their respective antimicrobial activity.

Table 2.7: Antimicrobial activity of crude solvent extracted (2 h) *Fucus serratus* tested using the disk diffusion assay.(n=3)

	Antimicrobial activity (5 mg/disk)			
	EtOAc	**Acetone**	**MeOH**	**H_2O**
MRSA 618	-	+	-	-
MRSA 619	-	+	-	++
MRSA 620	-	+	+	-
MRSA 621	-	-	+	-
MRSA 666	-	-	+	+
MRSA 667	-	-	-	+
MRSA 668	+	+++	+++	+++
MRSA 669	-	++	+++	++
MRSA 670	-	-	-	+
MRSA 671	-	+	-	+
MRSA 672	-	+	-	+
MRSA 673	-	++	++	++
MRSA 674	-	+	++	+
MRSA 675	-	-	++	+
MRSA 676	-	+++	++	+++
MRSA 677	-	-	-	+
MRSA 678	-	-	+	+
MRSA 679	-	++	++	+++
MRSA 680	-	-	-	+
MRSA 681	-	-	++	++
MRSA 682	-	++	++	+++
MRSA 683	-	++	++	+++
MRSA 684	-	-	+	+
MRSA 685	-	-	++	++
MRSA 686	-	+	+	+
MRSA 687 (mecC)	-	-	-	+
MRSA 688(mecC)	-	-	-	+
MRSA 689(GISA)	-	+	-	++
***E.coli* 625**	-	+	-	-
***E.coli* 626**	-	+	-	-
***A.baumani* 629**	-	-	-	-
***E.cloacae* 630**	-	-	-	++
***P.microbilis* 635**	-	-	-	-
***S.aureus* 631**	-	+	+	+
***S.aureus* 632**	-	-	-	-
***K.pneumoniae* 638**	-	-	-	-
***K.pneumoniae* 639**	-	-	-	-
***K.pneumoniae* 640**	-	-	-	-
***K.pneumoniae*(CRE)(NDM) 662**	-	-	-	-
***K.pneumoniae*(CRE)(OXA-48) 663**	-	-	-	-
***E.faecium* VRE (LZD-R) 664**	-	-	-	-
***S.epidermidis* LZD-R 665**	-	-	-	-
Positive control [a]	+++	+++	+++	+++
Negative control [b]	-	-	-	-

[a]Chloramphenicol antibiotic disk - 10 µg/disk. [b]Negative control - 50 µL of specific solvent. Inhibition zone reported as diameter of clear inhibition (including 6 mm disk) in mm; - indicates no inhibition, + indicates inhibition zone of 6 mm - 9.9 mm, ++ indicates inhibition zone of 10 mm - 14.9 mm, +++ indicates inhibition zone of 15 mm- 19.9 mm, ++++ indicates inhibition zone of 20 mm-24.9 mm, +++++ indicates inhibition zone of > 25 mm.

Table 2.8: Antimicrobial activity of crude solvent extracted (2 h) *Fucus vesiculosus* tested using the disk diffusion assay.(n=3)

	Antimicrobial activity (5 mg/disk)			
	EtOAc	**Acetone**	**MeOH**	**H_2O**
MRSA 618	+	++	++	+
MRSA 619	+	++	++	++
MRSA 620	++	++	+	+
MRSA 621	++	++	+	+
MRSA 666	+++	+++	+++	++++
MRSA 667	+++	+++	+	++++
MRSA 668	++++	++++	++++	+++++
MRSA 669	++++	++++	++++	+++++
MRSA 670	++	+++	+++	++++
MRSA 671	++	+++	++++	++++
MRSA 672	+++	+++	++	+++
MRSA 673	+++	+++	+++	+++++
MRSA 674	++	+++	+++	++++
MRSA 675	++	++	++	+++
MRSA 676	++++	+++++	++++	+++++
MRSA 677	++	++	+	++
MRSA 678	++	++	++	++++
MRSA 679	+++	+++	+++	+++++
MRSA 680	+++	++	++	++
MRSA 681	++	+++	+++	+++++
MRSA 682	++++	+++++	++++	+++++
MRSA 683	+++	+++	+++++	+++++
MRSA 684	-	+	+	++
MRSA 685	-	++	++	++
MRSA 686	+++	+++	+++	++++
MRSA 687 (mecC)	-	+	-	++
MRSA 688(mecC)	-	-	+	++
MRSA 689(GISA)	-	++	++	+++
***E.coli* 625**	-	-	-	-
***E.coli* 626**	-	+	-	-
***A.baumani* 629**	-	-	-	-
***E.cloacae* 630**	-	-	-	++
***P.microbilis* 635**	-	-	-	-
***S.aureus* 631**	+	+	+	++
***S.aureus* 632**	-	-	-	++
***K.pneumoniae* 638**	-	-	-	-
***K.pneumoniae* 639**	-	-	-	-
***K.pneumoniae* 640**	-	-	-	-
***K.pneumoniae* (CRE) (NDM) 662**	-	-	-	-
***K.pneumoniae* (CRE) OXA-48 663**	-	-	-	-
***E.faecium* VRE (LZD-R) 664**	-	-	-	-
***S.epidermidis*(LZD-R) 665**	-	-	-	-
Positive control [a]	+++	+++	+++	+++
Negative control [b]	-	-	-	-

[a]Chloramphenicol antibiotic disk - 10 µg/disk. [b]Negative control - 50 µL of specific solvent. Inhibition zone reported as diameter of clear inhibition (including 6 mm disk) in mm; - indicates no inhibition, + indicates inhibition zone of 6 mm - 9.9 mm, ++ indicates inhibition zone of 10 mm - 14.9 mm, +++ indicates inhibition zone of 15 mm- 19.9 mm, ++++ indicates inhibition zone of 20 mm-24.9 mm, +++++ indicates inhibition zone of > 25 mm.

From an exhaustive search of literature for activity using extracts from natural sources against GISA strains, it was found that one other study by Haste et.al investigated and noted the susceptibility of marinopyrrole; a bioactive marine natural product which is not reported as present in the Fucus species, against GISA strains (illustrated in Figure 2.6) (320). GISA strains refer to S. aureus with reduced susceptibility to glycopeptides such as vancomycin, whose mode of action relies on the disruption of peptidoglycan synthesis by binding to the d-alanyl-d-alanine at the free carboxyl end of the peptidoglycan stem peptide (194)(189). Once bound to this peptide, the enzyme peptidoglycan synthase cannot catalyse the synthesis of peptidoglycan in the cell wall due to the steric hindrance provided by antibiotics such as vancomycin. In this way, the building blocks for peptidoglycan are rendered inactive (194,195). GISA strains have been reported more frequently in recent times. A 2001 study by J. Liñares reported just 10 cases of GISA infection worldwide (321). A more recent 2016 survey of S. aureus strains isolated from burn wound patients in Bangladesh reported that 28 % of the 40 isolates tested were vancomycin resistant (294). As such, the susceptibility of the GISA strain of MRSA (689) to the water and acetone extract of both *Fucus serratus* and *Fucus vesiculosus* and the methanol extract of *Fucus vesiculosus*, detailed in Tables 2.8 and 2.9, demonstrates the potential of these extracts for use against AMR infections. These results are particularly promising as they indicated that the antimicrobial(s) responsible for this inhibition is neither reliant on the β-lactam ring (penicillin) or glycopeptide (vancomycin) modes of action.

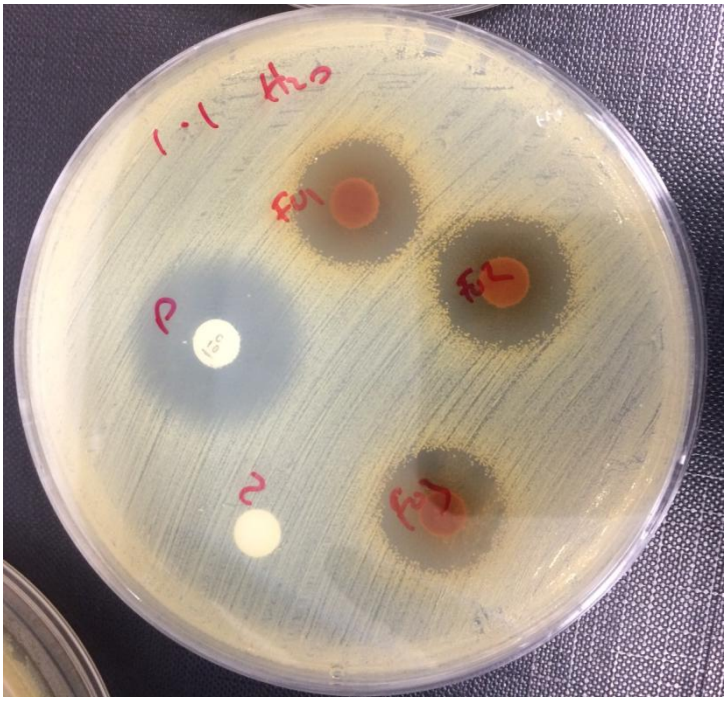

Figure 2.6: Plate showing *F. vesiculosus* water extract inhibiting MRSA 689 the GISA strain.

MRSA strains containing the mecC gene were discovered in a case of milk contamination from bovine mastitis in southwest England in 2007 (293). These strains were phenotypically confirmed as MRSA due to the isolates resistance to oxacillin and cefoxitin. However, upon an attempt to confirm the isolate strain as MRSA, the characteristic mecA gene was not present. The mecA gene is responsible for the production of the PBP2a (penicillin binding protein) which binds to penicillin at the active site and breaks the β-lactum ring in penicillin, rendering it inactive. In place of the mecA gene, a gene labelled mecC was discovered. MecC shares a 69 % DNA similarity to mecA, which is not enough to produce a positive result for MRSA by PCR (polymerase chain reaction) analysis. As such, mecC MRSA had until this time been creeping in under the guise of methicillin susceptible *S .aureus* and being treated as such (293). MecC strains of MRSA can be complicated to treat in that they can be misdiagnosed as methicillin susceptible *S. aureus* if tested using PCR and not using antimicrobial susceptibility tests against the indicator antibiotics: oxacillin and cefoxitin (293). The mecC strains of MRSA (687 and 688) which were screened as part of this investigation were inhibited by the water extract of both *F. serratus* and *F. vesiculosus*,

while MRSA (687) and MRSA (688) were inhibited by the acetone and methanol extracts of *F. vesiculosu*s, respectively. The potential of this extract as a treatment against bacterial infections containing MRSA is promising, particularly owing to the inhibition of both MecA and MecC strains of MRSA as there can be more than one strain type present in the infection.

Active extracts could potentially be used as part of a wound dressing formulation. Burn wounds are especially susceptible to infection on account of the fact that thermal injury can cause the underproduction of neutophils and cytokines by macrophages resulting in a decrease in natural bactericidal activity and immune system direction. Also the avascular nature of a burn can hinder the transport of lymphocytes and more obviously, the natural cutaneous barrier is broken (322). Due to the increased risk of infection, active wound dressings containing an antimicrobial may decrease the overall treatment time and cost of this treatment for burn wound victims.

Conversely, activity was demonstrated by *Fucus serratus* in this study, while crude water extracts established the most potent activity, methanol (Figure 2.7) and acetone extracts both contain consistent antimicrobials against the various strains of MRSA. All of the 28 strains of MRSA were inhibited by one or more of the acetone, methanol and water extracts for *Fucus serratus.* Water extracts of *Fucus serratus* were found to have antimicrobial activity against both the GISA and the mecC strains of MRSA, indicating potential for this extract to be used as an antimicrobial against these particularly troublesome strains. However, as the results from the time and autoclave studies indicated that the antimicrobial active compound(s) present in *F. serratus* were unstable, this inferior activity may be due to degradation of unstable compounds rather than a lack of potency.

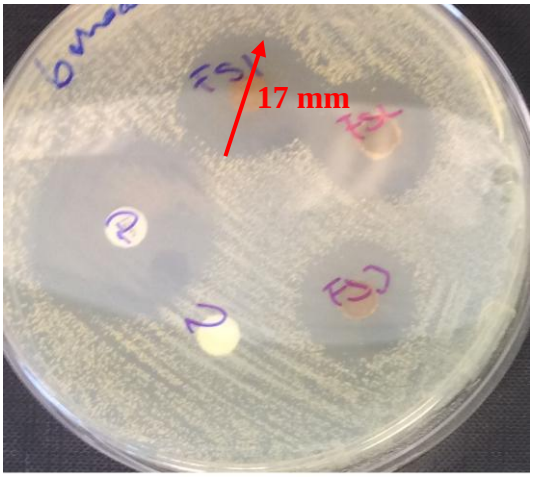

Figure 2.7: Antimicrobial extract of 5 mg methanol extract of *F. serratus* against MRSA 668.

Fucus vesiculosus extracts displayed a wide array of activity against the clinical MRSA strains used in this research. The ethyl acetate extracts displayed the least amount of activity, with no inhibition at all for two of the 25 mecA strains, the GISA and the two mecC strains. Acetone, methanol and water extracts inhibited every one of the mecA strains with water showing the largest zone of inhibition. Additionally, the water extracts were active against both the GISA and the two mecC strains of MRSA. The crude methanol extract was inactive against MRSA 687 which is one of the mecC strains and the acetone extract was inactive against MRSA 688, the second of the two mecC strains. The water extract of *Fucus vesiculosus* exhibited the most impressive activity, with many strains outperforming the positive control. An example of this is MRSA 668 is shown in Figure 2.8.

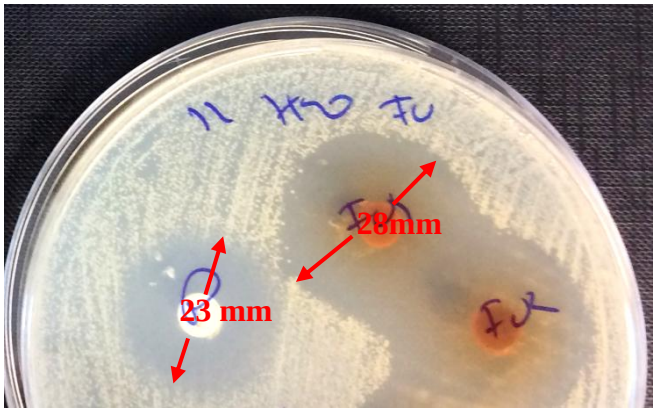

Figure 2.8: Disk diffusion of *Fucus vesiculosus* water extracts against MRSA 668.

None of the seaweed extracts were effective against the Gram positive *E. faecium* 664 or *S. epidermidis* 665, both of which are particularly prominent infections as they are resistant to the antibiotic linezolid. Similarly, none of the extracts from the two seaweeds were active against any of the clinical strains of *A. baumani*, *P. microbilis* or *K. pneumoniae*, including the CRE isolates.

Of the Gram negative strains tested, there were only two cases of antimicrobial activity exhibited from *Fucus vesiculosus* extracts. The two acetone extracts were active against the clinical *E. coli* 626 and the water extract was active against *E. cloacae* 630. These results are promising as antimicrobials that inhibit Gram negative strains are sought after due to the fact that many antibiotics that work against Gram negative are also active against Gram positive strains (283).

The extracts generated from *Fucus serratus* showed slightly more promising results in terms of Gram negative activity, as both of the *E. coli* strains 626 and 627 were susceptible to the acetone extract. In addition, the water extract for *Fucus serratus* was also active against *E. cloacae* 630.

Extracts from *Fucus vesiculosus* have been shown to inhibit the growth of the Gram negative *E.coli* in the past where a study established the methanol extract was shown to inhibit *E. coli* using the disk diffusion method with a zone of inhibition of 10 mm (323) and also in a study where a purified fraction of an extract completely inhibited *E. coli* grown using MIC techniques (53). To the authors' knowledge Gram negative

antimicrobial activity for *Fucus serratus* extracts has not been reported previously, this could be due to the instability of the extracts that have been displayed in the autoclave study carried out in section 2.3.5. Gram positive bacteria have been noted to be more susceptible to seaweed extract antimicrobials (323).

Water, being a 'green' solvent results in less toxic waste and is a desirable solvent for industries such as the pharmaceutical industry. Although there is a cost associated with the disposal of waste water from industrial practices, water and solvent combinations with a high percentage of water score lower using the EHS (environmental health and safety) scale to measure green solvents (324), making it a more desirable solvent for industry applications.

The display of antimicrobial activity exhibited by the water extracts of both *F. vesiculosus* and *F. serratus* in this study shows promise for these extracts to be used as a drug or supplement in a drug delivery system.

2.3.5 Time study

A time study was undertaken to refine the parameters of the solvent extraction process. Time increments of 1 h, 2 h, 12 h and 24 h were chosen and the seaweeds were extracted as before, (section 2.2.3) to compare yields and activities in order to determine the optimum extraction time. The time increments for this investigation were chosen based on the work of other studies, many of which use a 2 h (50,51,202) or 24 h (52,53) extraction time. One study went as far as to use 48 and 72 h extraction times (3). These were not used during this experiment due to the long duration of the extraction and the negligible increase in yield which was noted as part of that same study (3).

The efficiency of extraction is related back to cost in terms of electricity and time, therefore it is important to discover the lowest possible extraction time without compromising the yield and activity of the extract. As can be seen in the yields obtained for this study displayed in Table 2.9, there was no significant difference in yield generation between 1 h and 24 h solvent extraction. This data supports the use of lower extraction times as longer extraction times waste time and resources. The p values were greater than 0.05 indicating that there was no statistical difference between the yields

generated for extraction times of 1, 2, 12, or 24 h. Longer extraction times could also promote the extraction of undesirable compounds, which could be antagonistic to antimicrobial activity or just result in a more cumbersome sample cleanup. This was a similar result to a study by Rosell *et al.* which measured the yield against extraction times of 24, 48 and 72 h. It noted that there was no variation between 24 and 48 h extraction times, but there was a drop in activity at the 72 h extraction time point from 21-30 mm inhibition down to 11-20 mm inhibition of *S. aureus* using acetone extracts from *Nereocystis luetkeana* (3).

Table 2.9: Yields (%) of crude extract generated using water as the solvent for different time periods (n=3).

Seaweed	**1 h**	**2 h**	**12 h**	**24 h**
Fucus serratus	35.24 ± 2.02^{a}	36.27 ± 2.27^{a}	33.74 ± 1.73^{a}	35.02 ± 0.97^{a}
Fucus vesiculosus	30.63 ± 1.81^{b}	31.64 ± 0.28^{b}	33.21 ± 0.40^{b}	32.44 ± 0.98^{b}

($p<0.05$; One-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test). Data (n=3) are presented as the mean ± SD.

The antimicrobial activity of these extracts was also a significant factor in this study as antimicrobial compounds present in the seaweed could extract at a slower rate compared to other compounds depending on their solubility and, therefore, render the crude extract less or more active subsequent to extraction. The stability of the extract could also affect the antimicrobial activity post extraction as the antimicrobials can degrade in solvents. The antimicrobial activity for these samples was compared using the disk diffusion method outlined in section 2.2.4. MRSA 619, a clinical strain was used as indicator bacteria for this experiment. This bacteria was chosen due to its susceptibility to the crude water extract of both *Fucus serratus* and *Fucus vesiculosus* in the initial screening of the solvent extracts generated from their seaweeds.

The antimicrobial activity of these extracts is displayed in Table 2.10. As can be observed from this data, the activity of the *Fucus serratus* extract decreased over time

spent in the solvent, while the *Fucus vesiculosus* extract remained consistently active. This data indicates that extraction time is irrelevant in the case of activity for crude water samples belonging to *Fucus vesiculosus*, while *Fucus serratus* water extracts favour lower extraction times. This could possibly be due to deactivating interactions between the antimicrobial compound(s) in *Fucus serratus* and the water or oxygen molecules present in solution, or could just be an indicator that the antimicrobial compound(s) present in *Fucus serratus* are sensitive to environmental factors such as agitation, room temperature or light and are therefore, not stable (235). As mentioned, fucoidan is a water soluble polysaccharide present in both *F. serratus* and *F. vesiculosus*, however the structure of fucoidan can vary between the two seaweeds (302). Fucoidan from *F. vesiculosus* has been extensively noted to be bioactive (311,312,325,326), however there is very little on the bioactivity of fucoidan from *F. serratus*.

A study examining the extraction times of antimicrobial compounds from the seaweed *Nereocystis luetkeana* extracts tested against *S. aureus* at 24, 48 and 72 h extraction times noted that some activity was lost at longer extraction times (3). It was noted in this study that acetone extracts of *Nereocystis luetkeana* lost activity against *S. aureus* between the 48 and 72 h extraction time. This is possibly due to sample degradation in the solvent, which can be similarly observed in Table 2.8 of this study, where a decrease of the antimicrobial activity of *F. serratus* can be observed with an increase of extraction time.

This loss of activity for both the longer extractions and autoclaved extracts indicates that the active compound(s) present in *Fucus serratus* are less stable and different to those from *Fucus vesiculosus.*

Table 2.10: Antimicrobial activity of 5 mg of crude water extract of seaweeds against MRSA 619 using the disk diffusion method. (n=3)

Seaweed	1 h	2 h	12 h	24 h	2 h Autoclaved
Fucus serratus	+++	+	-	-	-
Fucus vesiculosus	++++	++++	++++	++++	++++

[a]Chloramphenicol antibiotic disk - 10 µg/disk. [b]Negative control - 50 µL of specific solvent. Inhibition zone reported as diameter of clear inhibition (including 6 mm disk) in mm; - indicates no inhibition, + indicates inhibition zone of 6 mm - 7 mm, ++ indicates inhibition zone of 7.1 mm - 8 mm, +++ indicates inhibition zone of 8.1 mm-10 mm, ++++ indicates inhibition zone of > 10 mm.

2.3.6 Autoclave study

The 2 h crude water extracts for these seaweeds were also autoclaved as a pilot investigation into the effect of heat on the extract and as a means of sterilising the seaweed extract. Any component in a pharmaceutical formulation must have a means of sterilisation. Heat is one of the most readily used forms of sterilisation, therefore, it is useful to know whether autoclaving deactivates the antimicrobial present in these seaweed extracts.

After autoclaving the solid samples, they were re-solubilised in a known volume of water so that 5 mg disks could be prepared for the disk diffusion assay. On addition of water, it was observed that a large insoluble brown mass remained and that the sample had developed a sweet smell. The autoclaved and non autoclaved samples are displaye in Figure 2.6. It was speculated that this mass could be due to the caramelisation of polysaccharides in the seaweed extract. The mass was broken up manually and the solution allowed to sonicate for 15 min in an attempt to solubilise it. Subsequent to sonication, the supernatant was loaded onto the disks and tested for antimicrobial activity against MRSA 619, as in section 2.24.

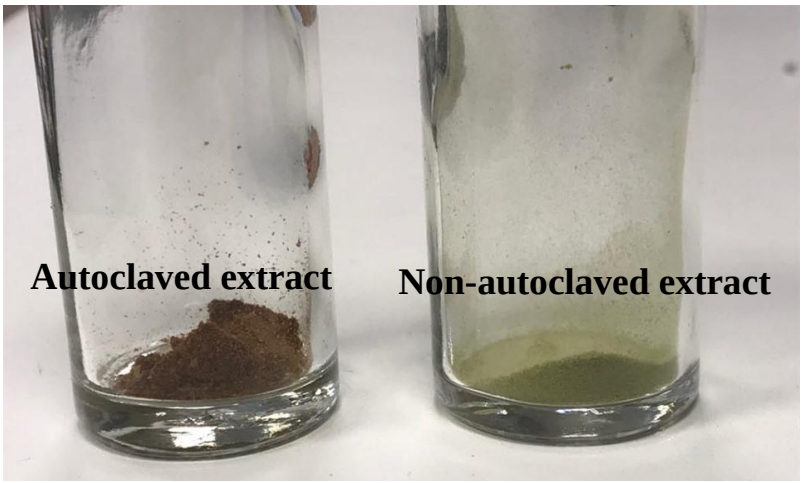

Figure 2.9: Autoclaved water extract from *F. vesiculosus* and non-autoclaved extract from *F. vesiuculosus.*

The results of this assay are displayed in Table 2.10 and Figure 2.10. As can be seen, the *Fucus serratus* extract lost activity subsequent to the autoclaving process. The *Fucus vesiculosus* extract, however, retained the same level of activity as before the autoclave process. Again, this is an indication that the antimicrobial(s) present in *F. serratus* are not stable. Due to the myriad of processes occurring within an autoclave, the degradation of antimicrobial activity could potentially be as a result of; the extract could be sensitive to oxidation which would be accelerated at the 121 ^{o}C operating temperature of the autoclave, compared to room temperature, it could be sensitive to moisture/hydrolysis, as there is steam present within the autoclave or the extract may just be thermo-labile. Although it is well know that natural antimicrobial compounds can be unstable after heating, however it was noted in one study, that the antioxidant capacity of antimicrobial phenolic compounds present in *Lentius edodes* actually increased upon the heating of this mushroom (327), indicating that not all of the antimicrobial compounds present in *Lentius edodes*, much like that displayed by *F. vesiculosus*, were thermo labile.

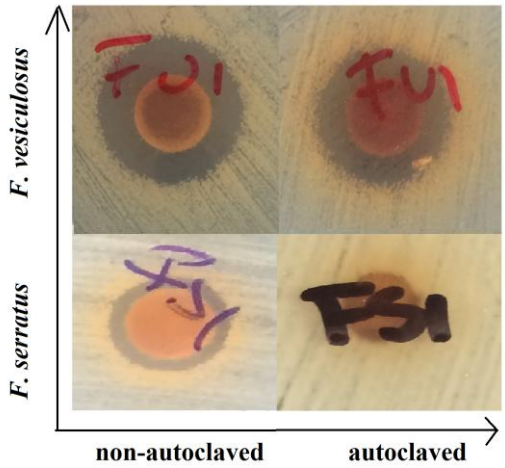

Figure 2.10: Antimicrobial activity of 5 mg of crude water extract of seaweeds against MRSA 619 using the disk diffusion method.

From these results, antimicrobial water extracts for *Fucus vesiculosus* appear to be robust enough to survive the autoclave process. This could potentially be used as a purification step to remove the non- antimicrobial compounds present in the insoluble mass which formed after autoclaving and thus requires further investigation. The preservation of activity after autoclaving does, however, give an indication that the active compound is not a protein, as proteins and chlorophyll structures are typically denatured at 49 °C (328). Fatty acids which are also a component in seaweed are stable enough to survive the autoclave process as they can degrade between 325 °C and 420 °C. However, these are non polar molecules and it is unlikely that they are present in high concentration in the water extracts (329).

2.3.7 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration(MBC) analysis

Due to the extent of the antimicrobial activity exhibited by the water extract of *F vesiculosus*, further characterisation was carried out on this extract alone. As such, MIC and MBC assays were performed on this extract and not on the organic solvent extracts.

Also, while it is possible to perform broth dilution assays on organic solvent extract, many extracts will not solubilise in the bacterial medium, as organic solvents are toxic to bacteria, water extracts are more suitably analysed using these methods.

The concentrations of the water extract from *Fucus vesiculosus* required for MIC, MBC are outlined in Table 2.11. These concentrations were found to inhibit/disrupt at least 80 % of MRSA (676) which was used as an indicator in this experiment.

Table 2.11: Concentrations (mg/mL) of water extract from *Fucus vesiculosus* required to inhibit >80 % of MRSA (676) (n=18) (± standard error).

	MIC_{80}	**MBC**
Concentration (mg/mL)	3.125±0.21	25.0

It was established that a dose of 25 mg/mL of water extract from *Fucus vesiculosus* will result in a bactericidal effect of colonies formed by an overnight culture of MRSA (676) adjusted to an optical density (625 nm) equivalent to 0.5 McFarland Standard or 10^7 - 10^8 cfu/mL. The MIC_{80} was found to be 3.125 mg/mL. This concentration of extract will inhibit at least 80 % of the culture.

An extract of fucoidan (water soluble polysaccharide from *F, vesiculosus*) was determined to have an MIC of 12.0 mg/mL for *Sargassum polycystum* and 6.0 mg/mL for S. *aureus* (330). This is not in disagreement with the MIC_{80} found in this study as 3.125 mg/mL, particularly as 20-30 % of *F.vesiculosus* is fucoidan (325) and fucoidan can be recovered at 9.65 % using cold solvent extraction similar to the method used in this study (331). Crude glycerine extracts from brown seaweed *Sargassum oligocystum* exhibited an MIC of 3.175, 3.175 and 9.556 mg/mL to inhibit *S. aureus, S. epidermidis* and *P. aeruginosa* (332), which again, is in the same order of magnitude of the results for this study.

The high level of activity displayed by extracts and compounds from *Fucus vesiculosus* found via both disc diffusion and MIC_{80} in this work has been documented in previous studies (292,312,333). For example a polyhydroxylated fucophloroethol extract from *Fucus vesiculosus* was found to inhibit the growth of *S. aureus* (53) among other strains, which shows potential for its use against MRSA. Fucoidan, which can be extracted from *Fucus vesiculosus*, has been shown to have antimicrobial effects against oral bacteria including *S. aureus* (312) and has also been proven to have a synergistic effect against MRSA when administered with the antibiotics oxacillin or ampicillin, increasing the efficacy of treatments by more than four times (334). There is little evidence of the antimicrobial nature of extracts of *Fucus serratus*. One study noted no inhibition of either *Fucus serratus* or *vesiculosus* against *S. aureus* or *E .coli*, however this study was performed on the seaweed itself and not on an extract (286). There is evidence that bacteria found on the surface of these seaweeds can produce compounds with a microbial antagonistic effect (282,335), however the method of preparation of as proteins and chlorophyll structures are typically denatured at 49 °C (328). these extracts, as well as autoclave studies detailed here (section 2.3.6), rule out the possibility of activity from marine-associated bacteria.

2.4 Conclusions

The broad range of activity for extracts of *Fucus vesiculosus* and *Fucus serratus* indicates that the antimicrobial compound(s) extracted from these seaweeds have the potential to be used as an antimicrobial agent against resistant strains of *S. aureus.* Water extracts from *Fucus vesiculosus* in particular exhibited promising results. Many of the *Fucus vesiculosus* extracts performed better than the positive control, including the water extracts against MRSA strains 669, 673,675 and 679 - 683, the acetone extract against MRSA 676, and the methanol extract against MRSA 683. It is also worth noting that these novel strains were obtained via collaboration with University Hospital Waterford, as such they are a current problem in the realms of medicine, adding novelty to the results.

As bacteria such as *S. aureus*, including MRSA, are opportunistic parasites often found to infect wounds, there is an obvious gap in the market for wound dressings impregnated with extracts such as those extracted as part of this study. The potency of the *Fucus vesiculosus* water extract is of particular intrigue as water is a non-toxic, green solvent. Due to the superior results of the water extract from *F. vesiculosus* when compared to other solvents, work will focus on the water extract in future studies, such as anti-biofilm and cytotoxicity.

This data also presents clues as to the class of compound responsible for activity in the aqueous fraction from *F. vesiculosus*. Water has a polarity index of 10.2 meaning that non polar compounds such as lipids are unlikely to be present in high concentrations. Proteins and polysaccharides however, are often soluble in aqueous solutions. The results of the autoclave study would indicate that the antimicrobial present in this extract is thermally stable, as antimicrobial activity was maintained in the sample subsequent to being held at 121 ^{o}C for 30 min. As proteins and chlorophyll structures are typically denatured at 49 ^{o}C (328), the antimicrobial compound is unlikely to be a protein. Therefore, it is speculated that the compound(s) responsible for the antimicrobial activity in the aqueous fraction of *F. vesiculosus* is potentially a polysaccharides.

The results retained in this section were treated as a flagship for further studies in bioactivity and incorporation of the most potent extract (water extract from *F.*

vesiculosus) into wound dressing formulations for the purpose of the development of a 'smart' drug delivery system (detailed in section 5). This study ensured that the water extract from *F. vesiculosus* was focused on and characterised in section 6 of this work.

Chapter 3: Investigation into antibiofilm and cytotoxic activity of water extracts from *F .vesiculosus*

3.1 Introduction

The aggregation of microorganisms in the wound matrix can facilitate the formation of biofilms, which encapsulate and thereby protect bacterial infections from the immune response (174) and traditional antimicrobial treatments (175). Typically, debridement is used to manage biofilm formation in wounds, as traditional antibiotic therapies can require 10-1500 times the dose to eradicate biofilm encapsulated infections (336). While debridement is an effective tool for maintaining a healthier wound bed, it is understandably uncomfortable for the patient and it has been noted that 57 % of wounds can regress in favour of the biofilm using debridement alone (336). As such, the use of antibiofilm agents which could potentially disrupt or prevent the formation of a biofilm as a tool for wound care could improve patient care and reduce wound treatment times (337). Antibiofilm agents could aid wound management by removing the biofilm burden and thereby allow infected wounds to be treated with antibiotics. Antibiofilm agents could allow antibiotics to be more effective by; degrading the extracellular polysaccharide matrix, disrupting quorum sensing and hindering initial attachment of the biofilm forming cells to surfaces (338).

For the development of an enhanced antimicrobial wound dressing formulation, any additional antibiofilm activity would be largely beneficial. There are many studies currently available which outline the antibiofilm activity of marine-associated bacteria (339) and algae (340–342). Ethanol extracts of A*vicennia marina* successfully limited the biofilms of both *Flavobacterium sp.* and *Bacillus sp* using 100 µL of extract at the concentrations they would naturally occur at within the seaweed (340). *Halidrys siliquosa* extracts were found to inhibit the biofilm formation of *Stalphlococcus sp., Streptococcus sp, Enterococcus sp., Psedonmonas sp., Stenotrophomonas sp. and Chromobacterium sp.* with concentration of 1.25 mg/mL to 5 mg/mL respectively (341). Another study using an ethanol extract of *Sargassum ilicifolium* and methanol extracts from *Sargassum sp.* and *Padina tetrastromatica* displayed antibiofilm activity against eight pathogenic bacteria (342).

This antibiofilm activity could potentially be due to the use of sub-lethal dosing observed in the environment to naturally disrupt and prevent the formation of a biofilm on the host organism, whilst not annihilating microbes that have ecologically relevant functions (343). As synthetic antibiotics such as, tetracycline (344), vancomycin (345)

and oxacillin (346), encourage the formation of biofilms there is potential for natural products as a source of biologically active compounds (343).

As such, it was speculated that the water extract from *F. vesiculosus* which outperformed other extracts as part of this thesis could also contain antibiofilm capabilities. Active extracts could potentially be used as part of a wound dressing formulation. Burn wounds are especially susceptible to infection, as thermal injury can cause the underproduction of neutophils and cytokines by macrophages resulting in a decrease in natural bactericidal activity and immune system direction. The avasular nature of a burn can also hinder the transport of lymphocytes and more obviously, the natural cutaneous barrier is broken (322). Due to the increased risk of infection, active wound dressings containing an antimicrobial may decrease the overall treatment time and cost associated with burn wound victims.

As the seaweed extract is intended for use in a topical formulation, it is important to assess any cytotoxic activity that may be associated with its use. Measuring the cytotoxicty of mammalian cell lines *in vitro* determines the viability of cells subsequent to being exposed to the extract (347). This can be an indication of the feasibility of wound dressings imbibed with the seaweed extract as a commercial product as the International Organization for Standardization, which is endorsed by the FDA, states that cytotoxicity is one of the criteria for biological evaluation of medical devices (348). It should be noted that these cell models are used as an *in vitro* analysis. While this does give an indication of how the extract will perform when in contact with these cells, they do not always reflect how treatments will behave *in vivo*. Therefore, the safety of the extracts cannot be guaranteed without further *in vivo* studies including clinical trials.

Cytotoxicity assays can also determine if the extract encourages cell proliferation by monitoring if cell viability is greater than 100 % (349). Other bioactive activities can be identified by analysing the cell proliferative activity of the extract. For example, wound healing as in the case of the natural product aloe vera (350).

3.2 Experimental proceedure

3.2.1 Biofilm prevention analysis

The method for Biofilm prevention was adapted from CLSI (formerly NCCLS) guidelines (351). Dried extracts were dissolved to a starting concentration of 50 mg/mL in Mueller Hinton Broth (MHB). 100 µL of this 100 mg/mL solution was then added in triplicate wells to a 96 well microtitre plate; serial twofold dilutions were then carried out on these samples. A row of control samples were also prepared for each of the dilutions, without the addition of bacteria. Other control samples included three wells of 100 µL of 10 µg/mL chloramphenicol as a positive control and six wells of 100 µL MHB; three as a media only controls and three as negative controls.

A 1 % inoculation of MRSA (676) was prepared in BHI and allowed to incubate overnight at 37 ºC. The subsequent cells were then washed in triplicate with MRD and adjusted to a McFarland standard of 10^7-10^8 colony forming units per mL as described in section 2.2.4. The adjusted solution was then diluted 1:100 *v/v* in MHB to a concentration of 100 mg/mL and 100 µL was added into each of the three rows of sample wells, the three positive control wells and the three negative control wells. 100 µL of sterile MHB was added into the remaining wells (sample controls, media only controls).

After overnight incubation at 37 ºC, the supernatants were transferred to a separate plate. Each well of the original plate, potentially containing a bacterial biofilm, was washed in triplicate carefully using sterile PBS and then 110 µL was added of MRD. Bacteria containing wells were carefully scraped into the MRD solution using 20 - 200 µL pipette tips. The resulting suspension was then carefully aspirated and transferred to a separate 96 well plate. Serial tenfold dilutions were made on bacteria containing samples and controls in MRD and then plated neat-10^{-7} to achieve plate counts. Positive and media only controls were plated neat only.

Plate counts were achieved for the supernatants using the same method. Biofilm prevention was then calculated as a percentage against the negative controls. This method was repeated in triplicate on different days and calculated using the equation:

Equation 3.1: % Biofilm Prevention = $100 - \left(\frac{\text{sample (cfu/mL)}}{\text{negative control (cfu/mL)}} \times 100 \right)$

3.2.2 Biofilm disruption analysis

The method of biofilm disruption was adapted from CLSI (formerly NCCLS) guidelines (351). The biofilm prevention assay differs from the biofilm disruption assay as is performed on an established biofilm. This assay therefore treats the plate after the addition and incubation of a biofilm forming bacteria, and not before as described in biofilm prevention analysis.

A 1 % inoculation of MRSA (676) was prepared in BHI and allowed to incubate overnight at 37 ºC. The subsequent cells were then washed in triplicate with MRD and adjusted to a McFarland standard of 10^7-10^8 colony forming units per mL, as described in section 2.2.4. The adjusted solution was then diluted 1:100 *v/v* in MHB and 100 µL added into all but one row and one column of a 96 well microtitre plate. 100 µL of sterile MHB was added into the remaining wells (sample controls, media only controls). These plates were incubated for 48 h at 37 ºC to ensure the formation of a biofilm.

Subsequent to incubation, treatments were prepared for the wells by dissolving dried extracts to a starting concentration of 50 mg/mL in MHB. Serial twofold dilutions were then carried out on these samples in MHB. Other control treatments prepared included 10 µg/mL chloramphenicol as a positive control and sterile MHB as a negative control.

100 µL of each of the twofold sample dilutions mentioned above was added into three bacteria containing wells and the row containing no bacteria. 100 µL of the chloramphenicol solution was added to three bacteria containing wells as a positive control and 100 µL of MHB was added to all other wells as a negative control. The plates were then left to incubate for a further 18 - 20 h at 37 ºC.

After overnight incubation at 37 ºC, each well was washed in triplicate carefully using sterile PBS and 110 µL was added of MRD. Bacteria containing wells were carefully scraped into the MRD solution using 20 - 200 µL pipette tips. The resulting suspension was then carefully aspirated and transferred to a separate 96 well plate. Serial tenfold

dilutions were made on bacteria containing samples and controls in MRD and then plated neat-10^{-7} to achieve plate counts. Positive and media only controls were plated neat only. Biofilm disruption can then be calculated as a percentage against the negative controls. This method was repeated in triplicate on different days and % biofilm disruption was calculated using equation 3.2.

Equation 3.2: % Biofilm Disruption $= 100 - \left(\frac{\text{sample (cfu/mL)}}{\text{negative control (cfu/mL)}} \times 100 \right)$

3.2.3 Cytotoxicity

3.2.3.1 HepG2 cells

HepG2 cells were thawed from glycerol stocks stored at -80 ºC and immediately added to 9 mL of Eagles Essential Minimum Medium (EMEM) with 10 % foetal bovine serum (FBS) and 1 % penicillin/streptomycin solution in a 15 mL falcon tube. After centrifugation at 200 g for 5 min, the supernatant was discarded and replenished with 5 mL of fresh medium pre warmed at 37 ºC. The cells were then added to a 25 cm^3 cell culture flask and allowed to incubate flat (as they are an adherent cell line) at 37 ºC in a 5 % CO_2 atmosphere until 80 % confluence was reached, replenishing medium every two days. Confluence was assessed visually under and inverted microscope (352).

Cells were split 1:3 *v/v*. Once 80 % confluence was reached, the cells were passaged by removing the medium and washing the flask with sterile PBS three times. Subsequent to the third wash, 5 mL of tripsin enzyme (Sigma-Aldrich) was added to the flask and incubated for 15 min at 37 ºC in a 5 % CO_2 atmosphere or until the cells were no longer adherent to the flask. Once the cells were in suspension, 5 mL of FBS-incorporated medium was added to inhibit the enzyme trypsin. This solution was then poured into a centrifuge tube and spun at 2000 g for 5 min. The supernatant was discarded and the cell pellet re-suspended in 15 mL of fresh medium to ensure the removal of the trypsin. Cells were then available for passage.

Once the cell population was high enough for assay (10 mL of $>1.5x10^6$ cells/mL determined by estimation based on initial cell concentration and number days incubated), the cells were concentrated down by first removing them from the flask as described above until they were suspended in a known volume of fresh medium. The population of this suspension was then counted using a haemocytometer. 5 µL of suspended cells were mixed in equal volume with 0.4 % Trypan Blue dye (Sigma-Aldrich) in a micro centrifuge tube. This solution was then placed in a haemocytometer; the four corner squares and centre square were counted and averaged on both sides of the haemocytometer. This figure was then multiplied by 2 to take into account the dilution factor for the dye and then multiplied by $1x10^4$ to account for the haemocytometer volume of $1x10^{-4}$ mL. The resulting figure had a unit of cells/mL. The established concentration was diluted using fresh medium until the desired concentration of $1.5x10^6$ cells was reached.

100 µL of suspended cells were seeded into ten out of twelve columns in seven out of eight rows of a 96 well microtitre plate, at a concentration of $1.5x10^6$ cells/mL, and allowed to incubate over a 48 h period. Prior to the removal of cells, extract treatments were prepared by dissolving dried extract in FBS free medium. Twofold serial dilutions were then performed in serum free medium leaving three columns clear for 100 µL medium only, 100 µL medium and cells only and 100 µL of 0.5 M and 0.25 M deoxycholic acid as a positive control split over one column (four wells 0.25 M and four wells 0.5 M). An example can be seen in Figure 3.1.

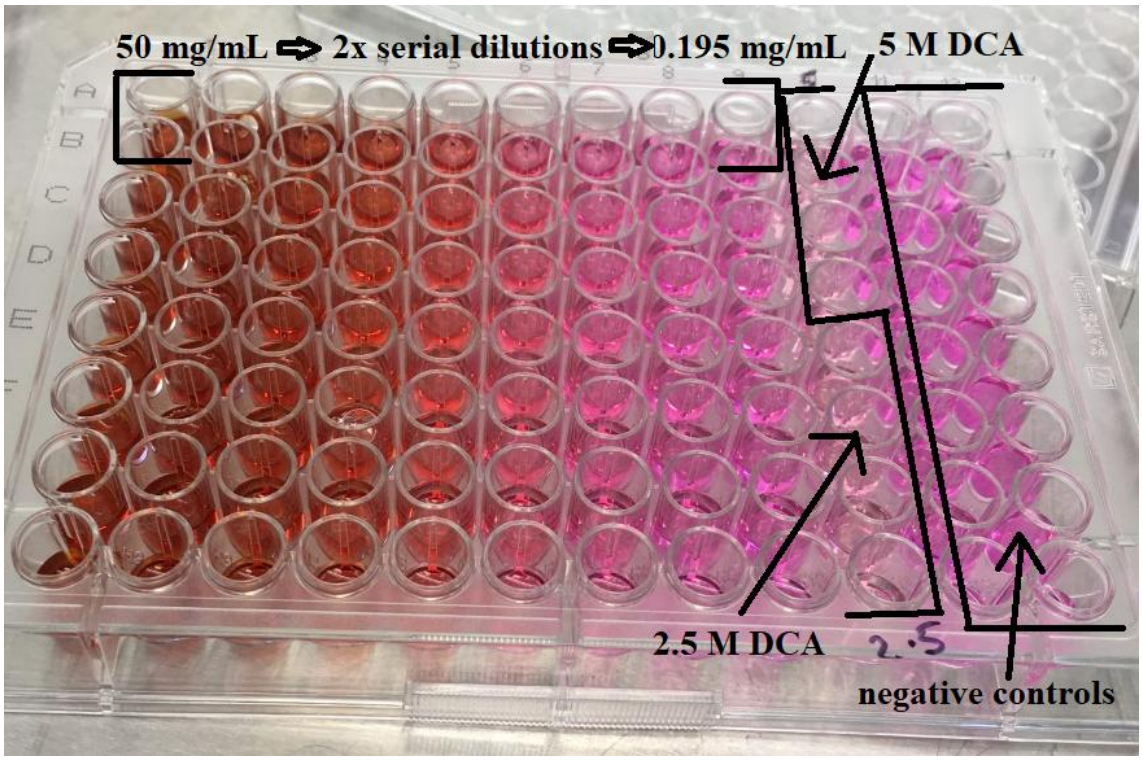

Figure 3.1: Sample setup for MTT assay.

The cells were allowed to incubate overnight at 37 ºC in a 5 % CO_2 atmosphere. After incubation, the wells were washed in triplicate with 100 µL PBS carefully so that adherent cells were not removed from the bottom of the wells during this process. The wells were replenished with 100 µL of pre warmed serum free medium at 37 ºC and 15 µL was added of MTT (Thiazolyl Blue Tetrazolium Bromide (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide - Sigma-Aldrich) to stain living cells, before incubating at 37 ºC in a 5 % CO_2 atmosphere. After 4 h, the plates were removed and 100 µL of solubilising buffer was added (446 mL isopropanol - Thermofisher Scientific, 50 mL Triton-X - Sigma-Aldrich and 4 mL HCl - Sigma-Aldrich). The plates were then incubated for a further 3 h at 37 ºC in a 5 % CO_2 atmosphere, to solubilise the MTT crystals. Subsequent to incubation, the plates were read spectrophotometrically on a mictrotitre plate reader at 570 nm (BioTek ELx800 Absorbance Microplate Reader) to determined the concentration of stained living cells. This method was repeated three times over three different weeks to achieve n=3. The above method was performed

completely under aseptic conditions in a Class 2 Biohazard cabinet dedicated for cell culture work. Cell % viability was calculated according to equations 3.3 and 3.4.

Equation 3.3: $\text{Absorbance}_{(\text{sample})} = \text{Absorbance}_{(\text{extract+cells})} - \text{Absorbance}_{(\text{extract})}$

Equation 3.4: $\text{Cell viability} = \left(\frac{\text{Abs}_{(\text{sample})}}{\text{Abs}_{(\text{cells})}} \right) \times 100\ \%$

3.2.3.2 HaCaT cells

HaCaT cells are human skin keratinocytes to give an indication of the toxicity of the extracts on human skin cells. HaCaT cells were assessed using the same protocol as HepG2 cells described above with the following adjustments:

- Medium: RPMI (Roswell Park Memorial Institute medium) with 10 % foetal bovine serum (FBS) and 1 % penicillin/streptomycin solution.
- Cell concentration: Cells were seeded into 96 well microtitre plates at a concentration of $7x10^3$ cells/mL.

3.2.3.3 THP-1 cells

THP-1 cells were thawed and revived as per section 3.2.3.1. using RPMI with 10 % foetal bovine serum (FBS) and 1 % penicillin/streptomycin solution as medium.

As THP-1 cells survive in suspension, they were seeded after revival at a concentration of $3x10^5$ cells/mL. Cultures were maintained between the concentrations of $3x10^5$ and $8x10^5$ so as to keep the culture populations of cells below that of $1x10^6$ cells/mL to ensure that they do not mutate due to over population. THP-1 cells were passaged by centrifuging the culture at 200 g for 5 min, and re-suspending the pellet in fresh medium

at the appropriate concentration. Cells were counted as per section 3.2.3.1 and medium was replaced every 2 days.

As THP-1 cells are in suspension, they were treated with PMA (phorbol-12-myristate 13-acetate - Sigma-Aldrich) at a concentration of 5 ng/mL medium prior to being seeded into ten out of twelve rows of a 96 well microtitre plate at a concentration of $1.5x10^5$ cells/mL and incubated for 48 h at 37 ºC in a 5 % CO_2 atmosphere to stimulate them to become adherent. Subsequent to the adhesion of the cells, they were assessed using the MTT assay using the protocol described in section 3.2.3.1.

3.2.4 Safety procedures for microbiological work

All microbiological work was carried out in a Class II pathogen lab with an emphasis on safety and containment of pathogenic substances. The Standard Operating Procedure (SOP) for the pathogen lab was followed, including: all items brought into the lab were autoclaved on exit. Only pathogen lab coats must be worn in the pathogen lab, gloves must be used at all times and hands washed at exit. All waste in pathogen lab must be autoclaved subsequent to disposal and benches washed down with 70 % *v/v* ethanol in water after use. All contaminated organic solvents were evaporated to dryness and diluted with virkon, before disposal.

3.2.5 Statistical analysis

Results are reported as the mean ± standard deviation. Comparisons between results were made using one-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test. All statistical analysis was performed using Minitab 17 software with a 5 % statistical significance level ($p<0.05$)

3.3 Results and discussion

3.3.1 Biofilm prevention and biofilm disruption analysis

The concentrations of the water extract from *Fucus vesiculosus* required for biofilm prevention and biofilm disruption are summarised in Table 3.1 and further in Figure 3.2. These concentrations were found to inhibit/disrupt at least 80 % of MRSA (676) biofilm which was used as an indicator bacteria in this experiment.

Percentage inhibition of the biofilm was calculated in relation to a negative, media only control, the results of which were determined to be the biofilm at 100 % viability. As such a percentage inhibition of 80 % results in 80 % of the negative only control being inhibited.

Table 3.1: Concentrations (mg/mL) of water extract from *Fucus vesiculosus* required to inhibit >80 % of MRSA (676) for biofilm disruption and prevention (n=18) (± standard error). (% inhibition is in relation to inhibition compared to a negative, media only control of 100 % cell viability)

	80 % Disruption	**80 % Prevention**
Concentration (mg/mL)	25.00±1.06	6.25 ± 0.31

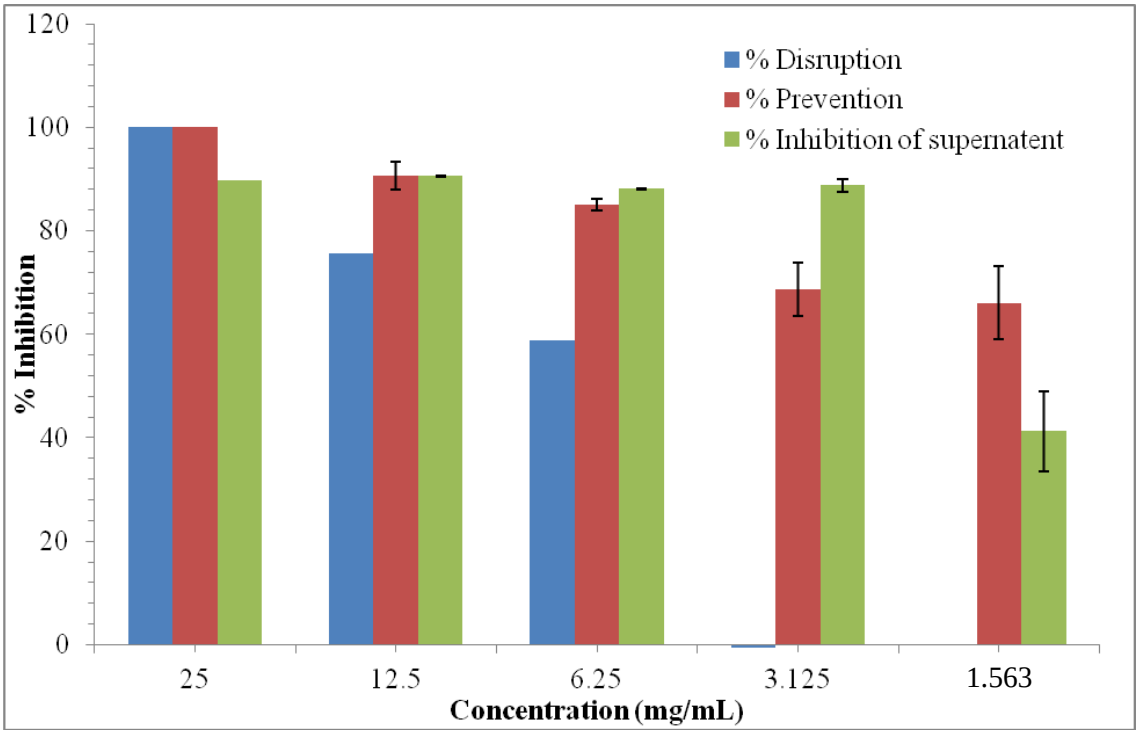

Figure 3.2: % Inhibition of MRSA (676) by the water extract of *Fucus vesiculosus* over a series of twofold dilutions in concentration (mg/mL) (n=18). The exact values for % inhibition are presented in Table 3.2.

Table 3.2: Concentrations (mg/mL) of water extract from *Fucus vesiculosus* required to inhibit >80 % of MRSA (676) for different microbial assays (n=18) (± standard error).

Extract Concentration mg/mL	**% Disruption**	**% Prevention**	**MIC**
25	99.96 ± 0.00	100 ± 0.00*	89.66 ± 9.73
12.5	75.59 ± 0.00	90.60 ± 2.69	90.55 ± 13.42
6.25	58.89 ± 0.00	85.11 ± 1.12	88.13 ± 17.98
3.125	-16351.18 ± 1.53	68.66 ± 5.22	88.85 ± 28.52
1.563	< -16351.18	66.10 ± 6.96	41.30 ± 12.27

Note: negative values indicate a promotion in bacterial growth.*standard error of 0.00 was due to no growth on any of the plates, therefore all replicates had the same result.

It was established that a dose of 25 mg/mL of water extract from *Fucus vesiculosus* will result in the disruption of a biofilm formed by an overnight culture of MRSA (676) adjusted to an optical density (625 nm) equivalent to 0.5 McFarland Standard or 10^7 - 10^8 cfu/mL. Similarly, 25 mg/mL had a bactericidal effect on the adjusted culture indicating that this dose is sufficient to completely kill the bacteria. This is described as the MBEC (minimum biofilm eradicating concentration) (353). While the biofilm disruptive action of the water extract of *F. vesiculosus* is promising, the 25 mg/mL concentration required to achieve 100 % MBEC is significantly higher than that of other extracts mentioned in the literature.

It was determined that a lower concentration of 6.25 mg/mL was required to prevent the formation of a biofilm with MRSA (676), highlighting the potential of this seaweed extract as a prophylactic treatment to infection. This can more clearly be seen in Figure 3.1, where the prevention of the biofilm consistently required less extract than was required to disrupt the biofilm, but more than what was needed to inhibit suspended bacteria (MIC_{80}) not sheltered by the biofilm. As such, this study reinforces the fact that cultures encompassed in biofilms are harder to treat than planktonic bacteria (170).

Marine ecosystems such as seaweed are a novel source of biofilm disrupting and prevention compounds (354). Toluene extracts of *Chondrus crispus* were noted to inhibit bacterial attachment leading to the biofilm formation of *Caloplaca. marina* at the much lower concentration of 50-200 ppm and *Marinobacter hydrocarbonoclasticus* at a concentration of 100-200 ppm (355). Also, the antibiofilm nature of the aqueous extract from *F. vesiculosus* found as part of this study has been noted in several studies. Further antifouling effects of *F. vesiculosus* extracts (of which antibiofilm is included as anti-settlement) have been established against the marine microbial foulers; *Cytophaga sp., B. aquaimaris, Cobetia marina* and *Ulvibacterr litoralis* (290,356) indicating that these results are in line with other studies. However, these studies focus on the ability of *F. vesiculosus* as a host organism to resist the formation of biofilms and extracts generated from the seaweed. Also, it should be noted that after an extensive search, the use of extracts of *F. vesiculosus* to reduce the biofilm of MRSA was not found in the literature.

Interestingly, fucoidan (which can be extracted from *F. vesiculosus*) at a concentration of 250 µg/mL was found to completely suppress the growth of planktonic bacteria and biofilm formation of some orally sourced Gram positive bacteria (312). Again, this could indicate the potential of seaweed extracts to be used as a complimentary therapy in conjunction with established antibiotics.

As stated in section 3.1, mechanisms of biofilm disruption typically rely on the degradation the extracellular polysaccharide matrix, disrupting quorum sensing in the biofilm and hindering initial attachment of the biofilm forming cells to surfaces (338). Typically, premature biofilms require less antibiotics to clear that those required to disrupt a mature biofilm (357), as such, it is unsurprising that biofilm prevention was achieved at a lower concentration (6.125 mg/mL) than biofilm disruption (25 mg/mL) in this study. Additionally an antibiofilm mode of action which includes the prevention or hindering of initial attachment suggests that biofilm prevention is more straightforward than biofilm treatment, however, further studies would be required to determine the antibiofilm mechanism of action for the aqueous extract of *F. vesiculosus*.

The relatively high concentrations of 25 mg/mL could have implications for the toxicity of the treatment against human cells. The absence of a cell wall in mammalian cells typically results in mammalian cells being more sensitive to cell death than bacterial cells (358), this sensitivity can translate to a sensitivity to adverse effects on exposure to substances.

The biofilm disrupting and preventing nature of the water extract from *F. vesiculosus* against MRSA 676 could potentially be an indication of the mode of action of the antimicrobial present in the seaweed, as biofilm producing bacteria are notoriously more difficult to treat using antibiotics (175). This could pave the way for a potential application of *F. vesiculosus* extracts in tandem with a lower dose of antibiotics. This and the susceptibility of AMR strains of *S. aureus*, coupled with the safe use of seaweed edibles for millennia (359), make these extracts particularly promising in terms of therapeutics.

Interestingly, it was noted that a dose of 3.125 mg/mL caused an increase of cell viability to 16,351 % of the negative control which can be seen in Table 3.2 . Doses lower than this were not diluted enough to be counted, indicating that their cell viability was >16,351.0 % by over a factor of 100. The exact cause of this increase is unknown.

However, it has been speculated that the hormesis effect is responsible for this phenomenon. The hormesis effect describes the often observed dose response that was demonstrated as part of this study, whereby higher doses have a toxic effect and lower doses have a promoting effect on cell viability (360). This stimulation of cell viability at a low dose was similarly observed in a study analysing aminoglycoside antibiotics (361) with the three commercially available antibiotics, tobramycin, ciprofloxacin and tetracycline (362).

Water, being a 'green' is a desirable solvent to industries such as the pharmaceutical industry. The display of antimicrobial activity exhibited by the water extract of *F. vesiculosus* against MRSA 676 in this study shows promise for these extracts to be used as a drug or supplement in a drug delivery system. The merit of the tested extracts' antibiofilm activity in conjunction with its antimicrobial effects, could further aide in the treatment of wounds by protecting them against biofilm formation. This could establish an increased vulnerability of the infection to antimicrobial treatment in addition to possibly preventing the uncomfortable debridement of the wound.

3.3.2 Cytotoxicity

As the extract was intended for use in a wound formulation, cytotoxicity was carried out on three different cell lines, the results of which are detailed below in Figure 3.3 and Tables 3.3 and 3.4. The percentage of cell viability was calculated against a negative, meduim only control, which was taken to be 100 % cell viability.

Table 3.3: Concentrations (mg/mL) of water extract from *Fucus vesiculosus* required to inhibit >80 % of the mammalian cells (n=21) (± standard error).

	THP-1	**HepG2**	**HaCaT**
Concentration (mg/mL)	0.3906±3.97	<0.1953	>0.1953

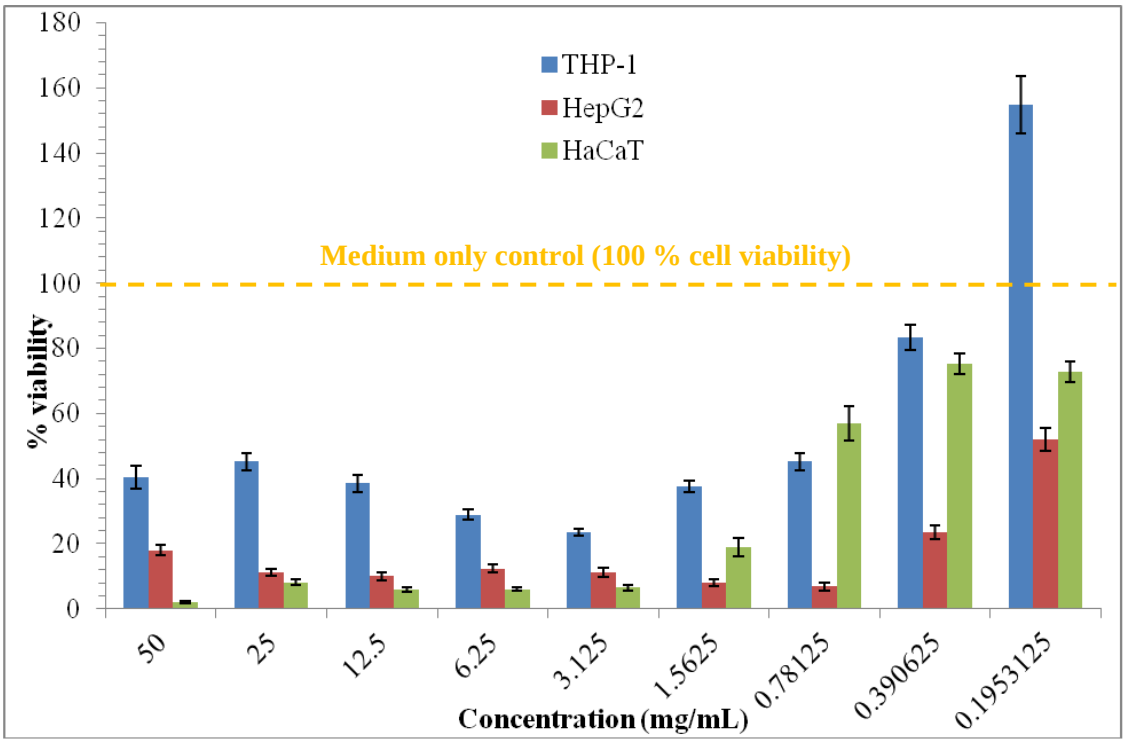

Figure 3.3: Cytotoxicty of water extract from *F. vesiculosus* against THP-1 (blue), HepG2 (red) and HaCaT (green) cell lines.(n=21)

Table 3.4: Cell viability concentrations (mg/mL) of water extract from *Fucus vesiculosus* for mammalian cells (n=21) (± standard error).

Extract Concentration mg/mL	Cell viability %		
	THP-1	**HepG2**	**HaCaT**
25	40.42 ± 3.39	17.95 ± 1.67	2.00 ± 0.32
12.5	45.24 ± 2.59	11.13 ± 1.01	8.14 ± 1.00
6.25	38.48 ± 2.56	10.03 ± 1.25	5.83 ± 0.62
3.125	28.93 ± 1.57	12.33 ± 1.13	5.95 ± 0.54
1.563	23.45 ± 1.03	11.11 ± 1.40	6.44 ± 1.00
0.7813	37.65 ± 1.77	8.02 ± 0.98	18.78 ± 2.82
0.3906	45.22 ± 2.56	6.86 ± 1.17	56.86 ± 5.22
0.1953	83.35 ± 3.97	23.47 ± 2.22	75.21 ± 3.11

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is a yellow tetrazolium salt which turns purple when it undergoes reduction and converts into formazan salt (363). Enzymes such as dehydrogenases which are present in living cells, can catalyse the reduction of MTT in the cells to a purple colour. Much like the other assays, this can be quantified using a UV-Vis spectrophotometrically. This method is sensitive and is the ATCC recommended method of use. It works on both adherent and suspended cells which is a major benefit. MTT however is highly toxic and therefore presents a health and safety hazard (364).

THP-1 cells are a human monocyte cell line that was initially isolated from a patient with acute monocytic leukaemia (365). Monocytes effectively protect against microbial infiltration of Gram negative pathogens (366). As monocytes circulate in the blood as part of the immune system, they invade the wound site and become activated macrophages which can aid the repair of the wound by releasing growth factors (126).

Marcorphages can also contribute to the phagocytosis of pathogens (366). As these cells are present in the blood, they were used as an indicator as to whether the extract would be cytotoxic to monocytes and, therefore, would hinder the immune system and healing of the wound bed (366). The seaweed extract demonstrated cytotoxic activity against THP-1 cells down to a concentration of 0.7813 mg/mL as is displayed in Figure 3.3. Concentrations lower than 0.7813 mg/mL, were not cytotoxic and were even growth promoting at 0.153 mg/mL. This increase in cell proliferation could be due to the hormesis effect, which was observed in the biofilm section of this chapter. This was not unexpected, as THP-1 cells were noted to have undergone the typical biphasic concentration response curve for other substances such as silver nanoparticles (367) and macrocylic trichothecenes (368). This phenomenon could potentially have a positive impact on the use of the water extract from *F. vesiculosus* in wound dressings. If incorporated at low doses, this extract could potentially encourage cell proliferation and thereby, wound healing.

HaCaT cells are immortalised human skin keratinocytes (369) and as such were considered a good indication of whether the bioactive extract would be cytotoxic to human skin. The water extract of *F. vesiculosus* was determined to be cytotoxic using the MTT assay. As there was under 80 % cell viability for all concentrations tested down to < 0.195 mg/mL and the MIC of the crude extract is 3.125 mg/mL, this extract is more cytotoxic to HaCaT cells than it is antimicrobial. It was noted that the HaCaT cells followed a typical dose response for toxic compounds, where lower concentrations tested had a lower % viability.

HepG2 cells are an immortalised cell line extracted from a human hepatocellular carcinoma (370) and were chosen as an indication for toxicity of the extract on the liver. HepG2 cells followed the same trend as HaCaT cells, however, none of the tested doses resulted in greater than 80 % cell viability, indicating that these cells are particularly sensitive to the cytotoxic effect of the crude seaweed extract.

It has been noted extensively in the literature that fucoidan (which is water soluble and is present at 9.8 % dry mass in *F. vesiculosus*) is an effective anti-tumour agent (331,371–373). It has been shown to be toxic against Lewis lung carcinoma cells (LCC) and melanoma B16 cells (MC) above 0.2 mg/mL (372), HT-26 and HCT116 (human colon cancer cell lines) above 20 µg/mL concentrations (371). This is not out of line

with the results shown above as the HaCaT cells fall just below the limit of 80 % at 75 % viability at 0.19 mg/mL and the THP-1 are non toxic at this concentration. The HepG2 cells are still vulnerable to the extract at this concentration. It has been detailed that fucoidan has anti-tumour causing apoptosis and attacks the growth mechanisms in cancer, but not toxic to non-cancer cell lines (374,375).

The cytotoxic effect of this extract could potentially be due to the fact that it is a crude extract, and therefore contains impurities that have the potential to be cytotoxic. Repeating this investigation work on a purified extract could result in different results for cytotoxicity. The attempted purification of this extract will be carried out in section 6 of this work, however, due to the mass of sample that would be required to repeat these biological assays on a purified extract, the development of a method to maximise the extraction efficiency was subsequently focused on.

While it cannot be presumed that the cytotoxic effect observed as part of this work is solely dependent on the cytotoxic effects that fucoidan has demonstrated, it is possible that the cytotoxic component is not toxic to non-cancer cell lines. As such, further studies on non-immortalised cell lines would be required to definitively identify the cytotoxicity of the water extract of *F. vesiculosus.*

3.4 Conclusions

The water extract of *F. vesiculosus* was analysed for biofilm preventative and biofilm disruptive capabilities, as biofilm growth has a major impact on the management and treatment of wounds. It was found that a biofilm grown using MRSA (676) was disrupted by the extract at a concentration of 25±1.06 mg/mL and prevented at a concentration of 6.25±0.31 mg/mL, which was determined to be promising in terms of wound management. To our knowledge, the antibiofilm activity of *F. vesiculosus* extracts against MRSA biofilms has not been noted in the literature. This novel result could have positive consequences for the use of this extract to treat wound infections which are likely to contain methicillin susceptible *S. aureus. (*MSSA) or MRSA.

The seaweed extract demonstrated cytotoxic activity against THP-1 cells at a concentration of 0.3906 mg/mL, lower concentrations of which appeared to have a cell proliferative effect on the cell line. This phenomenon could be due to the hormesis effect. It should be noted that 0.3906 mg/mL is below the concentration of MIC for the pathogens tested, indicating that the crude extract would be unfit for use in a drug delivery system. However, as the extract was crude and therefore contained impurities which could potentially be responsible for this cytotoxicity, further work post purification would be required to determine if the antimicrobial compound(s) from *F. vesiculosus* are cytotoxic.

HaCaT and HepG2 cells were susceptible to the cytotoxic effects of the water extract from *F. vesiculosus* at all concentrations tested, indicating that this extract is particularly toxic against these cell lines down to a concentration of <0.1953 mg/L for both cell lines. As this extract should contain a significant amount of fucoidan, it was speculated that this observed cytotoxic effect could be due to the presence of fucoidan in the sample, and may not be cytotoxic to these cell lines. As all cell lines tested were cancerous, as such, further work would be required by repeating this assay on primary cell lines, to confirm the cytotoxicity of the extract.

As the bioactivity of the water extract from *F. vesiculosus* has been established, further work developing a method for the extraction of this bioactive fraction was carried out.

Chapter 4: The development of a microwave assisted extraction methodology for the extraction of antimicrobials from seaweeds

4.1 Introduction

The established antimicrobial and anti-biofilm activities of the aqueous crude extract of the seaweed *Fucus vesiculosus* as part of this thesis indicated that this extract has the potential to be used as an antimicrobial agent, particularly in the topical treatment of wound infections via bioactive wound dressings. This extract displayed antimicrobial activity against a range of pathogens, many of which were clinically isolated from infected wounds, indicating its potential against wound infections. Similarly, in the previous section of this work, the aqueous extract from *F. vesiculosus* was determined to disrupt a biofilm at a concentration of 25 mg/mL, and prevent biofilm formation at 6.25 mg/mL. The formation of biofilms in a wound infections can hinder wound healing (337), indicating that anti-biofilm agents could prove useful in an infected wound.

The extraction process of this antimicrobial comprised of the agitation of dried, powdered seaweed in water for 2 h at a ratio of 1:100 (*w/v*). This process has the advantage of being relatively 'green', but costly in terms of time, which in turn has an electrical and, therefore, monetary cost associated with it. Microwave assisted extraction (MAE) has been reported to be more efficient for the extraction of bioactive compounds from natural sources than some other methods. For example, a review found that on average MAE takes 1-30 min while Soxhlet extraction has a duration of 3-48 h (61). Traditional methods such as Soxhlet extraction have many disadvantages when compared to modern methods of extraction such as; high volume of organic solvents and the use of high temperatures over extended periods of time which has costs associated with it in terms of personnel and electrical energy (376). Methods such as MAE tend to require smaller volumes of solvents, shorter extraction periods and are capable of being streamlined into automation (66).

Microwaves work by accelerating electromagnetic waves in the radio frequency range towards the sample. The frequency of the waves causes dielectric materials, such as water and other solvents, to undergo dipole rotation. This causes friction on a molecular level and results in the heating of the sample (60). The extraction of bioactives via MAE is more efficient than that of solvent extraction owing to the penetration of the solid powdered seaweed by electromagnetic radiation, thus resulting in changes in cell structure which facilitates the extraction of compounds (61). Other suggestions surrounding the accelerated extraction of bioactives via MAE involve the pressure

generated by the evaporating solvent in closed vessel systems resulting in stress on the plant/seaweed cells which, in turn, results in the breaking of the cell and thus the release of its contents into the extraction solvent (62).

Although MAE is an economical, environmentally friendly technique for extracting secondary metabolites from natural products (69), it has been observed that MAE can degrade compounds (such as polysaccharides (377)) and as such, could result in a loss of the bioactive compounds that are sought. As degradation of bioactive compounds is a documented issue, developing the MAE of antimicrobial extracts from *F. vesiculosus* while preserving the already established activity, is a task that would benefit this project. Investigating the MAE of the antimicrobial compounds responsible for the activity demonstrated by *F. vesiculosus* has the potential to generate higher extract yields in shorter extraction times. Also, it is worth noting that a study by Bagherian *et. al.* investigated the qualitative and quantitative changes made by MAE of pectin from grapefruit, observing that MAE improved both of these factors (378), opening up the potential for MAE to increase the antimicrobial activity established as a part of this work.

The extraction of bioactives from seaweeds using MAE has been documented, However, as a 2015 study on the extraction of bioactives from seaweed using MAE pointed out, there is limited literature detailing the extraction of antimicrobials using these methods (379). Since this paper in 2015, several papers have been published on the MAE of bioactives from seaweed, such as, antioxidant phlorotannins from *Carpophyllum flexuosm*. Research has found that MAE generated a higher extraction yield than conventional hydrothermal extraction, room temperature extraction and solid-liquid extraction (380). A different study tested aqueous extracts extracted via MAE from *Polysiphonia, Ulva* and *Cladophora* against *Staphylococcus aureus* and *Escherichia coli* and found no bacterial inhibition, regardless of the fact that the water extract from *Polysiphonia* harvested in every season was shown to have antimicrobial properties against *S. aureus* (286). This was attributed to degradation of the antimicrobial. However, there have been many examples of bioactives such as polyphenols from *Enteromorpha prolifera* (381), fucoidan from *F. vesiculosus* (62,69), and fucoxanthin from *Laminaria japonica, Undaria pinnatifida* and *Sargassum fusiforme* (382) which were successfully extracted using MAE, with maintenance of

antimicrobial activity. Brown seaweed polysaccharides were similarly reported to have been extracted using MAE with a 40-60 % yield for prebiotics (383).

The parameters investigated as part of this study were; time, temperature, volume and power. There are other variables that could possibly be investigated, such as, feed:solvent ratio which was maintained at 1:100 w/*v* for the duration of this study. This meant the results were comparable to those generated using basic solvent extraction, which was performed in section 2 of this work. Feed:solvent ratio could also be investigated by increasing/decreasing the particle size of the seaweed powder, thereby altering the surface area in contact with the solvent. However, this would be an impractical variable and would render this method incomparable to solvent extraction conditions, which was undertaken using a particle size of <850 nm.

The CEM MARS 5 system used in this experiment has no stirring facility, so it should be noted that basic convection via the heat generated and the rotation of the microwave vessels in the turntable at 8.5 rpm (384) are the only means of agitation that the samples received. Matrix moisture is another variable which is quoted in literature (61), however, as the extraction solvent for this experiment is water, this factor remains irrelevant.

The objective of this study was to develop a method for the extraction of bioactive compound(s) from *F. vesiculosus* using the technique of MAE. Both the recovery of extract and the antimicrobial activity were the important factors on which this method was based. A loss in antimicrobial activity of the extract could signify a degradation of the compound(s) or that the conditions for extraction are not appropriated for the recovery of this crude extract Similarly, if yields are significantly lower than those generated using solvent extraction in section 2, this will result in an MAE method which is not effective when compared to solvent extraction. Ultimately, this method was developed to generate the highest amount of antimicrobial extract from the seaweed mass via MAE; this was performed in the interest of using less raw seaweed, and therefore, improving the sustainability of the research.

4.2 Experimental Procedure

4.2.1 Design of Experiment

There are several variables related to the assisted extraction of antimicrobials from seaweeds via microwaves including: time (min), temperature (°C), volume (mL) and power (W). There are several possible levels within these variables resulting in a very large number of experiments to determine the most favourable extraction parameters. Some variables are limited by the hardware. For example, the Mars 5 CEM microwave used in this experiment has only three options for power, limiting the levels of this experiment to three. The operation manual for this system (384) suggests that no greater than 0.5 g of dry feed should be extracted in any tube, which limited the volume variable to 50 mL, as a 1:100 *w/v* ratio of feed:solvent was maintained to achieve a relatable comparison of the MAE to previous solvent extraction methods used. This ratio was also maintained to ensure that MAE extracts could be directly compared to each other, resulting in a simplified analysis. To avoid the overloading of microwave tubes, an upper limit of 40 mL was chosen.

The variables of time and temperature were chosen subsequent to a review of the literature (61). In addition to several trial microwave runs which indicated that the microwave tubes would not reach or be maintained at a temperature of 85 °C. As such four variables of both time and temperature were chosen and are displayed in Table 4.1.

Table 4.1: The experimental variables for the accelerated microwave extraction of antimicrobials from seaweed.

Time (min)	Temperature (°C)	Volume (mL)	Power (W)
1	30	10	300
2	45	20	600
5	60	30	1200
10	75	40	

It was calculated that the above investigation would result in 192 microwave experiments. Due to the anticipated labour involved in completing such a high number of experiments, a factorial design of experiment was generated using minitab software, bringing the number of experiments down to 52 as can be seen in Table 4.2. The software also randomised the order of the experiments so that any trends associated with operator training or day-to-day variables would be negated.

Table 4.2: Parameters for factorial design of accelerated microwave assisted extraction of antimicrobials from *Fucus vesiculosus*, with randomised experiment order.

Run Order	Order from initial 192 points	Time (min)	Temp (°C)	Volume (mL)	Power (W)
1	190	10	75	40	300
2	48	1	75	40	1200
3	155	10	30	40	600
4	182	10	75	10	600
5	95	2	75	40	600
6	168	10	45	40	1200
7	186	10	75	20	1200
8	60	2	30	40	1200
9	87	2	75	10	1200
10	147	10	30	10	1200
11	130	5	60	40	300
12	139	5	75	30	300
13	175	10	60	30	300
14	23	1	45	40	600
15	41	1	75	20	600
16	161	10	45	20	600
17	129	5	60	30	1200
18	18	1	45	20	1200
19	144	5	75	40	1200
20	180	10	60	40	1200
21	10	1	30	40	300
22	88	2	75	20	300
23	148	10	30	20	300
24	70	2	45	40	300
25	37	1	75	10	300
26	157	10	45	10	300
27	189	10	75	30	1200
28	50	2	30	10	600
29	9	1	30	30	1200
30	27	1	60	10	1200
31	102	5	30	20	1200
32	78	2	60	20	1200
33	69	2	45	30	1200
34	83	2	60	40	600
35	122	5	60	10	600
36	152	10	30	30	600
37	111	5	45	10	1200
38	92	2	75	30	600
39	107	5	30	40	600
40	137	5	75	20	600
41	97	5	30	10	300
42	73	2	60	10	300
43	55	2	30	30	300
44	173	10	60	20	600
45	19	1	45	30	300
46	32	1	60	30	600
47	116	5	45	30	600
48	28	1	60	20	300
49	112	5	45	20	300
50	2	1	30	10	600
51	169	10	60	10	300
52	14	1	45	10	600

Further to the design of the experiment, it was necessary to decide the concentration that was most appropriate for testing for antimicrobial activity. This was tested in triplicate using the solvent extract with well diffusion as described in Chapter 3.2.3. with a dose of 1, 3, 5, and 10 mg/disk against optically adjusted MRSA 676 as an indicator bacteria.

4.2.2 Production of microwave accelerated extracts

The apparatus for the Mars 5 CEM microwave consists of a rotating carousel with the capacity to hold 16 x 50 mL Teflon microwave tubes. All of the tubes in the carousel must be filled with the same amount of water so that the microwaves are distributed evenly to each tube. The microwave extracts were generated by weighing the amount of *Fucus vesiculosus* that corresponded to a 1:100 *w/v* ratio of seaweed:volume into a microwave tube and filling it with the appropriate volume of deionised water for the specific experiment being run. The samples were then loaded into the carousel and any remaining carousel slots were filled with tubes containing a volume of water corresponding to the volume of that specific experiment.

The microwave was programmed to the set of variables outlined in Table 4.2. A ramp time of 3 min was deemed sufficient after a trial experiments and this was included in the program to ensure that the tubes reached their desired temperature.

Once the run was finished, the carousel was removed from the microwave and the tubes containing the samples were decanted into centrifuge tubes. These centrifuge tubes were made up to a volume of 50 mL using deionised water to balance the centrifuge, and centrifuged at a speed of 4500 rpm for 4 min until the seaweed particles had formed a solid pellet. The supernatant was subsequently decanted into pre-weighted bottles and frozen at - 20 ^{o}C before being freeze dried and stored.

4.2.3 Antimicrobial activity dose concentration

Prior to the assessment of antimicrobial activity via the disk diffusion method (described below), a small concentration study was carried out to determine how much

extract was to be used per disk in this screen. Dried water extracts were aseptically dissolved in the solvent of their extraction at a concentration of 100 mg/mL. These solutions were then spotted in 10 µL aliquots until the desired concentration of 1, 3, 5 and 10 mg per disk were achieved. These disks of increasing concentration were then tested against a clinical strain of MRSA (MRSA 618) and MRSA NTCC using the disk diffusion method described below.

4.2.4 Antimicrobial activity of crude seaweed microwave extracts

The antimicrobial activity of the extracts generated above was assessed using the well diffusion assay. This method comprised of inoculating MHA (Mueller Hinton agar- Sigma Aldrich) with a bacteria grown overnight in (Brain Heat Infusion- Sigma Aldrich) BHI broth (1 mL broth per 100 mL agar) and aseptically cutting 6 mm circular wells into the set agar. The wells were then filled with 50 µL of seaweed microwave extract diluted in sterile deionised water to a concentration of 100 mg/mL of freeze dried extract, this corresponded to 5 mg of seaweed extract per well. A 50 µL negative control of sterile deionised water and a positive control of 50 µL of 0.5 mg/mL chloramphenicol (Sigma ≥98 % (HPLC)) were loaded onto each plate along with the seaweed extract. The positive control was prepared by making a 10 mg/mL stock solution of chloramphenicol in ethanol (Lennox, 96 %) and diluting it to a 0.5 mg/mL solution in sterilised glassware using sterile deionised water as a diluent.

4.2.5 Safety procedures for microbiological work

All microbiological work was carried out in a Class II pathogen lab with an emphasis on safety and containment of pathogenic substances. The Standard Operating Procedure (SOP) for the pathogen lab was followed, including: all items brought into the lab were autoclaved on exit. Only pathogen lab coats must be worn in the pathogen lab, gloves must be used at all times and hands washed at exit. All waste in pathogen lab must be autoclaved subsequent to disposal and benches washed down with 70 % *v/v* ethanol in water after use. All contaminated organic solvents were evaporated to dryness and diluted with virkon, before disposal.

4.2.6 Statistical analysis

Results are reported as the mean ± standard deviation. Comparisons between yields were made using one-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test. All statistical analysis was performed using Minitab 17 software with a 5 % statistical significance level ($p<0.05$).

4.3 Results and Discussion

4.3.1 Antimicrobial activity dose concentration

The concentrations of 1, 3, 5, and 10 mg/disk were analysed using the disk diffusion method to observe any increasing dose response. This was preformed to ensure the concentration used did not influence diffusion, which may affect the observation of bacterial inhibition at a maximum dose. The results of this study are presented in Table 4.3. and can be clearly visualised in Figure 4.1.

Table 4.3: Dose response for antimicrobial activity of the water extract of *Fucus vesiculosus* against MRSA 676.

	1 mg	**3 mg**	**5 mg**	**10 mg**
Zone of inhibition (mm)	6.17 ± 0.41^{a}	9.33 ± 0.52^{b}	10.17 ± 0.41^{c}	13.50 ± 0.55^{d}

Values with the same superscript letters indicate a p value of <0.05, n=6.

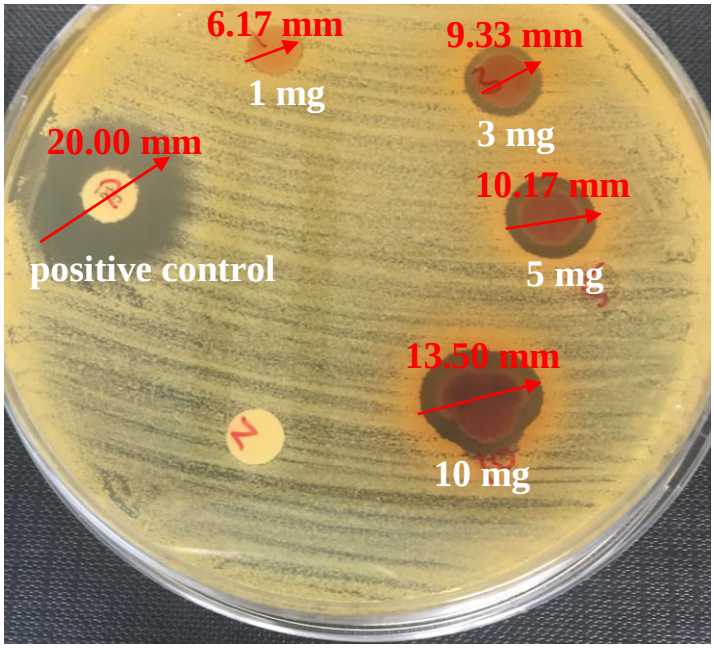

Figure 4.1: Water extract of *F. vesiculosus* at 1, 3, 5, and 10 mg against MRSA 676 using disk diffusion.

The concentrations of 1, 3, 5 and 10 mg was used in this work, even though a range of 10 µg (307), to 5 mg (111), was shown to successfully assess antimicrobial activity from seaweed extracts. However for this study it was thought that at lower doses, the disk diffusion method was not sensitive enough to detect sought after antimicrobial activity.

Based on the results displayed in Table 4.3, 5 mg was chosen as the dose that was to be used on the microwave extracts. This was chosen based on a number of factors. While 1 mg gave antimicrobial activity it was ruled out as an appropriate dose for this experiment as the diameter of the disk is 6 mm.10 mg could have potentially been used also as it gave an activity of 13.5 mm which is well over the disk diameter of 6 mm and statistically different to that of 5 mg. However, this concentration was determined to be too high based on the interest of not wasting extract this left the 3 mg and 5 mg extracts. While these concentrations have similar but statistically different activity, 5 mg was selected so that it could be comparable to the data discussed in Chapter 2.

This study was carried out with the intention of ensuring that the extract would continue to exhibit a dose response. As diffusion based antimicrobial assays can be dependent on the ability of the antimicrobial to diffuse through the polar agar, antimicrobial inhibition for this assay may not always follow a dose response (385). This can be due to: the polarity or size of the extract components being incompatible with the polarity or pore size of the agar (386), and the lowering concentration of antimicrobial bioactives with continuous diffusion.

4.3.2 Antimicrobial activity of microwave extracts

The antimicrobial activity of the microwave digested extract was consistent throughout the range of experiments conducted on the samples. This confirmed that the compound(s) responsible for antimicrobial activity in *F. vesiculosus* was stable at temperatures as high as 75 °C. The stability of these compounds was initially indicted by the establishment of antimicrobial activity after autoclaving a crude, solvent extracted water extract in section 2.3.5. The consistency of the antimicrobial activity can be observed in Table 4.4 (16 mm inhibition zone for all samples including 2 h solvent extract) and also in Figure 4.2 suggesting that the compound(s) responsible for antimicrobial activity does not degrade between room temperature and 75 °C. This is a significant result, as it illustrates the robust nature of the compound(s) responsible for antimicrobial activity. As such, the compound(s) responsible for this antimicrobial is heat stable and water soluble. Many phenols present in seaweeds are water soluble and have been noted to contain bioactivity such as polyhydroxylated fucophlorethol from *F. vesiculosus* which was found to be antimicrobial against *E. coli, S. aureus* and *P. aeruginosa* (53). Other Fucophlorethols and phlorotannins which would be water soluble were also extracted from *F. vesiculosus* and found to antimicrobial such as, trifucotriphlorethol A (387). Low molecular weight phlorotannins (phloroglucinol, diphlorethol, trifluhalol A, dieckol, trifucol and difucophlorethol A) were found to degrade at 50 °C (35). This would indicate that these phlorotannins could degrade at 75 °C, this was noted over a ten week storage period meaning that they could potentially remain stable for a ten minute extraction.

Fucoidans have been noted to be antimicrobial and anti-biofilm, and are also present in *F. vesiculosus* (312), these water soluble polysaccharides are noted to be stable up at 80 °C during a four hour extraction(388) indicating that they could survive this heating process and may well be responsible for the antimicrobial activity seen in this work.

Table 4.4: Antimicrobial activity of 5 mg microwave generated extracts against MRSA 676 using the well diffusion method after microwave assisted extraction.

Run [T,T,V,P]	Activity	Run [T,T,V,P]	Activity	Run [T,T,V,P]	Activity
10,75,40,300	+++	**5,75,40,1200**	+++	**5,45,10,1200**	+++
1,75,40,1200	+++	**10,60,40,1200**	+++	**2,75,30,600**	+++
10,30,40,600	+++	**1,30,40,300**	+++	**5,30,40,600**	+++
10,75,10,600	+++	**2,75,20,300**	+++	**5,75,20,600**	+++
2,75,40,600	+++	**10,30,20,300**	+++	**5,30,10,300**	+++
10,45,40,1200	+++	**2,45,40,300**	+++	**2,60,10,300**	+++
10,75,20,1200	+++	**1,75,10,300**	+++	**2,30,30,300**	+++
2,30,40,1200	+++	**10,45,10,300**	+++	**10,60,20,600**	+++
2,75,10,1200	+++	**10,75,30,1200**	+++	**1,45,30,300**	+++
10,30,10,1200	+++	**2,30,10,600**	+++	**1,60,30,600**	+++
5,60,40,300	+++	**1,30,30,1200**	+++	**5,45,30,600**	+++
5,75,30,300	+++	**1,60,10,1200**	+++	**1,60,20,300**	+++
10,60,30,300	+++	**5,30,20,1200**	+++	**5,45,20,300**	+++
1,45,40,600	+++	**2,60,20,1200**	+++	**1,30,10,600**	+++
1,75,20,600	+++	**2,45,30,1200**	+++	**10,60,10,300**	+++
10,45,20,600	+++	**2,60,40,600**	+++	**1,45,10,600**	+++
5,60,30,1200	+++	**5,60,10,600**	+++	**Solvent Extract (2 h)**	+++
1,45,20,1200	+++	**10,30,30,600**	+++		

[a]Chloramphenicol antibiotic disk - 10 µg/disk. [b]Negative control - 50 µL of specific solvent. Inhibition zone reported as diameter of clear inhibition (including 6 mm disk) in mm; - indicates no inhibition, + indicates inhibition zone of 6 mm - 9.9 mm, ++ indicates inhibition zone of 10 mm - 14.9 mm, +++ indicates inhibition zone of 15 mm-19.9 mm, ++++ indicates inhibition zone of 20 mm-24.9 mm, +++++ indicates inhibition zone of > 25 mm. [TTVP] = [Time, Temperature, Volume, Power].(n=3)

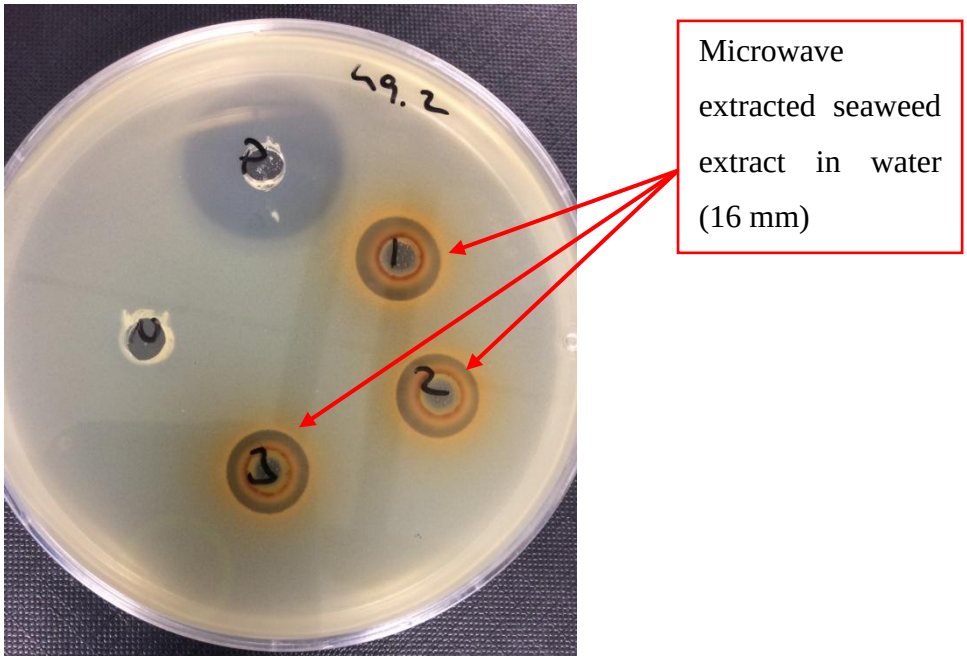

Figure 4.2: Well diffusion of microwave extract from *F. vesiculosus* showing typical antimicrobial activity.

Table 4.3 illustrates that solvent extraction and microwave assisted extraction maintains antimicrobial activity irrespective of the extraction method used and further confirms the stability of the antimicrobial compound(s) at higher temperatures. As such, the respective yields generated for each of the methods and conditions needed to be examined in order to establish optimum extraction conditions. This conclusion was reached in other investigations such as the extraction of phenols from tea, where the antioxidant activity was not affected by microwave conditions (389). However, it should be noted that in an experiment testing the antimicrobial effects of several seaweed water extracts, no activity was noted against *E. coli* or *S. aureus,* for the water extract of *Polysiphonia lanosa* (379), regardless of the fact that it has been found to be active against *S. aureus* in all seasons in other studies (286). This loss of activity could be due to antimicrobial degradation or due to difference in geographical location where the seaweed was harvested.

4.3.3 Microwave extract yields

As already mentioned, the mass of the extract generated by microwave assisted extraction was used to determine the optimum extraction conditions due to the consistent activity established by the extracts. The yields generated as part of this experiment are outlined in Table 4.5

Statistical analysis was used to determine the optimum extraction conditions using one-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test with 95 % confidence on each of the variable conditions. The raw data was inputted into Minitab 17 statistical software, with each experiment carried out in triplicate. As antimicrobial testing by well diffusion displayed consistent results between all extracts, the desirable outcome for this method is to find the conditions which give the highest yield. As the yields, detailed in Table 4.4 vary between 23.54 % and 63.43 %, they are deemed an appropriate criterion on which to base this method. Particularly, as yields were also used in other studies generating extracts from natural products using MAE, such as the extracting of polysaccharides from pumpkin (390) and polyphenols from Dalmation sage leaves (391). Due to the variables in this study, yields will be discussed separately in terms of each variable before determining the optimal conditions using MAE for this extraction.

Table 4.5: Percentage yields obtained for extracts generated using microwave assisted digestion.(n=3)

Run [T,T,V,P]	Yield (%)	Run [T,T,V,P]	Yield (%)	Run [T,T,V,P]	Yield (%)
10,75,40,300	43.63±1.71	**5,75,40,1200**	30.95±0.48	**5,45,10,1200**	35.55±4.69
1,75,40,1200	34.34±2.72	**10,60,40,1200**	29.39±0.71	**2,75,30,600**	31.35±1.11
10,30,40,600	38.71±2.26	**1,30,40,300**	31.23±0.19	**5,30,40,600**	28.46±1.45
10,75,10,600	50.16±3.58	**2,75,20,300**	32.24±1.50	**5,75,20,600**	35.49±1.42
2,75,40,600	35.20±1.26	**10,30,20,300**	34.17±1.10	**5,30,10,300**	39.14±2.90
10,45,40,1200	45.24±0.16	**2,45,40,300**	27.65±0.16	**2,60,10,300**	34.31±0.64
10,75,20,1200	63.43±1.78	**1,75,10,300**	39.62±1.58	**2,30,30,300**	30.10±1.52
2,30,40,1200	34.93±1.75	**10,45,10,300**	40.64±1.68	**10,60,20,600**	32.85±1.85
2,75,10,1200	33.32±0.91	**10,75,30,1200**	35.14±0.92	**1,45,30,300**	28.00±1.04
10,30,10,1200	30.48±0.09	**2,30,10,600**	40.15±0.76	**1,60,30,600**	31.68±0.60
5,60,40,300	31.70±0.32	**1,30,30,1200**	23.54±0.54	**5,45,30,600**	30.99±0.035
5,75,30,300	31.68±0.20	**1,60,10,1200**	41.29±0.52	**1,60,20,300**	30.78±1.17
10,60,30,300	31.57±0.16	**5,30,20,1200**	29.39±3.20	**5,45,20,300**	30.99±0.068
1,45,40,600	29.49±0.62	**2,60,20,1200**	32.12±1.18	**1,30,10,600**	31.27±0.38
1,75,20,600	31.51±0.34	**2,45,30,1200**	31.43±0.98	**10,60,10,300**	35.02±4.52
10,45,20,600	30.45±0.57	**2,60,40,600**	28.84±1.50	**1,45,10,600**	36.08±1.31
5,60,30,1200	31.37±0.59	**5,60,10,600**	35.15±1.13	**Solvent Extract (2 h)**	28.31±1.54
1,45,20,1200	31.06±0.50	**10,30,30,600**	29.53±1.26		

4.3.3.1 Time

The time spent extracting the samples is a key factor in this experiment, as less time would incur lower cost in terms of labour and electricity and make the methodology more suitable for scale-up. Typically, MAE extractions take place anywhere between a few minutes up to an hour (392). However, several studies have warned of the possibility of degrading the antimicrobial via thermal decomposition, oxidation and hydrolysis reactions facilitated by long microwave extractions (61,392,393). Longer extraction times are associated with higher yields in MAE as there is more time for the solvent to extract the powdered seaweed and solubilise any available solutes in the sample (61). In the literature, times points such as 1-3 min for the MAE extraction of antioxidants from natural products (394), 10-50 min for the extraction of polysaccharides (29 min was determined to be optimal) (390) and 1-10 min for the extraction of polyphenols (391) were selected. However, a study using MAE to extract ginsenosides in Panax ginseng found that after 10 min of extraction time at high pressure, yields did not increase in a statistically significant manner (395). For these reasons, four (1, 2, 5, 10 min) time points were chosen of relatively low values so that the potential for degradation and oxidation reactions were reduced and so that the extraction time would be significantly lower than that of solvent extraction (2 h), hence justifying the use of MAE over solvent extraction.

Other studies into the method development of MAE for natural bioactive compounds generally found that lower extraction times were most favourable. A method developed for the MAE of fucoidan from *F. vesiculosus* found that 1 min was enough to extract the fucoidan at 120 psi, and that extraction yields were not influenced by time (69). A similar study by the same author found that fucoidan yields generated from 20 min autohydrolysis were not statistically different from those generated via 1 min MAE with no change in antioxidant activity assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) methods, indicating that bioactivity was not affected by MAE (396). A study on the MAE of bioactives from *Ascophyllum nodosum* found that a 15 min extraction time at 90 °C gave the highest extraction yield. It is worth noting that a 15 min extraction time gave a 15 % increase in yield when compared to the lowest extraction time of 5 min (71). Similarly, an experiment extracting pectin from grapefruit established that 6 min was optimum for

MAE resulting in the highest yield and that 2 min MAE presented the same yield as a 90 min conventional extraction in a hot acidic solution (378).

Conventional extraction methodologies are often comprised of Soxhlet extraction and solvent extraction such as the methods used in Chapter 2 of this work. These are often time consuming and can require a great deal of energy, such as is the case for Soxhlet which requires the constant reflux of solvent over a specified time period.

Solvent extraction studies have used extraction times of 24 h, such as fatty acids from *Nereocystis luetkeana* (3), polyhydroxylated fucophlorethol from F. vesiculosus (333), and antimicrobial bioactives from *Valonia aegrophila, Ulva pertusa, Halimeda opuntia, Callerpra raccllossa* and *H. tuna* (52). Phenolic compounds were also extracted from *Laminaria digitata* using a 24 h solvent extraction (397). Shorter solvent extraction times of 2 h have also been used in the extraction of bioactives from natural sources. For example the 2 h solvent extraction that was used in one study to extract antioxidants from *Enteromorpha prolifera* (398) and 2 h was also used to extract antimicrobials from seaweeds (273).

Extraction times for Soxhlet can vary. An investigation using different extraction methods to extract astragalosides from *Radix astragali* roots found that a relatively short 4 h Soxhlet reflux at 90 ^{o}C was the optimum. Another study involving the Soxhlet extraction of soybean germ in hexane used the significantly shorter time of 4 h extraction for Soxhlet extract (51). However, the typical extraction times using Soxhlet extraction were stated to be 24 - 48 h as part of a review article by Herrero *et al.* (78). Leaving Soxhlet extraction clearly not as efficient in terms of time when compared to MAE.

The results for yields with respect to time outlined in Table 4.6 confirm that the higher time interval of 10 min exhibited the highest yields on average. These were shown to be statistically significantly higher than the yields for 1, 2, and 5 min as can be observed in the one-way ANOVA performed on the data in Figure 4.3 and Tukey's pair comparison in Figure 4.4. In the one-way ANOVA, the intervals for the 10 min data points do not overlap with that of 1, 2, or 5 min with respect to the 'y' axis. The intervals for 1, 2, and 5 min, however, all overlap, meaning that these points are not statistically different from each other. This is denoted in Table 4.5 by the use of a common superscripted letter. Figure 4.3 and 4.4 display the interval plot and Tukey's multiple comparison test, which

confirms the statistical similarity between the yields at 1, 2, and 5 min, while showing the statistical difference between those and the 10 min yield which was statistically the highest.

Table 4.6: Mean % yields ± standard deviation for the time variable.

	Time			
Levels (min)	1[a]	2[a]	5[a]	10[b]
Mean % yield	32.37 ± 4.70	32.96 ± 3.62	32.67 ± 3.57	38.11 ± 9.23

Data is presented as the mean ± SD; Data that do not share a common superscript are statistically different (p<0.05; One-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test).(n=3)

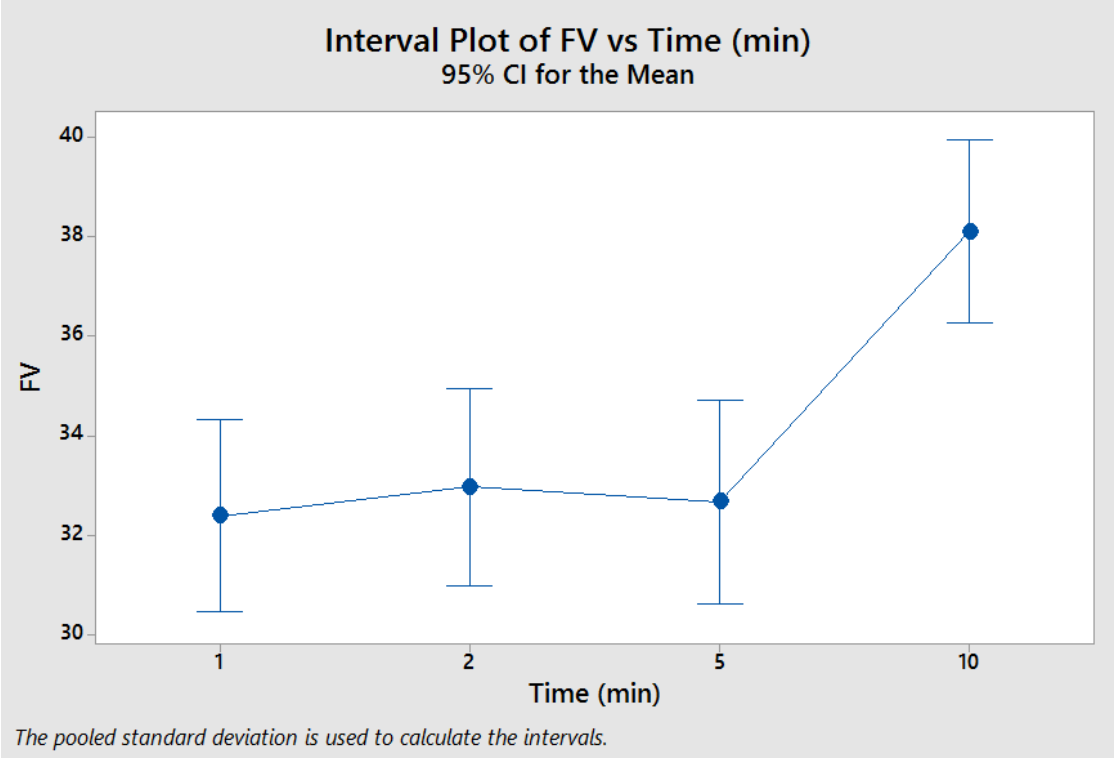

Figure 4.3: Interval plot comparing % yield of extract with the four time variables.

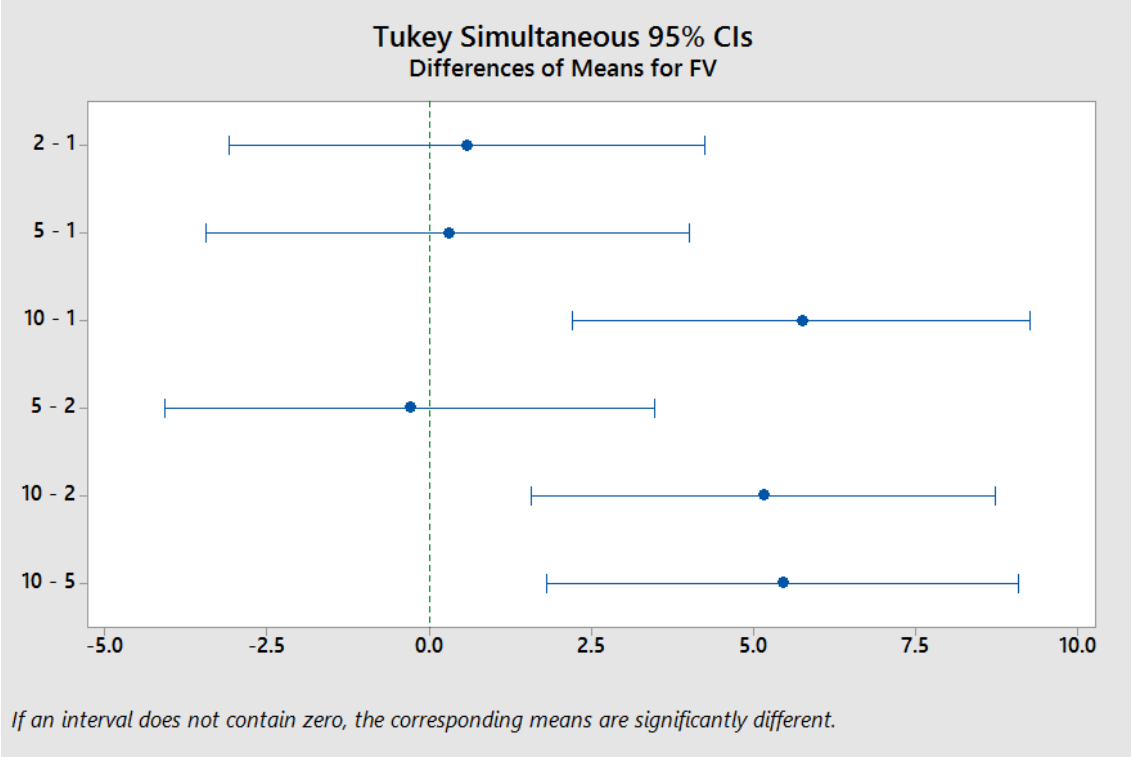

Figure 4.4: Plot detailing the differences of the means between various time variables.

This data does not correspond to the results found by Rodriguez *et al.* (73), which established that at high pressures, 1 min extraction time was not statistically different to that of 16 min or 31 min at 120 psi. The variation observed here could be a result of the different factors varied as part of this investigation. This study used the variant of temperature and not pressure, which could possibly be a reason for this difference, along with the fact that the algal ratios were varied for that experiment, but not in the study carried out under this project (69). However, a group extracting fucoidan from *A. nodosum* did find that yields increased between 5 min extraction using temperature and not pressure as a variable in MAE and 15 min, (the increase between 15 and 30 min was deemed absent of statistical significance) (71).

A maximum point of 10 min was decided on for this study as it was the intention to significantly decrease the energy requirements. It was calculated that running the Mars 5 system at its full capacity of 1200 w would cost just €0.027 for the 10 min run time using Bord Gais Energy in July 2019 (399).

4.3.3.2 Temperature

The temperature that the antimicrobial compounds are extracted has an effect on the solubility of compounds in the extraction solvent and, therefore, is interlinked with the yield that will be generated (61). Higher temperatures will allow more solutes in *F. vesiculosus* to dissolve in water. However, higher temperatures could also potentially cause molecules to degrade or rearrange, resulting in the possibility of loss of bioactivity (61,377,392). This was observed in a study extracting flavonol glycosides from *Ginkgo foliage* where bioactivity declined after 15 min MAE (400). One review also noted that higher extraction temperatures also have limitations in terms of longer cool down periods that are required before sample handling (401).

Considering the results obtained in section 2.3.5 of this work which demonstrated the ability of the antimicrobial activity in the water extract of *F. vesiculosus* to survive the autoclave process, which is 121 °C for 15 min, indicating that the antimicrobial compound(s) could be robust enough to survive an extraction at 75 °C. With this in mind, trial runs were carried out on the microwave to test the temperature which the system is capable of reaching and sustaining. It was shown that the microwave would only reach 109 °C. Owing to this, a range of four temperatures including; 30, 45, 60, and 75 °C were chosen with the yields of these experiments summarised in Table 4.7. 75 °C was chosen as an upper limit for these experiments due to a trial run that was put on the CEM Mars 5 system to investigate the systems' ability to reach and maintain a set temperature. It was determined that the system could not reach and maintain 90 °C comfortably. With this in mind, an upper limit of 75 °C was selected to ensure that the system could maintain the setting.

A study extracting fucoidan from *F. vesiculosus* found that moderate temperatures of 62.5 °C were optimum for MAE (62). This is promising as a study carried out on the thermal stability of fucoidan and laminarin (both present in *Fucus*) found that these compounds can survive being cooked to 180 °C in meat (402). Other examples include; the extraction of stilbenes from grapes which was successfully optimised at 125 °C (403), and the extraction of flavanoids from *Radix astragali* roots at 110 °C (404). One review found that the temperatures which were chosen to extract bioactives from natural products using MAE tended towards temperatures between 60 - 140 °C (392).

The increased capacity for solvent systems to dissolve analytes at higher temperatures indicates that greater temperatures are generally associated with higher yields (61,405). However, some studies have noted that an increase in temperature generally accompanies an increase in yield until an optimum is reached, at which point yields will begin to decrease (61,406). It is speculated that this is due to decomposition of target compounds resulting in poorly solubilised degradation products (406).

Table 4.7: Mean % yields ± standard deviation for variations of temperature.

	Temperature			
Levels (°C)	30[a]	45[a]	60[a]	75[b]
Mean	32.57 ± 4.51	33.055 ± 5.059	33.205 ± 4.243	37.50 ± 8.99

Data is presented as the mean ± SD; Data that do not share a common superscript are statistically different ($p<0.05$; One-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test)(n=3).

As expected, the higher extraction temperature of 75 ° C resulted in the highest mean yield. The results of the one-way ANOVA outlined in Figure 4.5 indicates that 75 ° C was the only temperature which was statistically significantly greater than the others. This can be seen more clearly in Figure 4.6 where only pair comparisons including 75 ° C do not intercept zero. Consequently, 75 ° C was established as the extraction temperature which generated the greatest yield.

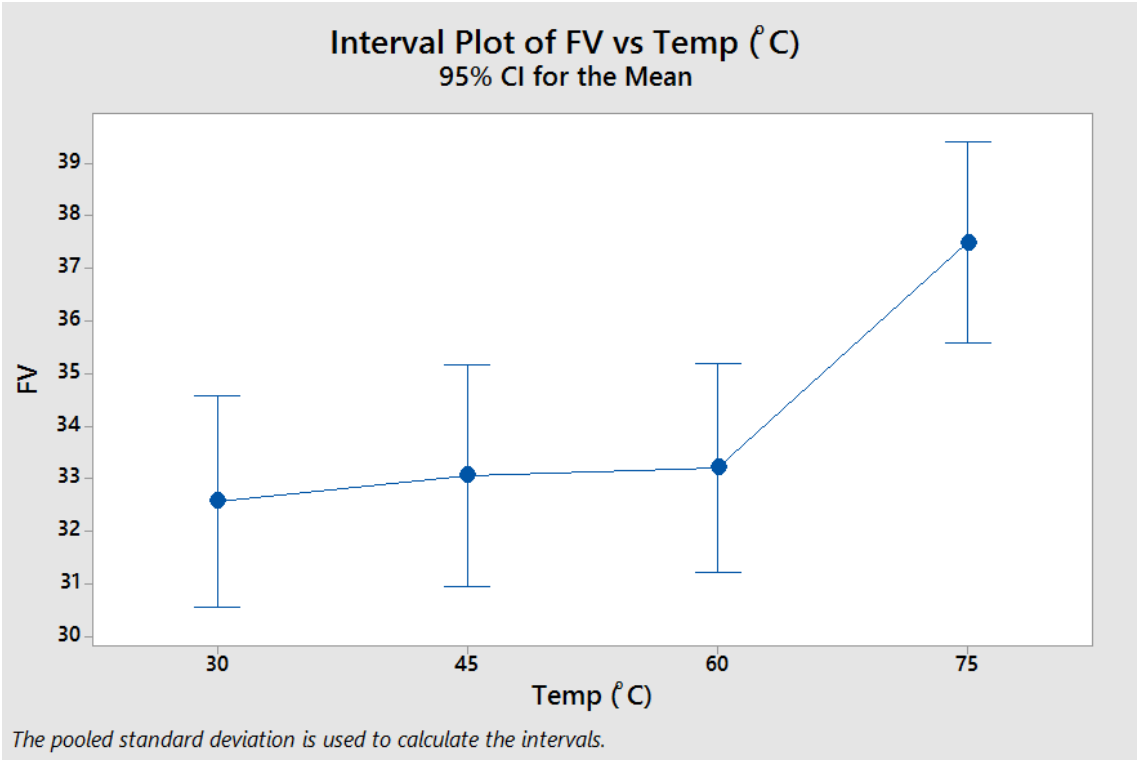

Figure 4.5: Interval plot comparing % yield of extract with the four temperature variables.

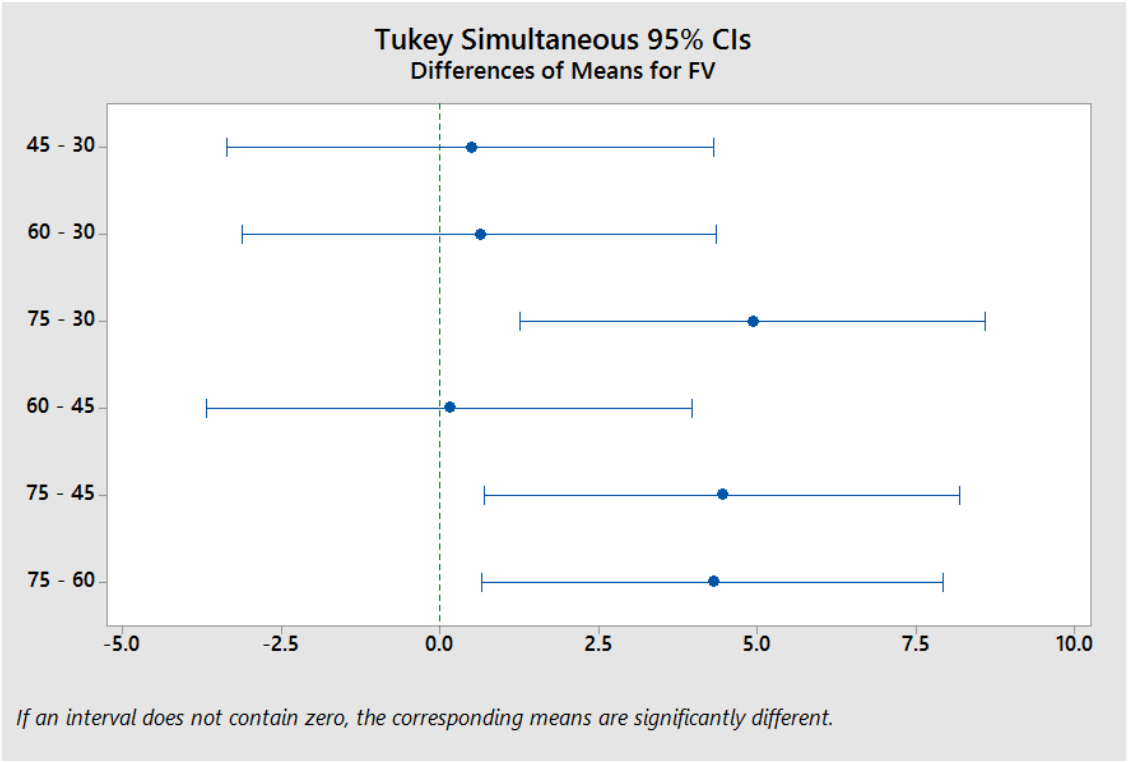

Figure 4.6: Plot detailing the differences of the means between various temperature variables.

4.3.3.3 Volume

In general, greater importance is placed on the solvent:feed ratio and the surface contact between the two than volume on its own as a variable. As mentioned above, the solvent:feed ratio was maintained at 1:100 *w/v* seaweed:solvent to make the results more comparable with those generated via solvent extraction. As the particle size of the powder also remained uniform throughout the experiment and the microwave tubes are of a constant diameter, the water content was maintained as constant as possible. There were four levels chosen for volume including; 10, 20, 30 and 40 mL, which were selected based on the 50 mL maximum capacity of the microwave tubes and the Mars CEM 5 system operating manual which dictates that no less than 10 mL of solvent should be used in the system (384). These volumes were also chosen in consideration of a review by Eskilsson *et. al.* into analytical scale MAE which states that volumes of 10 - 30 mL are typical for this technique.

The results for the yields with respect to volume detailed in Table 4.8 established that the 10 mL and 20 mL were the most favourable options in terms of improving yields. This is supported by the one-way ANOVA and Tukey's pairwise comparison test performed which established that 10 mL had the highest mean % yield of all the levels and that 20 mL was not statistically different from 10 mL (p= 0.139). The volume of 10 mL was, however, statistically different 30 and 40 mL tested, as established in Figure 4.7 and 4.8. Therefore, the volume of those tested which generated the highest yield was either 10 or 20 mL.

Table 4.8: Mean % yields ± standard deviation for the volume variable.

	Volume			
Levels (mL)	10^{a}	20^{ab}	30^{d}	40^{cd}
Mean	37.76 ± 4.87	34.75 ± 9.00	30.69 ± 2.61	33.28 ± 5.35

Data is presented as the mean ± SD; Data that do not share a common superscript are statistically different (p<0.05; One-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test)(n=3).

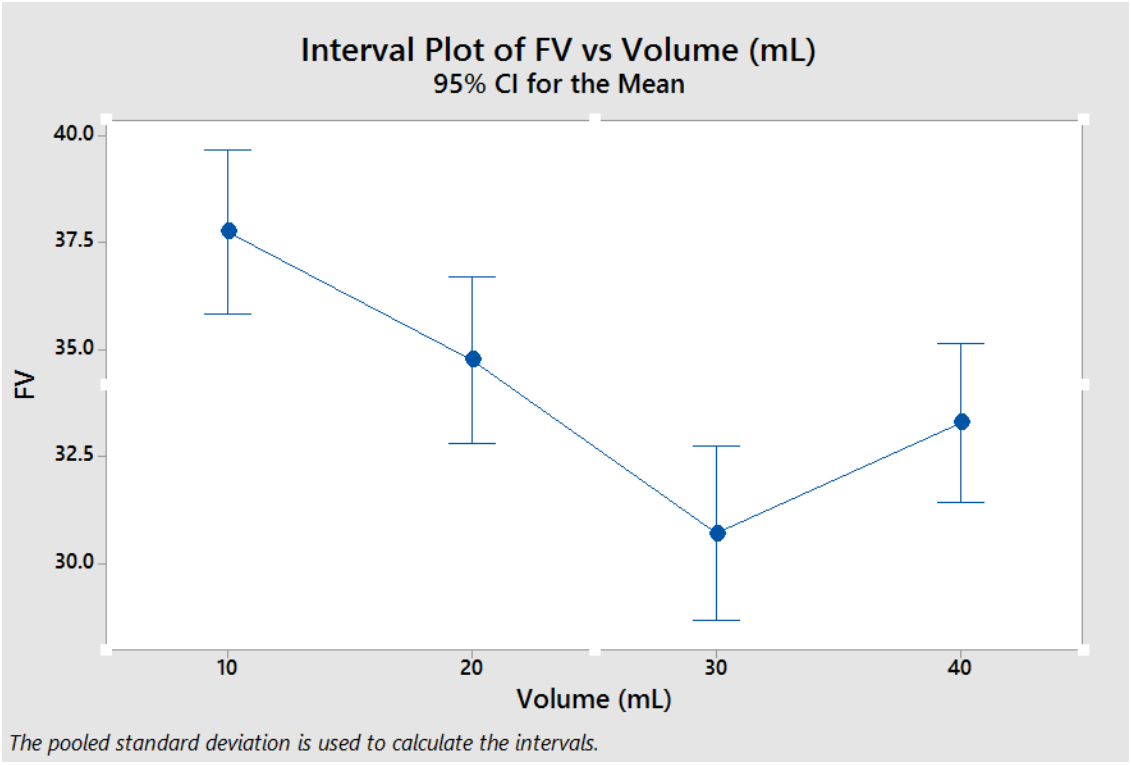

Figure 4.7: Interval plot comparing % yield of extract with the four volume variables.

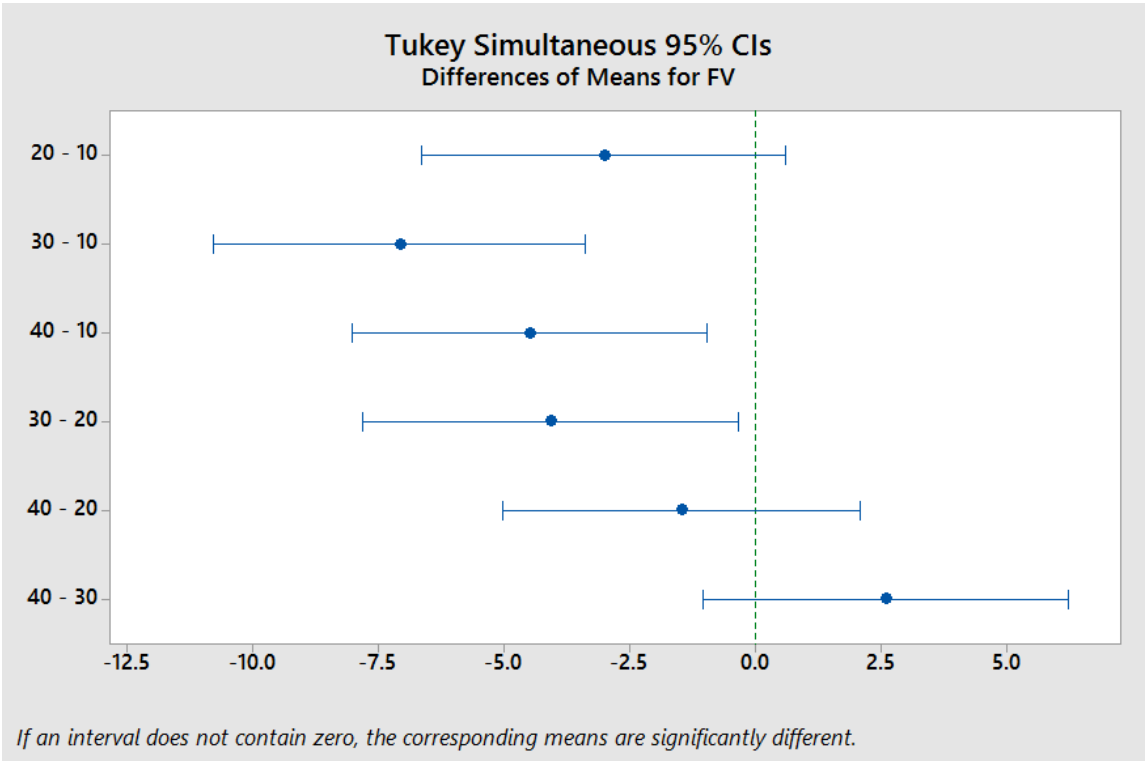

Figure 4.8: Plot detailing the differences of the means between various volumes.

Most noteworthy is the display of statistical significance between the data generated as part of this study. This work established that the lower volumes used of 10 mL or 20 mL were the most favourable volumes in this MAE. A possible explanation for this could be that while the feed:solvent ratio remains the same at 1:100 *w/v*, the contact between the solvent and the seaweed powder would be relatively higher at lower volumes. This can be observed in the diagram in Figure 4.9. As the diameter of the microwave vessels was the same throughout this study, there was a greater volume of solvent not in direct contact with the seaweed powder at higher volumes. As stated earlier, the slow rotation of the microwave stage and basic convection are the only mechanisms of agitation used, meaning there could be a difference between the amounts of extract dissolved in the top half of the tube as opposed to the solvent in close contact with the seaweed powder.

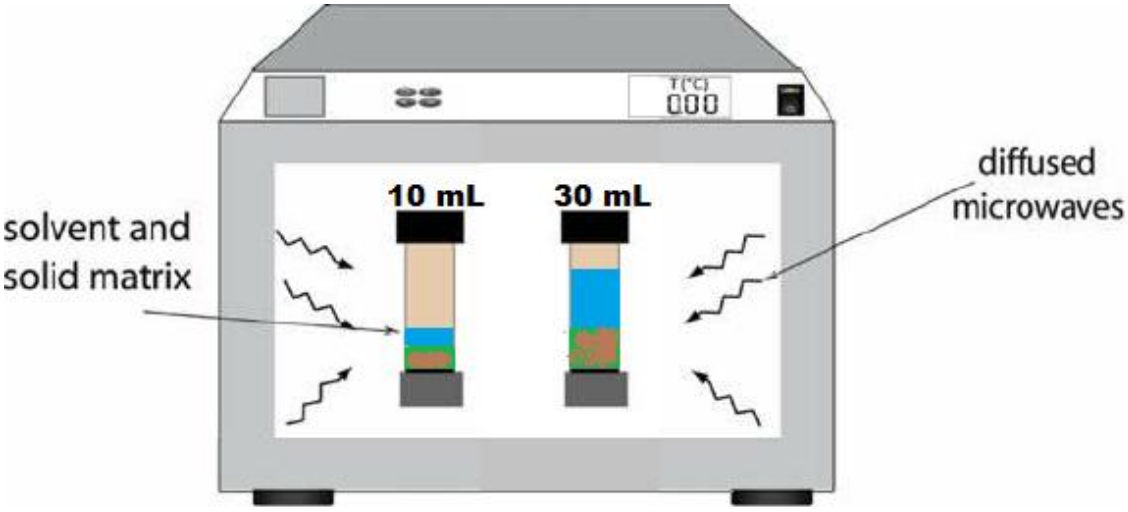

Figure 4.9: Schematic diagram of microwave depicting two different volumes in vessels with a ratio of 1:100 *w/v* solvent:seaweed powder.

This phenomenon is noted in the literature, whereas conventional extraction techniques achieve a higher recovery with the use of more solvent, MAE can give lower yields with the increased use of solvent (407,408). This agrees with a study extracting flavonoids from the Chinese herb *Radix puerariae*, which found that yield increased with solvent (70:30 *v/v* EtOH:H_2O) volume up to a volume of 35 mL and decreased dramatically

thereafter (407). It was speculated that this could be due to the ethanol which can cause water to swell the material and prevent further extraction of bioactive into the solvent (407).

4.3.3.4 Power

Levels for power were chosen based on the 3 available presets on the CEM Mars 5 system used. These power levels were: 300, 600, or 1200 W and yields obtained are displayed below in Table 4.9.

Table 4.9: Mean % yields ± standard deviation for the power variable.

	Power		
Levels (W)	300 [a]	600 [a]	1200[a]
Mean	33.59± 4.84	33.50± 4.95	35.52 ± 8.64

Data is presented as the mean ± SD; Data that do not share a common superscript are statistically different (p>0.05; One-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test)(n=3).

Power was stated to be one of the most important factors for the method development of MAE, as power dictates the amount of energy available to the solvent and, therefore, influences the rate at which dielectric solvents flip polarity (61). It is also one of the factors that directly contributes to cost, as running the microwave at high powers over extended periods of time would be expensive in terms of energy, regardless of the low cost associated with MAE (393,409). Regardless of this, the results of this study (Figure 4.10 and 4.11) show that power was not a statistically significant factor in this investigation.

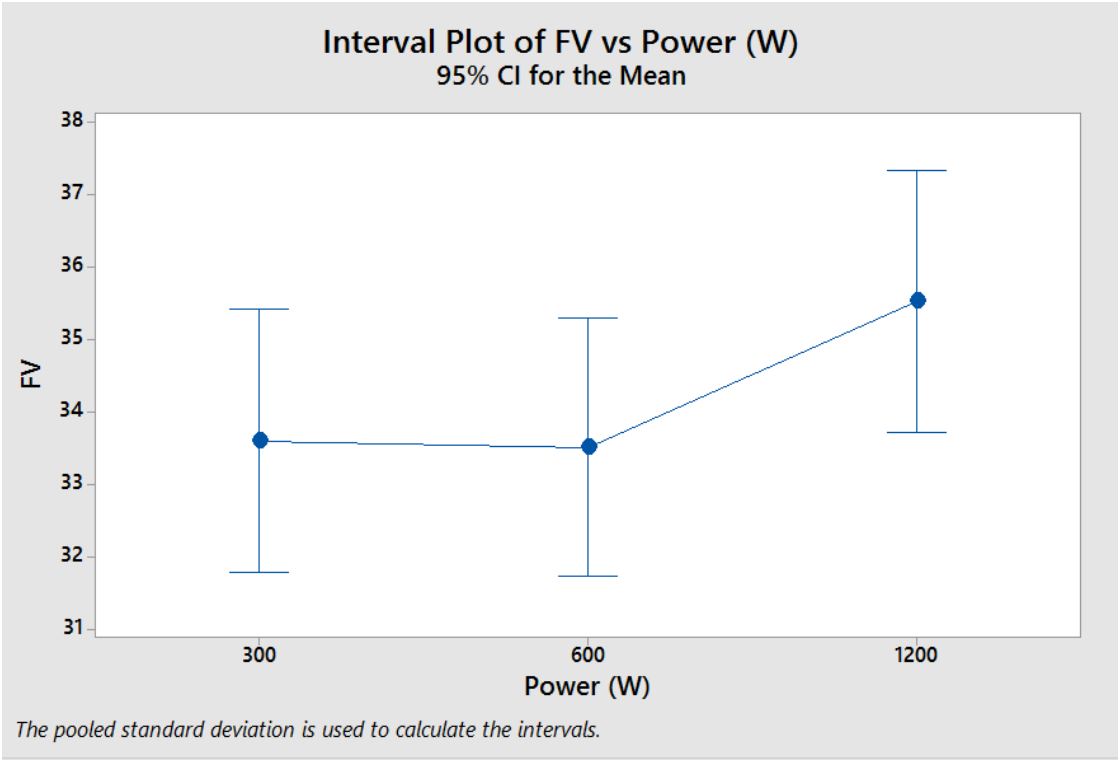

Figure 4.10: Interval plot comparing % yield of extract with the three power variables.

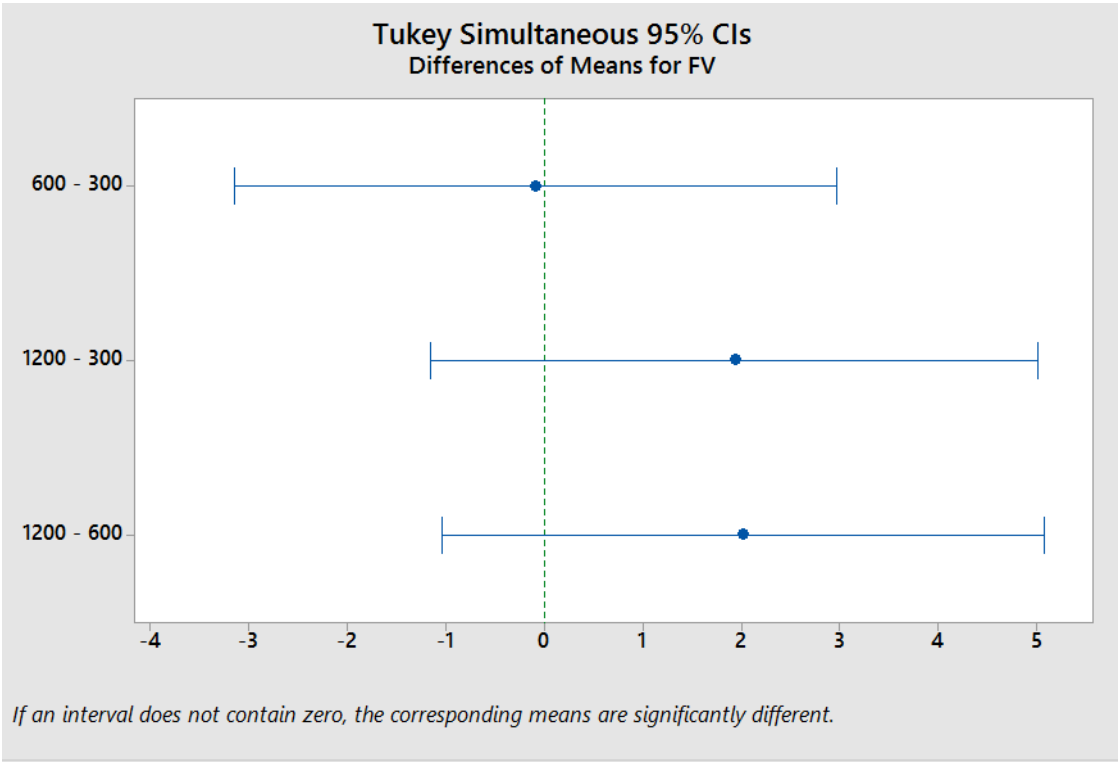

Figure 4.11: Plot detailing the differences of the means between power variables.

The absence of statistical significance of the power variable on the yields generated via MAE is especially clear through a review of the interval plots above. This was not an expected result, as it contradicts many studies insisting that an increase in power correlates to an increase in yields generated via MAE (61,392). Although it has been noted that this is typically up to a point after which the increase becomes insignificant or degradation begins to occur, oftentimes decreasing yields (392). There are studies which have come to the conclusion of power as a statistically non-significant factor for MAE. Experiments using MAE to extract notoginseng saponins from *Panax notoginseng* (410); the extraction of hydrocarbons in marine sediments (411); and the extraction of flavonoids from *Saussurea medusa* (412) all determined that power was not a statistically significant condition in the method development of MAE methods.

The absence of statistical significance of power as a factor may also be due to the fact that temperature and power are very closely linked. Studies reiterate that higher powers cause an increase in temperature (61,392,406). Similarly, the use of high temperatures drives power needs up due to a drop in surface tension and viscosity in the sample (413). The CEM MARS 5 microwave used as part of this experiment has three main power settings of 300, 600 and 1200 W which were used as factors in this experiment. However, the system adjusts itself to reach and maintain a specified temperature (30, 45, 60, 75 ^{o} C). As such, the use of power as a factor may have been too interlinked with the achievement of the set temperature and, therefore, may have been irrelevant.

Ruling out power as a significant factor for the method development of the extraction is a beneficial result for the use of microwave assisted extraction as it infers that the lowest power setting of 300 W can be used instead of higher power settings. This would save money in terms of electricity and is an attractive quality for industry.

4.3.4 Method Development

The factors resulting in the highest yield were determined from the variables tested and are summarised in Table 4.10. These levels reduce a 2 h solvent extraction time down to 13 min (10 min run time, plus 3 min ramp time) while maintaining antimicrobial activity of the extract (Chapter 3.3.1). To examine the most favourable method in terms of yield, a one-way ANOVA and Tukey pairwise comparison test between the raw data

for the yields generated for solvent extraction at 2 h compared to the yields established as part of the microwave assisted extraction study was conducted.

Table 4.10: Factors for microwave assisted extraction of antimicrobials from *F. vesiculosus* resulting in the highest yield.

Factor	**Time (min)**	**Temperature (^{o}C)**	**Volume (mL)**	**Power (W)**
Level	10	75	10/20	N/A*

*power variable determined to be absent of statistical significance.

The statistical analysis between the extraction yields generated using 2 h solvent extraction verses that of microwave assisted extraction are displayed in Figure 4.12 and 4.13. The one-way ANOVA performed shows that the microwave assisted extract generated higher yields which are statistically different from those of the solvent extraction ($p=0.001$). This is confirmed by Tukey's pairwise comparison which does not intercept zero and, therefore, supports microwave assisted extraction as the better of the two methods for the extraction of antimicrobial bioactives from *F. vesiculosus.*

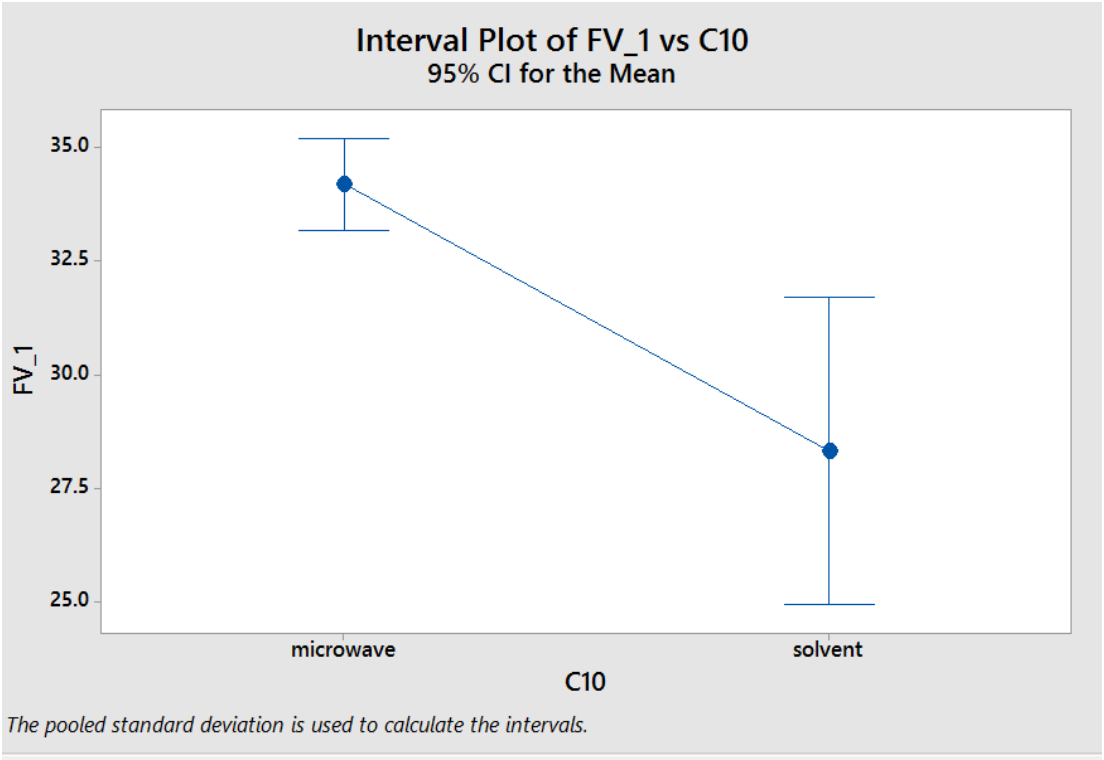

Figure 4.12: Interval plot for comparing solvent extraction yields with MAE.

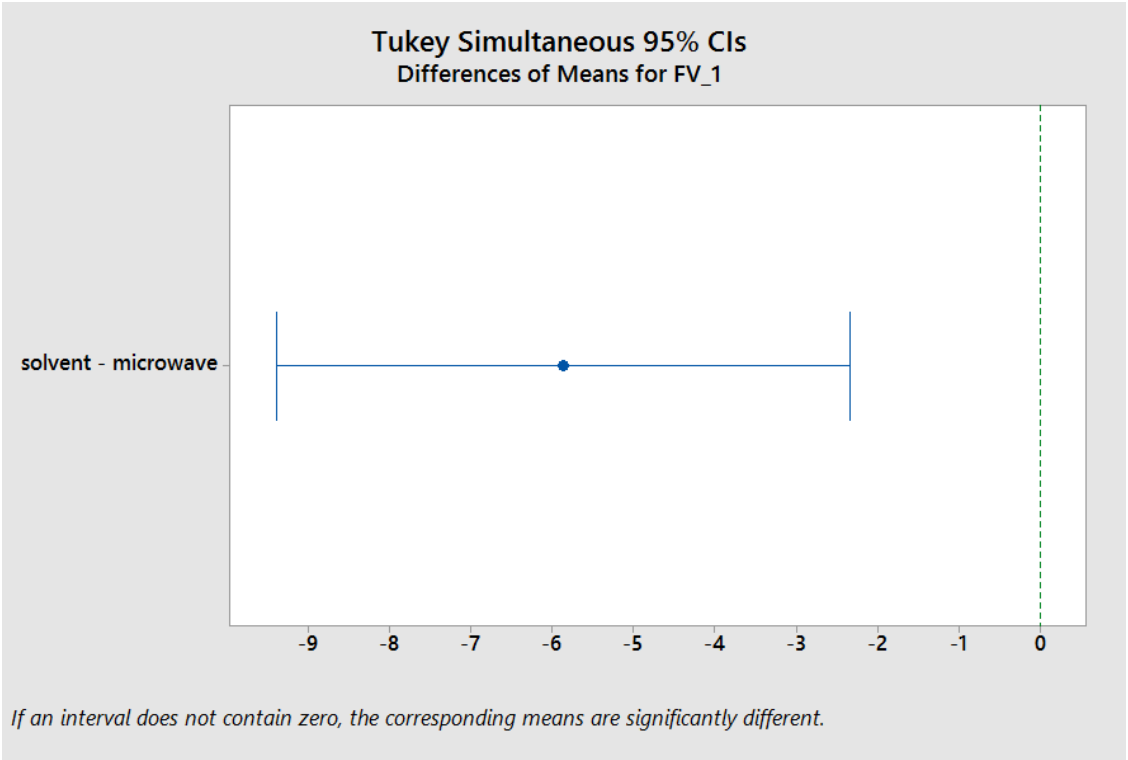

Figure 4.13: Tukey pairwise comparison test between solvent extraction (2 h) yields against those generated using MAE (10 min).

To further establish the potential use of microwave assisted extraction, the data obtained for the most favourable extraction conditions was compared with that generated using 2 h solvent extraction, Table 4.11.

Table 4.11: Data for factors generating the highest yield with microwave assisted extraction and 2 h solvent extraction (from Tables 4.3 and 4.4)

Method [time, temp, vol, power]	**% yield ± standard deviation**	**Antimicrobial activity**
MAE[10,75,10,600]	50.16 ± 3.58	+++
MAE[10,75,20,1200]	63.43 ± 1.78	+++
Solvent Extraction (2 h, 19°C)	28.31 ± 1.54	+++

The same one-way ANOVA and Tukey pairwise comparison was performed on this data and is displayed in Figure 4.14 and 4.15. These plots clearly show that the yield generated using microwave assisted extraction is both higher than that of 2 h solvent extraction and statistically different ($p = 0.00$). When the mean of the microwave assisted extraction conditions in Table 4.11 (58.12 ± 7.592) is compared to that of 2 h solvent extraction (28.31 ± 1.541), microwave assisted extraction offers a 51.29 % yield with no detectable compromise in antimicrobial activity (Table 4.4).

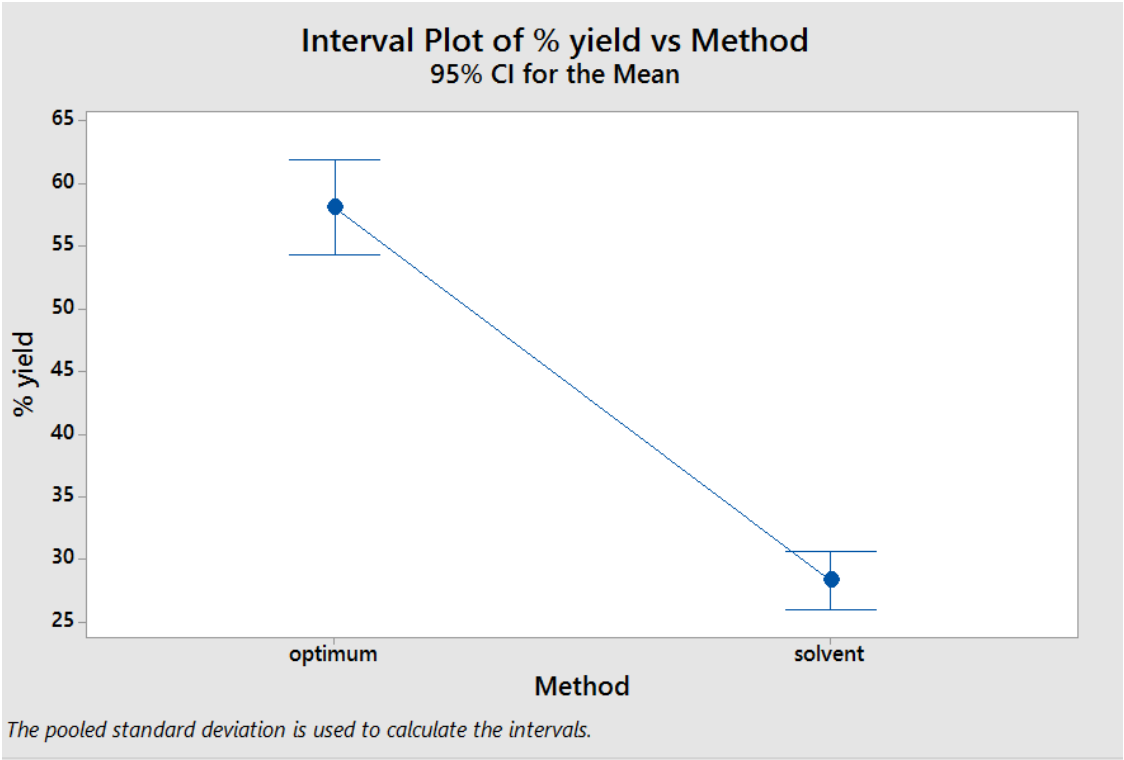

Figure 4.14: Interval plot comparing solvent extraction yields with the most favourable MAE methods.

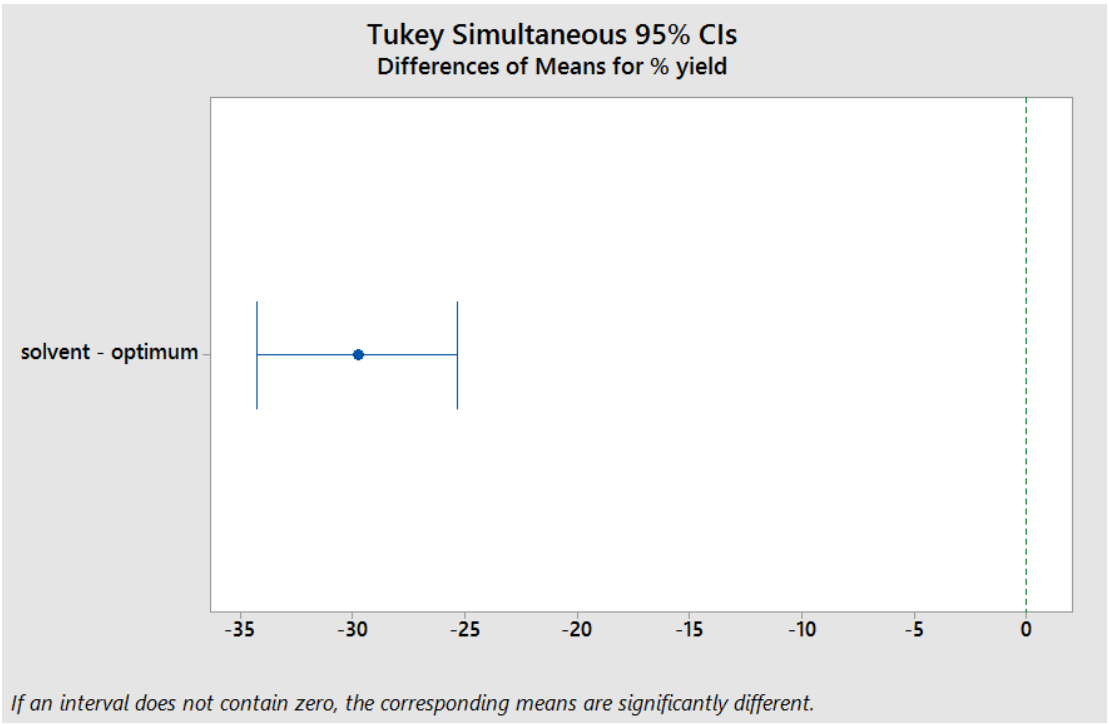

Figure 4.15: Tukey pairwise comparison test between solvent extraction (2 h) yields against those generated using the developed method for MAE.

4.4 Conclusions

This study assessed various conditions of microwave assisted extraction of antimicrobials from *F. vesiculosus* in water in order to establish the highest % yields and antimicrobial activity. The antimicrobial activity of the extracts was established not to be effected to any detectable level by either the method of extraction or the amount of extract generated using this method. As such, the % yield generated for each of the conditions was used to determine the most favourable conditions for microwave assisted extraction. This assessment was carried out using Minitab 17 statistical software to perform both a one-way ANOVA and Tukeys pairwise comparison test. By this process, most favourable conditions for the microwave assisted extraction were determined to be a 10 min run time, at 75 ^{o} C with 10/20 mL of solvent. It was also determined that the power setting did not have any statistical significance on the yields generated with 300 W.

The yields generated for microwave assisted extraction including those of the developed method were then compared with the yields generated from a 2 h solvent extract which were established as part of a study carried out in section 2 of this work. These comparisons showed that microwave assisted extraction consistently resulted in a higher, statistically significant, yield compared to 2 h solvent extraction. The yields generated using the microwave conditions developed throughout this section, resulted in a 51 % higher yield than that of 2 h solvent extraction in 89.2 % less time. This result saw a large reduction in the time taken to extract the antimicrobial which in turn would reduce the cost of producing the extract in terms of hours of labour needed to generate extract.

As the extract yield was improved by 51 % with no notable loss in antimicrobial activity, there was a saving in terms of dry matter of seaweed. This would in turn reduce the waste associated with extracting these antimicrobials. Furthermore, these results are interesting as they affirm the stability of the antimicrobial compound(s) under microwave conditions as the antimicrobial compound(s) survived the 75 ^{o}C extraction temperature. This is valuable information as it can rule out the identity of the compound(s) being of thermolabile origin.

Chapter 5: Development of an antimicrobial wound dressing

5.1 Introduction

Wound infections are often treated using oral or intravenous antibiotics which have side effects associated with the systemic delivery of these drugs (414). Targeted drug delivery to the local effected area has been a topic of discussion and research in recent years, due to the reduction of potential systemic interactions and interferences connected to treatments (125). Meta analysis has indicated that a combination of systemic and topical antibiotics are a better form of treatment (415,416). Such an approach would have the benefit of reducing the concentration of systemic antibiotics used in wound treatment. Arguably, the management of external indications such as wounds, burns and their infections have more obvious and relatable methods for targeted drug delivery such as; active or 'smart' wound dressings (256,275).

There are a range of active wound dressings currently on the market, many of which rely on the use of silver as an antimicrobial agent. While silver and silver nanoparticles are effective antimicrobial compounds which remain stable in wound dressing formulations (212,417), there are issues surrounding the side effects of their use. Concerns have been raised owing to the established cytotoxic effects of silver ions. While cytotoxity in relation to bacteria is desirable in a wound dressing, both keratinocytes and fibroblasts, which aid in wound healing, are also susceptible to silver ions (204,418,419). As such, doubt has been cast upon the appropriateness of silver in wound dressings, as it could potentially hinder the healing process. There are further concerns with the use of silver, as there were noted cases of argyria (blackening of the area exposed to silver) and increases in aspartate aminotransferase, alanine aminotransferase, and gamma-galactosyl transferase liver enzymes (214) which are indicators of hepatotoxicity (420).

Natural products are an attractive alternative to synthetic antimicrobial agents, a point reinforced by consumer market attraction for formulations containing natural remedies as bioactives (18). This is reflected in the FDA's approval of honey as a wound dressing in 2007 (216,421) giving rise to a range of commercially available wound dressings incorporating honey as n antimicrobial, beginning with Derma Sciences Medihoney® (237). Another natural product used as an active ingredient in commercially available wound dressings is AmeriGel® (approved by the FDA (422))which contains an oak extract which also acts as an antimicrobial agent and has been found to be effective

against MRSA (423). There are studies which incorporate other natural products known to have antimicrobial capabilities such as thymol from thyme (424), Ocimum oil from *Ocimum gratissimum* (425) and essential oils such as; tea tree, lavender, geranium and patchouli oils which were effective against MRSA (426).

To date, an antimicrobial water extract of *P. lanosa* was formulated into a hydrogel wound dressing was the only noted use of seaweed extract as an antimicrobial in a wound dressing in the literature (202), highlighting the need for further studies. The lack of literature surrounding wound dressings incorporating antimicrobial seaweed extracts brings to light the novelty surrounding this area.

Wounds have different characterisations and as such require different environments to promote their individual healing. For example, bed sores may be necrotic in nature and require a dressing, such as bacterial cellulose, which promotes the autolytic debridation of dead tissue from the wound, by rehydrating the eschar (237), whereas, burns can produce excess exudate which requires an absorbent dressing such as a hydrogel (270).

Wound infections are typically sloughy in nature and contain excess exudate which provides a media for bacterial infection to grow. As such, an ideal wound dressing should:

- Provide mechanical protection (256,427).
- Provide a moist but not wet environment for optimum wound healing (428).
- Remove excess exudate to inhibit the maceration of healthy tissue and to provide a less attractive growth medium for bacteria (275).
- Allow for gaseous exchange (256).
- Not adhere to the wound; preventing troublesome dressing removal/changes (256).
- Be biocompatible and non-toxic to healthy tissue (427).
- Be antimicrobial (206,275,423).
- Be cost-effective.

As the water extract from *F. vesiculosus* was established to be, antimicrobial (section 2) and both disruptive and preventative to biofilm formation (section 3) it was suspected that this extract would be appropriate for inclusion into a novel wound dressing formulation, encompassing the above criteria.

Prior to the development of these dressings, a preliminary study was carried out to examine the use inert wound dressings soaked in the water extract from *F. vesiculosus* against the established, commercially available antimicrobial wound dressings Algivon (honey), Inadine (iodine) and Aquacel® (silver).

5.2 Experimental Procedure

5.2.1 Preliminary wound dressing study

Aseptically, 6 mm^2 of the commercially available wound dressing Aquacel® with no active ingredient was cut and soaked in a 1 mg/10 µL solution of seaweed extract dissolved in sterile deionised water. Positive controls of commercially available silver woven antimicrobial Aquacel®, iodine (Inadine) and honey (Algivon) were prepared by aseptically cutting 6 mm^2 of the active dressings. A negative control was prepared by soaking 6 mm^2 of the un-active Aquacel® in sterile deionised water.

The antimicrobial activity for these wound dressings was tested using a modified disk diffusion method, as outlined in section 2.2.4 of this work, except that the dressings were used in the place of a paper disk against MRSA strain 676. The same dressings were placed on plates which were freshly swabbed with MRSA 676 adjusted according to section 2.2.4

5.2.2 Preparation of antimicrobial alginate wound dressings

A method detailing the formulation of all-natural composite wound dressing films was adapted for this formulation (244). Sodium alginate (0.3g) (Sigma-Aldrich) was added to 10 mL of sterile deionised water in a 100 mL beaker. The resulting solution was stirred and heated (<100 ^{o}C) on a hot plate (within the aseptic zone of a flame) to ensure the complete dissolution of the sodium alginate. Once the sodium alginate was dissolved, the solution was allowed to cool to room temperature before adding 0.1 mL of sterile glycerol (Sigma-Aldrich) and stirring to ensure that the solution was homogeneous. This solution was transferred aseptically into five 1 mL portions in sterile Eppendorf tubes. Different masses (0.05 g, 0.025 g and 0.001 g) of crude water seaweed extracts were added to three of the tubes resulting in three different concentrations of extract (1 mg, 5 mg and 10 mg per 200 µL). 100 µL of 10 mg/mL chloramphenicol (Sigma-Aldrich) was added to one of the remaining Eppendorf tubes as a positive control and the final tube was treated as a negative control, where 100 µL of sterile deionised water was added to the formulation. 10 µL of medium molecular

weight polyethylene glycol was then added to all five of the tubes and vortexed to mix the solutions.

200 µL of each of the solutions was pipetted aseptically onto a 1.5 cm x 1.5 cm square surface of a sterile microscope slide in a sterile petri dish in duplicate, an example of which can be seen in Figure 5.1. The films were then allowed to set overnight and tested using the antimicrobial method for bioautography (50), detailed further in section 5.2.2. where the samples were overlaid with media inoculated with MRSA 676 and allowed to incubate overnight. The diameter of the zone of inhibition from any resulting bacterial inhibition was then measured.

Figure 5.1: Placement of negative control film wound dressings on microscope slide prior to antimicrobial testing.

5.2.3 Preparation of antimicrobial gelatin/chitosan hydrogel

A method by Wang *et al.* for the preparation of honey, chitosan and gelatin hydrogel sheets was adapted for the inclusion of the water extract from *F. vesiculosus* instead of honey as the active ingredient (429). 2 g of gelatin (Sigma-Aldrich) was weighed out and dissolved aseptically into 4 mL of sterile, deionised water using a magnetic stirrer on a hot plate at approx. 60 °C. Similarly, a solution of 0.05 g medium molecular weight chitosan and 0.05 g of glacial acetic acid was dissolved in 3.8 mL of sterile deionised water and stirred aseptically on a hot plate at ≈ 60 °C until the chitosan was completely dissolved in solution. Simultaneously a solution of crude water extract from *Fucus vesiculosus* was made up by dissolving 0.2500 g of extract in 4 mL of sterile deionised water.

The full contents of the chitosan/glacial acetic acid solution was added to the gelatine solution and stirred at 60 ^{o} C to ensure the solution was homogenously mixed to form a hydrogel. At this point the dissolved seaweed extract was added and mixed with the hydrogel solution at 60 ^{o}C. Subsequent to this, the loaded hydrogel solution was pipetted aseptically in 200 µL aliquots into nine wells of a sterile 96 well microtitre plate and allowed to set aseptically at room temperature. A further 8 mL of this solution was pipetted into a 60 mm petri dish and allowed to set for inspection.

The hydrogel mix was made up two more times, 1 mL of 10 mg/mL chloramphenicol and 3 mL of sterile deionised water was added to one as a positive control and 4 mL of sterile deionised water was added to the other as a negative control. 200 µL of each of these solutions was pipetted into three wells of the same 96 well microtitre plate and allowed to set. A further 8 mL of each solution was pipetted into a 60 mm petri dish and allowed to set for inspection.

The antimicrobial activity for these hydrogels was tested using a modified disk diffusion method outlined in section 2.2.5. of this work except that the hydrogels cast in the 96 well microtitre plate were used in the place of a paper disk against MRSA strain 676.

5.2.4 Preparation of antimicrobial alginate/chitosan dressings

Alginate and chitosan are known to polymerise and have been used in wound dressing preparations in the literature (236,430,431). As such, a dressing using these starting materials was formulated. A 1 % *w/v* solution of sodium alginate was made up in 1 % *v/v* glacial acetic acid (Sigma-Aldrich) diluents prepared using sterile deionised water. This solution was stirred until dissolution using a magnetic stirrer on a hotplate within the aseptic zone of a flame (430). This solution was labelled 100 % alginate. A 1 % *w/v* solution of medium molecular weight chitosan (Sigma-Aldrich) was prepared using the same 1 % *v/v* glacial acetic acid solution. The sample was stirred until the complete dissolution of the chitosan was observed using a magnetic stirrer on a hotplate at approximately 60 ^{o}C within the aseptic zone of a flame (430). This solution was then labelled as 100 % M.M.W. chitosan. A 1 % *w/v* solution of low molecular weight chitosan (Sigma-Aldrich) was prepared using the same 1 % *v/v* glacial acetic acid

solution. The sample was stirred until the complete dissolution of the chitosan was observed using a magnetic stirrer on a hotplate at approximately 60 °C within the aseptic zone of a flame (430). This solution was then labelled as 100 % L.M.W. chitosan.

These stock solutions were mixed to obtain solutions at the following concentrations:

- 100 % alginate
- 75:25 % alginate:M.M.W.chitosan
- 50:50 % alginate: M.M.W.chitosan
- 25:75 % alginate: M.M.W.chitosan
- 100 % M.M.W.chitosan
- 75:25 % alginate: L.M.W.chitosan
- 50:50 % alginate: L.M.W.chitosan
- 25:75 % alginate: L.M.W.chitosan
- 100 % L.M.W.chitosan

These ratios were chosen to represent a of range formulation combinations for each of the starting polymers.

10 mL of each solution was pipetted aseptically three times into three sterile McCartney bottles. One of the three samples had 0.25 g of crude water extract from *Fucus vesiculosus* equivalent to 5 mg/200 µL added to it. The second of the three samples was added with 1 mL of 10 mg/mL chloramphenicol as a positive control. The final sample was left unchanged and used as a negative control. This process was repeated three times.

With each of the resulting 27 samples, 200 µL was pipetted aseptically in triplicate into a sterile 96 well microtitre plate to cast the dressings. The remaining 9.4 mL was transferred aseptically into sterile 60 mm petri dishes to cast.

The 96 well microtitre plate and petri dishes containing solution were frozen overnight at - 20 °C and freeze dried until there were no signs of ice or moisture in the dressings.

The dressings were tested for their antimicrobial activity using a modified disk diffusion method outlined in Chapter 2.2.4 of this work, except that the dressings cast in the 96 well microtitre plate were used in the place of paper disks.

5.2.5 Development of seaweed incorporated antimicrobial alginate/chitosan dressings

A similar formulation to that described above in section 5.2.4. The sodium alginate, L.M.W chitosan and M.M.W. chitosan solutions differed in that they were made up in 1 % lactic acid (Sigma-Aldrich) solution due to the strong smell of acetic acid.

The two stock solutions which were made, comprising of the most flexible and most brittle performing formulations from the previous formulations were:

- 50:50 % alginate:M.M.Wchitosan
- 50:50 % alginate:L.M.W.chitosan

These two stock solutions were mixed to obtain solutions at the following concentrations with 0, 0.5, 1, 2, 5, 10 and 15 % propylene glycol (Sigma-Aldrich):

10 mL of each solution was pipetted aseptically three times into three sterile McCartney bottles. One of the three samples was added with 0.25 g of crude water extract from *Fucus vesiculosus* equivalent to 5 mg/200 µL. The second of the three samples was added with 1 mL of 10 mg/mL chloramphenicol as a positive control. The final sample was left unchanged and used as a negative control. This process was repeated three times.

With each of the resulting 14 samples, 200 µL was pipetted aseptically in triplicate into a sterile 96 well microtitre plate to cast the dressings. The remaining 9.4 mL was transferred aseptically into sterile 60 mm petri dishes to cast.

The 96 well microtitre plate and petri dishes containing solution were frozen overnight at - 20 ^{o}C and freeze dried until there were no signs of ice or moisture in the dressings. The dressings were tested for their antimicrobial activity using a modified disk diffusion

method outlined in section 2.2.4 of this work, except that the dressings cast in the 96 well microtitre plate were used in the place of paper disks.

5.2.6 Antimicrobial release profile of developed alginate/chitosan dressings

A solution of 0.03 M KH_2PO_4 (Sigma-Aldrich) was made up and pH adjusted to 6.5 using 0.1 M NaOH drop wise. This solution was then autoclaved to sterility and allocated aseptically into 5 mL sterile sample tubes, which were subsequently incubated at 37 °C. The dressings prepared as part of chapter 5.2.5 were then placed in individual tubes in triplicate for each dressing formulation. Three buffer only solutions were treated as a negative control, while commercially available honey (Algivon), silver (Aquacel®) and iodine (Inadine) dressings were placed individually in solution in triplicate to act as positive controls.

100 µL aliquots were removed aseptically from each sample at time points over a 72 h period (0.5, 1, 2, 3, 4, 5, 24, 48, 72 h) while maintaining incubation at 37 °C. These aliquots were subsequently added with MRSA 676 as an indicator bacteria and allowed to incubate overnight at 37 °C before being tested spectrophotometrically in a similar fashion to MIC analysis carried out in section 2.2.5.

5.2.7 Safety procedures for microbiological work

All microbiological work was carried out in a Class II pathogen lab with an emphasis on safety and containment of pathogenic substances. The Standard Operating Procedure (SOP) for the pathogen lab was followed, including: all items brought into the lab were autoclaved on exit. Only pathogen lab coats must be worn in the pathogen lab, gloves must be used at all times and hands washed at exit. All waste in pathogen lab must be autoclaved subsequent to disposal and benches washed down with 70 % *v/v* ethanol in water after use. All contaminated organic solvents were evaporated to dryness and diluted with virkon, before disposal.

5.2.8 Statistical analysis

Results are reported as the mean ± standard deviation. Comparisons between dressings were made using one-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test. All statistical analysis was performed using Minitab 17 software with a 5 % statistical significance level ($p<0.05$)

5.3 Results and Discussion

5.3.1 Preliminary wound dressing study

A preliminary study was carried out by soaking non-active commercially available wound dressings (Aquacel®) in seaweed extract and testing them against the commercially available antimicrobial wound dressings Aquacel®, iodine (Inadine) and honey (Algivon). While the concentrations of active ingredient in each preparation is not known, wound dressings are applied on a basis of wound area, as such, the same area (6 mm^2) of the dressings were used so that a direct comparison could be made. These dressings were tested using the disk diffusion method, outlined in section 2.2.4, over a 72 h period to assess the efficiency of each dressing for delivering antimicrobial compounds over time. This study was intended as a proof of concept study to assess the suitability of the seaweed extract based antimicrobial wound dressings when compared to antimicrobial dressings which are currently on the market and was carried out according to a similar study by Samuel J. Hooper *et.al* (432), except that the disc diffusion method was set up according to CSLI and not BSAC (British Society for Antimicrobial Chemotherapy) guidelines. The results of this study are shown in Figure 5.2 and can be clearly seen in the petri dishes in Figure 5.3.

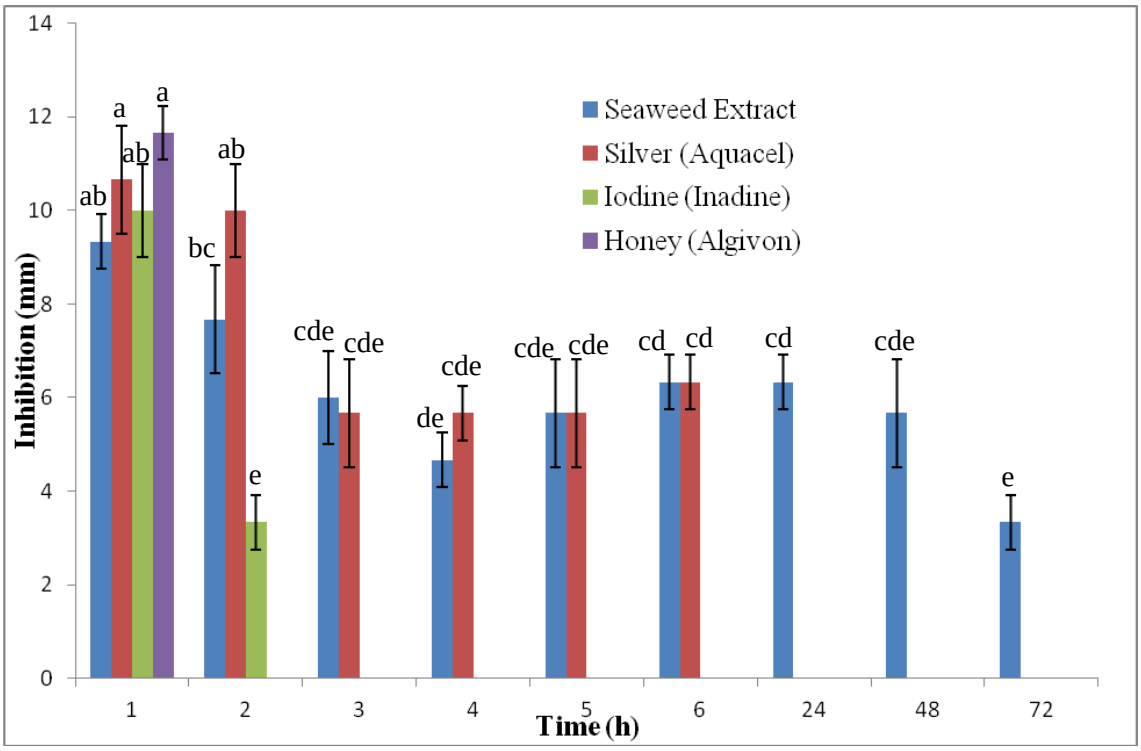

Figure 5.2: Disk diffusion of non-active Aquacel® soaked in seaweed extract as the antimicrobial agent compared to commercially available antimicrobial wound dressings Aquacel®, iodine (Inadine) and honey (Algivon) over time against MRSA 676. Values with the same superscript letters indicate a P value of <0.05 (n=3).

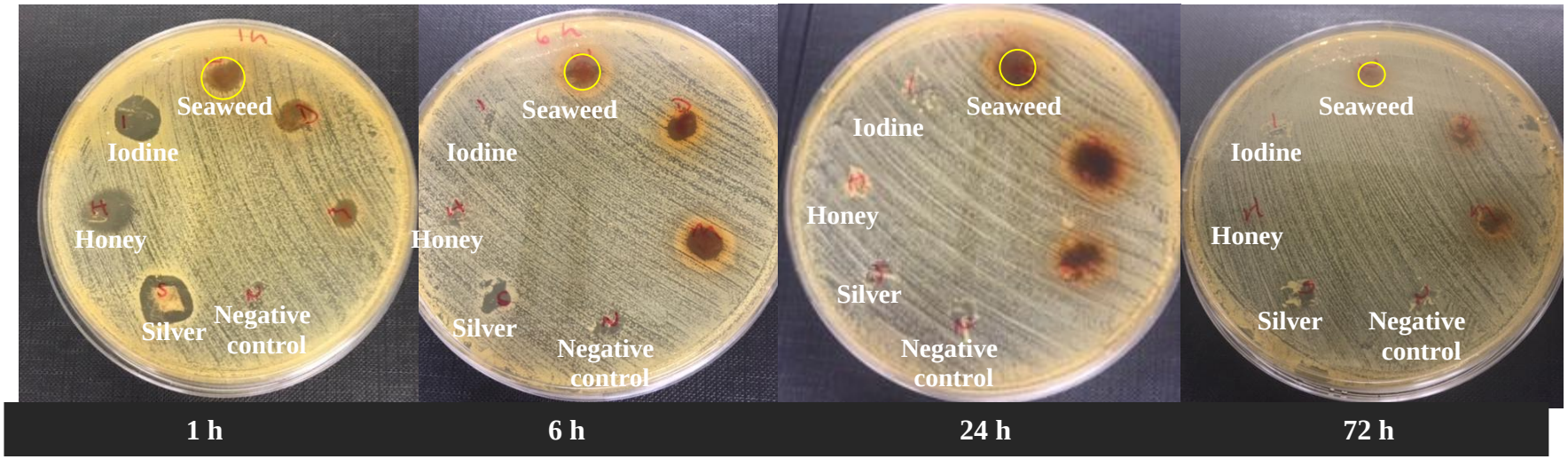

Figure 5.3: The change in antimicrobial activity of a seaweed based bioactive wound dressings versus Aquacel®, iodine (Inadine) and honey (Algivon) over time against MRSA 676 using the modified disk diffusion method.

From Figure 5.3. it can be seen that the honey based dressing (Algivon) released its antimicrobial for only the 1 h time period. This could potentially be due to the limitations of the method used to evaluate this dressing based on its mode of action. Honey works as an antimicrobial wound dressing in several ways; as the honey is very high in sugar content, it inhibits microbial growth via an osmotic affect (433) as it lacks the free water necessary for microbes to grow. Additionally, the pH of raw honey is 3.2-4.2 which is an overly acidic environment for most wound pathogens to thrive (434). Enzymes in raw honey produce the oxidation agent H_2O_2, which acts as an antimicrobial as it causes oxidative stress and DNA degradation of bacterial cells (435), H_2O_2 is also an aid to the debridement of the wound (434). These modes of action are dependent on a high concentration of honey being in contact with the microbial contamination, which may be a reason for Algivon performing statistically as well as other wound dressings in the first hour of this assay. However, the activity of Algivon dropped dramatically after this point to no notable inhibition of MRSA 676 after the 1 h period. Potentially as honey is water soluble, it may have diffused into the agar and lost a high concentration of active to the first agar plate.

Molecular iodine (Inadine), is known as a strong oxidation agent. Its mode of action is thought to a rise from iodine's ability to bind to unsaturated carbon bonds in fatty acids and nucleotides preventing hydrogen bonding and compromising the cell wall structure and cytoplasm for the microbes resulting in cell death (436). At low doses, iodine is affected by organic matter (437) which could have resulted in iodine being no longer able to inhibit MRSA 676 after a 2 h time point. As diffusion of the active ingredient into agar is essential for microbial inhibition in the disk diffusion method, much of the I_2 concentration may have been lost through the transfer of the dressing to a new plate. While this loss of antimicrobial activity via diffusion is a limitation of this assay, antimicrobial compounds must also be capable of diffusing into wound exudates, which are similarly contaminated with microbial organic matter and would dilute the concentration of the agent.

The silver based wound dressing Aquacel® functioned better than Inadine overall and better than Algivon from the 2 h point onward (Figure 5.2). Active Aquacel® inhibited microbial growth up to 6 h using this assay, which was 4 h longer than the Inadine and 5 h longer than Algivon commercial wound dressings. The mode of action of silver wound dressings comes from the ionisation of elemental silver with exposure to air or

wound exudates (212). As silver ions are highly reactive they can disrupt bacterial cell function by binding to thiol groups in enzymes and proteins. Cell membranes are also disrupted in bacteria, making them antimicrobial to a broad range of bacteria. (212). The ability of silver ions to bind preferentially with phosphate groups in nucleic acids to the detriment of DNA and RNA in bacterial cells is also a factor in its mode of action (207).

The burst release in antimicrobial activity demonstrated by the initial inhibition of MRSA 676 after 1 h, was displayed by all of the dressings. A burst release followed by sustained release of the antimicrobial is favourable for the treatment of wounds suffering from bio burden. Conversely, a prolonged release is advantageous for prophylactic treatment of wounds (254). Considering the results displayed in Figure 5.2, Algivon and Inadine were would be more suited to the treatment of infected wounds rather than as a preventative treatment. Aquacel® and the seaweed based wound dressings however, displayed both a burst and sustained release in this study, indicating that both of these treatment are more versatile in that they would be suited as both treatment and preventative treatment of infected wounds.

The seaweed extract soaked wound dressings displayed inhibition of MRSA 676 for the duration of the 72 h study, which was 66 h longer than the next longest wound dressing, (silver Aquacel®). This was a promising result in terms of the effectiveness of the seaweed based wound dressing over a long period of time and as a preliminary proof of the concept that water extracts from *Fucus vesiculosus* are a viable option for use in an antimicrobial wound dressing formulation with a long effective application. The Irish Health Service Executive (HSE) recommends that wounds be assessed 48 - 72 h after initial debridement and assessment (438) and, the application of a wound dressing to diminish the bioburden in the wound in this period. Thereafter, guidelines also suggest that wounds dressings need to be changed every 72 h during wound management (439). A dressing which remains active for the duration of 72 h would, therefore, reduce the need for redressing wounds within this time frame, saving costs associated with wound management.

5.3.2 Alginate film wound dressing

The method for production of these alginate based films was modified from a study which encapsulated natural essential oils as antimicrobials into alginate films (244). Alginate absorbs moisture and permits vapour transfer, making it a suitable material for the synthesis of a wound dressing.

The films were synthesised as described in section 5.2.1 and were tested for antimicrobial activity. Although the films did partially set to the extent that scratching the surface on which they were cast resulted in a permanent effect, they remained fluid. This is a poor indication for the structural stability of the films, which would have to be stable enough to be packaged and transported in a commercial setting.

The cast films held their shape subsequent to the addition of inoculated agar to the petri dishes and were incubated overnight. The antimicrobial results for these films are shown in Table 5.1.

Table 5.1: Antimicrobial activity of the alginate film wound dressing against MRSA 676.(n=3)

Concentration	**1 mg**	**5 mg**	**10 mg**
Inhibition	-	+	+++
Positive control[a]	++++	++++	++++
Negative control[b]	-	-	-

[a]Chloramphenicol antibiotic - 10 µg/film. [b]Negative control - 50 µL of specific solvent. Inhibition zone reported as diameter of clear inhibition (including 6 mm disk) in mm; - indicates no inhibition, + indicates inhibition zone of 6 mm - 7 mm, ++ indicates inhibition zone of 7.1 mm - 8 mm, +++ indicates inhibition zone of 8.1 mm- 10 mm, ++++ indicates inhibition zone of > 10 mm.

Although these results look promising, the films appeared to dissolve in the surrounding agar upon incubation. Therefore, though the inhibitions zones are reflective of the

bacteria in the agar cleared by the films, they also include voids formed in the agar, which are demonstrated in Figure 5.4.

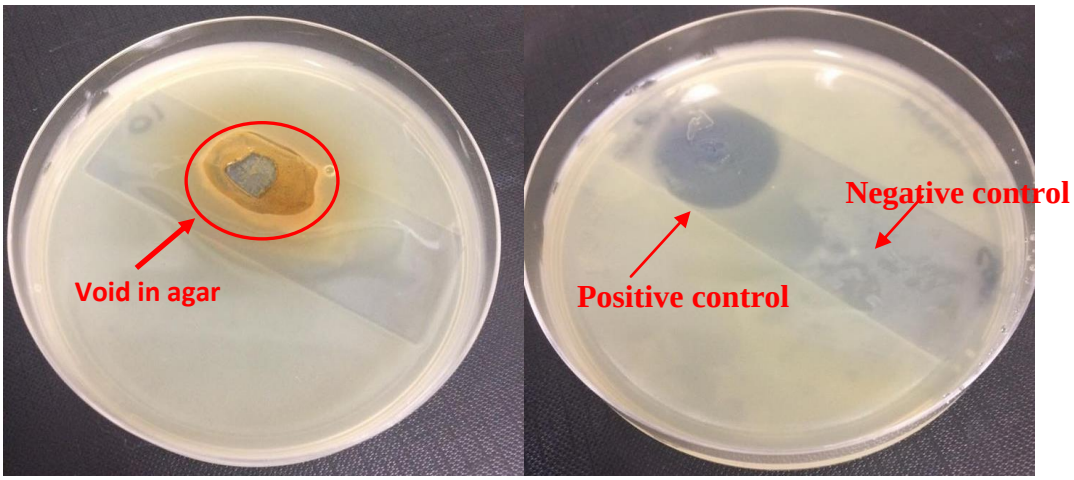

Figure 5.4: Right: antimicrobial assay of alginate films against MRSA 676 using 10 mg of crude water extract from *F. vesiculosus.* Left: positive and negative control for alginate film wound dressings. (n=3)

These voids were not present in the positive and negative control slides as shown in Figure 5.4, indicating that it is the addition of the seaweed extract which caused this difference. Agar is made using water soluble polymers extracted from seaweeds such as *F. vesiculosus* (440), and as such, it can be expected that water extracts would readily dissolve and diffuse into the media. This could possibly be an explanation for these voids. As the films did dissolved, they may be more suited for an antimicrobial liner in a wound dressing rather than a dressing on their own.

This does not definitively mean that the films would not work *in vitro*, only that this assay does not accurately portray the ability of the films as an antimicrobial. It was also noted that higher doses of extract gave comparatively lower zones of inhibition when compared to the traditional well or disk diffusion methods. This may be due to the fact that the films were cast over a 1.5 cm^2 square area as opposed to the 6 mm^2 round area of a disk or well for the same dose. This would have consequences for the zone of

inhibition as there was more surrounding agar directly in contact with the film for the crude extract to diffuse into.

5.3.3 Antimicrobial gelatin/chitosan hydrogel

Gelatin/chitosan hydrogel wound dressings were prepared and tested for antimicrobial activity using a modified disk diffusion method, where the 5 mg/200 µL plugs were placed on the media instead of disks. The resulting hydrogel maintained its shape as can be seen in Figure 5.5.

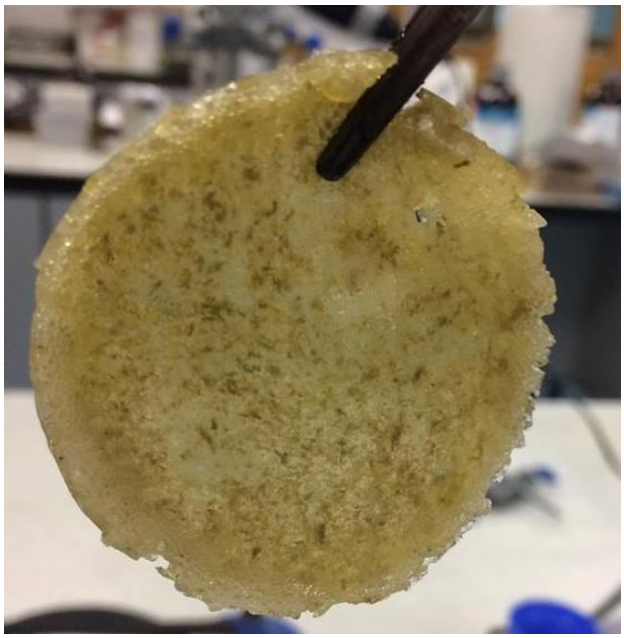

Figure 5.5: Hydrogel incorporated with 25 mg/mL crude seaweed extract.

The results of the antimicrobial analyses are displayed in Figure 5.6. As can be seen, the hydrogel plugs which were originally solid as in Figure 5.5, had dissolved upon incubation. This could have been due to the moisture in the agar dissolving the dressing or the temperature of the incubator (37 °C) which may have facilitated the breakdown of the dressing. The dissolution could possibly be a combination of both of these factors, but regardless, the dissolution of the dressing was predicted to be due to the hydrogel nature of the formulation.

The dissolved plugs did not exhibit any zone of inhibition against MRSA 676. They did, however, appear to inhibit the bacteria in contact with the hydrogel. This is also true of the negative control, indicating that the dressing formulation itself exhibited the antimicrobial activity. The antimicrobial activity of chitosan has been documented, and implicated in its usefulness as a wound dressing, which could be the source of this contact inhibition (441,442).

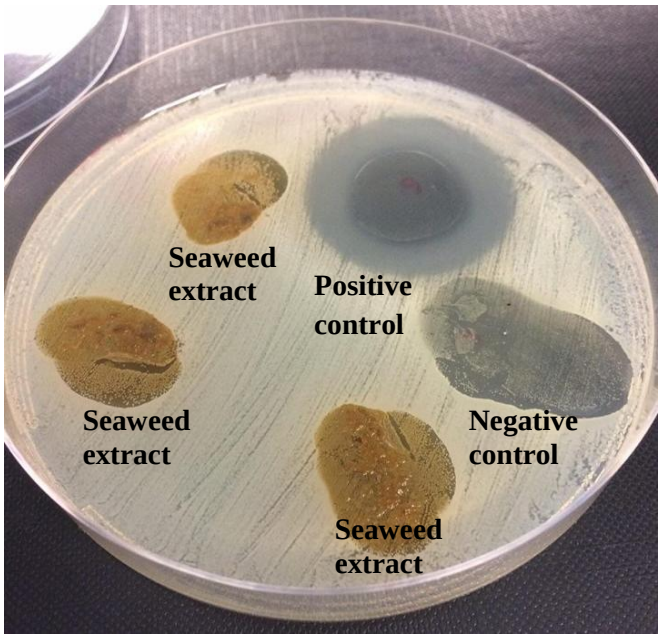

Figure 5.6: Antimicrobial assay for 25 mg/mL hydrogel plugs using a modified disk diffusion method against MRSA 676.

5.3.4 Antimicrobial alginate/chitosan dressings

Ten wound dressing formulations of different alginate/chitosan ratios were synthesised and tested against MRSA 676 as an indicator to their activity. The resulting dressings were sturdy and flexible as displayed by Figure 5.7 and 5.8 although, further development was required to improve their flexibility and also to reduce the strong smell of vinegar which is associated with acetic acid, which is addressed in the following section.

Figure 5.7: 25:75 % low molecular weight chitosan:alginate dressing with 25 mg/mL seaweed extract.

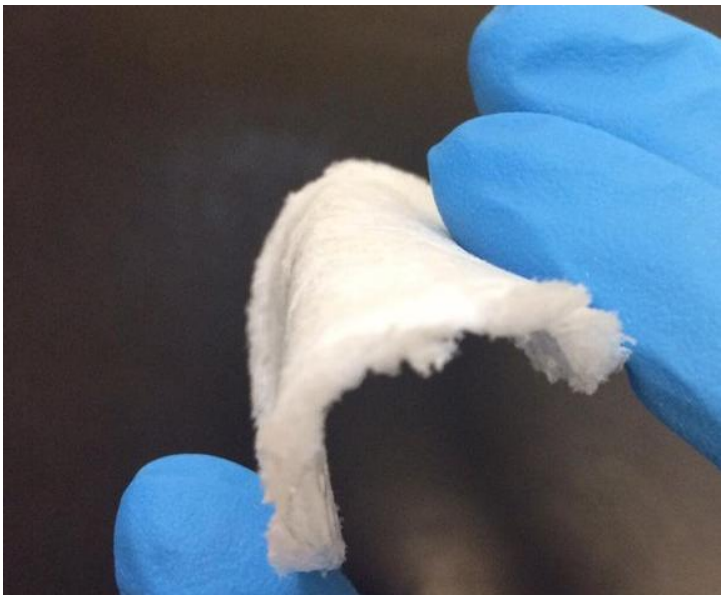

Figure 5.8: Demonstration of negative control 25:75 % low molecular weight chitosan:alginate dressing flexibility.

The wound dressings were cast in 200 µL aliquots in a 96 well microtitre plate to form plugs for their antimicrobial testing via a variation of the disk diffusion method (Chapter 2.2.4); the results of which can be seen in Table 5.2.

Table 5.2: Antimicrobial susceptibility of water extract from *F. vesiculosus* incorporated into various ratios of alginate:chitosan wound dressings and tested against MRSA 676.

	Ratio of alginate:chitosan				
Alginate: L.M.W.Chitosan	1:0	7.5:2.5	5:5	2.5:7.5	0:1
Seaweed extract	++	+	+	-	-
Positive control[a]	++	+	++	-	-
Negative control[b]	-	-	-	-	-
Alginate: M.M.W Chitosan	1:0	7.5:2.5	5:5	2.5:7.5	0:1
Seaweed extract	++	++	++	++	-
Positive control[a]	+++	+++	++	+++	-
Negative control[b]	-	-	-	-	-

[a]Chloramphenicol 10 µg/plug. [b]Negative control - alginate:chitosan dressing. Inhibition zone reported as diameter of clear inhibition (including 6 mm disk) in mm; - indicates no inhibition, + indicates inhibition zone of 6 mm - 9.9 mm, ++ indicates inhibition zone of 10 mm - 14.9 mm, +++ indicates inhibition zone of 15 mm- 19.9 mm, ++++ indicates inhibition zone of 20 mm-24.9 mm, +++++ indicates inhibition zone of > 25 mm. L.M.W. indicates low molecular weight chitosan. M.M.W. indicates medium molecular weight chitosan. (n = 3).

As can be seen from Table 5.2, the seaweed extract incorporated dressings did maintain antimicrobial activity for most of the dressings. The molecular weight and ratio of chitosan added to the formulation appeared to be a factor in the efficacy of the antimicrobial dressing. This was true even for the positive controls, which also resulted

in no bacterial inhibition, indicating that the issue lies in the formulation, and not the addition of the seaweed extract (Figure 5.10). There are a number of potential explanations for this phenomenon. Dressings containing a higher ratio of alginate had better antimicrobial inhibition, however, these would have less diffusion issues due to the fact that the Mueller Hinton agar used for growing this bacteria is made using water soluble seaweed extracts (agar) as gelling agents (443). As alginate and agar are both similarly hydrophilic, there is little likelihood that diffusion issues would be present in these plugs. However, diffusion could potentially be a problem in plugs containing a higher ratio of chitosan, as chitosan is noted to have poor water solubility (442,444) which is a general indication of its hydrophilicity .

Although these diffusion issues would indicate that the 100 % alginate dressing formulations are superior, plugs with a higher ratio of alginate tended to deliquesce (shown in Figure 5.9) during antimicrobial testing, a characteristic that would make for a poor wound dressing. The resulting soft pulp that forms subsequent to the exposure of the 100 % alginate wound dressings to moisture would be a disadvantageous wound dressing as, wound dressings are required to provide mechanical protection of the wound (256,427). These dressings also assume a wet form and therefore would not be able to provide a moist but not wet environment (428) that wound require for healing. They could potentially be used along with a gauze-like backing as an antimicrobial liner. Antimicrobial fillers and liners can be beneficial in the treatment of wounds as they generally have less adherence to the wound bed (445), preventing further trauma on removal. Many antimicrobial wound dressings require lamination to the wound site using other structures, including the honey based Algivon (446), and iodine based Inadine used in section 5.3.5 and 5.3.6 of this study.

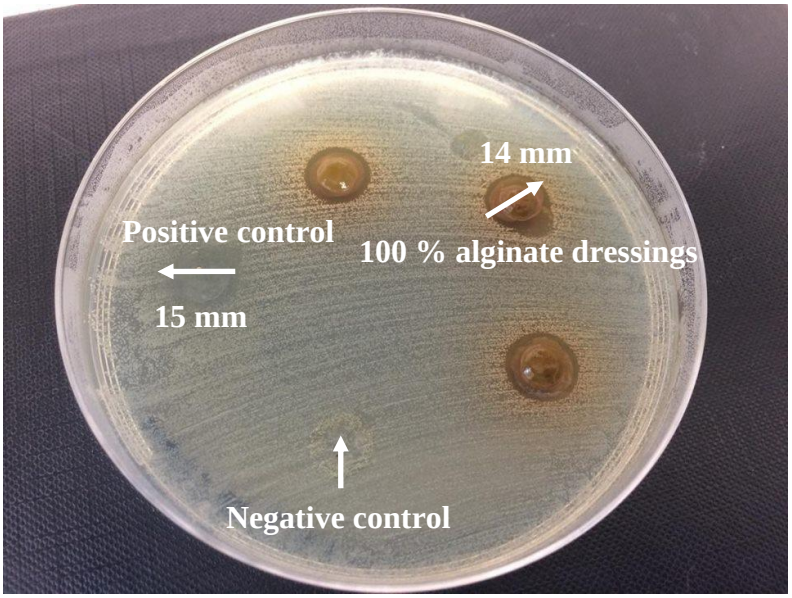

Figure 5.9: 100 % alginate plugs containing 25 mg/mL of the water extract from *F. vesiculosus* antimicrobial tested against MRSA 676 with 10 µg chloramphenicol as a negative control.

Medium molecular weight chitosan appeared to be the better formulation due to the resulting dressing's antimicrobial efficacy. Also, it was noted that both low and medium weight chitosan made less flexible dressings that were more likely to lose integrity and crumble as can be seen in Figure 5.10. These characteristics are undesirable, as again one of the primary functions of a wound dressing is to prevent further mechanical trauma to the wound whilst protecting it from external physical and microbial burdens by creating a barrier between the wound and the environment (256,427). In addition to the unsuitability of these formulations as wound dressings, the crumbling aspect of their structure could provide additional difficulty to wound treatment by supplying foreign particulates which could embed themselves in the wound bed.

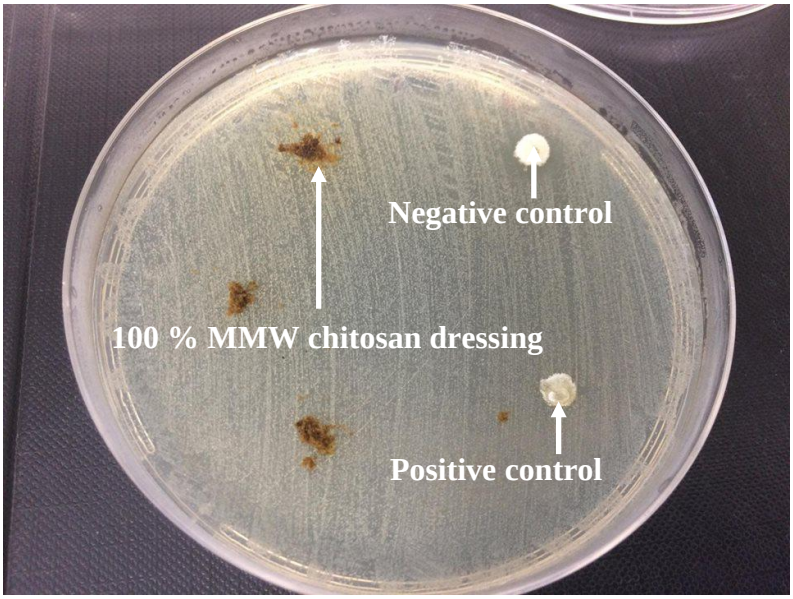

Figure 5.10: 100 % medium molecular weight chitosan containing 25 mg/mL of the water extract from *F. vesiculosus* antimicrobial tested against MRSA 676 with 10 µg chloramphenicol as a negative control.

As such the most durable plugs were the 50:50 % alginate: low molecular weight chitosan and 25:75 % alginate: low molecular weight chitosan as can be seen in Figure 5.11. These plugs maintained their cylindrical shape, however, only the 50:50 % ratio plugs established antimicrobial activity against MRSA 676. Considering these results it was determined that the 50:50 % alginate: low molecular weight chitosan, as these dressings maintained both antimicrobial activity against MRSA 676 and maintained their desired shape. As the texture of these dressing is not prone to disintegration, they could provide appropriate mechanical protection for wounds while their maintaining antimicrobial activity. However, these formulations were not flexible and therefore could provide issues on the employment of the dressings to the natural curvature of the body. The use of the strong smelling acetic acid in the diluent for the chitosan and alginate stock solutions also was a matter to be addressed in future formulations.

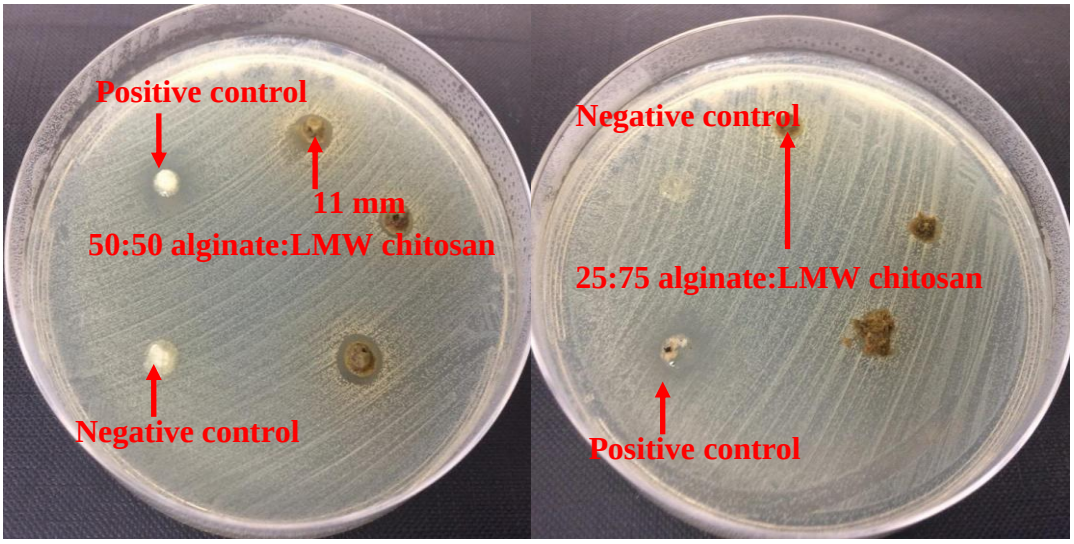

Figure 5.11: 50:50 % alginate:low molecular weight chitosan wound dressings and 25:75 % alginate:low molecular weight chitosan wound dressings in modified disk diffusion method against MRSA 676.

Due to the issues with texture that arose for these formulations, further development of the formulation was undertaken.

5.3.5 Development of antimicrobial alginate/chitosan dressings

Due to the overly rigid and inflexible texture and unpleasant smell of the alginate/chitosan wound dressings, further development of the formulation was required. As the strong odour of the dressings was caused by the use of glacial acetic acid in the polymer diluent, it was postulated that the use of a different biocompatible acid such as lactic acid would make for a more suitable substitute. The brittle texture of the dressingwas also addressed though the use of a biocompatible plasticiser. Propylene glycol was chosen for the formulations due to its hydrophilic nature, antimicrobial activity (447), and low potential for toxicity (448). It was added over a concentration

range of 0-15 % to identify the concentration which improved the flexibility of the formulation.

Studies have been found to use anything from 0.5 % to 30 % plasticiser (431,448–450) for these polymers, albeit in different types of formulations. A sodium alginate/chitosan composite membrane was developed with a range of plasticiser concentrations 0-30 % using glycerol as the plasticiser (431). As expected, the tensile strength of the formulation reduced with an increase in concentration of plasticiser and conversely the elongation rate and break strength increased, indicating that flexibility improved with the optimum concentration of glycerol being 10 % (431). As such, a concentration range of 0 - 15 % was chosen as a reasonable range to formulate based on these studies and, under the condition that the resulting formulations were too brittle or wet, more or less plasticiser could be added in future formulations.

The results of the antimicrobial testing of these formulations using the modified disk diffusion method on MRSA 676 as an indicator bacterial strain are given in Table 5.3 and in Figure 5.12.

Table 5.3: Antimicrobial susceptibility of water extract from *F. vesiculosus* incorporated into alginate:chitosan wound dressings with various % of propylene glycol and tested against MRSA 676.

	% Plasticiser							Commercial Dressings		
Alginate: L.M.W.Chitosan1:1	**0**	**0.5**	**1**	**2**	**5**	**10**	**15**	**Honey**	**Iodine**	**Silver**
Seaweed extract	$++^{e,f,g}$	$+++^{b,c,d,e,f}$	$++^{d,e,f,g}$	$++^{d,e,f,g}$	$++^{d,e,f,g}$	$++^{f,g}$	$++^{d,e,f,g}$	$++++^{a}$	$++^{c,d,e,f}$	$++^{c,d,e,f}$
Negative control[a]	-	$+^{e,f,g}$	$+^{g}$	$+^{g}$	$+^{e,f,g}$	$+^{g}$	$+^{g}$			
Alginate: M.M.W.Chitosan1:1	**0**	**0.5**	**1**	**2**	**5**	**10**	**15**			
Seaweed extract	$++^{e,f,g}$	$++^{d,e,f,g}$	$++^{d,e,f,g}$	$+++^{a,b,c,d}$	$+++^{a,b,c,d}$	$++++^{a,b,c}$	$++++^{a,b}$			
Negative control[a]	-	$+^{e,f,g}$	$+^{e,f,g}$	$+^{g}$	$+^{e,f,g}$	$+^{g}$	$+^{g}$			

[a]Negative control - alginate:chitosan dressing with % propylene glycol. Inhibition zone reported as diameter of clear inhibition (including 6 mm disk) in mm; - indicates no inhibition, + indicates inhibition zone of 6 mm – 7.9 mm, ++ indicates inhibition zone of 8 mm – 9.9 mm, +++ indicates inhibition zone of 10 mm- 11.9 mm and ++++ indicates inhibition zone of >12 mm. L.M.W. indicates low molecular weight chitosan. M.M.W. indicates medium molecular weight chitosan. (n=3). Values with the same superscript letters indicate a P value of <0.05.

As can be seen in Table 5.3, negative control formulations containing propylene glycol exhibited antimicrobial activity, this was due to the documented antimicrobial activity of propylene glycol (447). It was thought that the use of a biocompatible antimicrobial would enhance the antimicrobial activity of the formulations.

Formulations with M.M.W. chitosan preformed as well or better than formulations containing L.M.W. chitosan as part of this modified disk diffusion assay. While M.M.W. chitosan formulations with 10-15 % propylene glycol were found to be the superior formulations in terms of delivering antimicrobial activity, it was observed that these formulations contained too much plasticiser as they lost their shape and took on an almost fluid consistence. Similarly, formulations containing 5 % propylene glycol presented with a similar, rigid unacceptable consistency. Other M.M.W. formulations (0-2 %) were brittle in texture and as such retained the flaws of their plasticiser free predecessors.

In general, the texture of the L.M.W. chitosan dressings were better than those of their M.M.W. counterparts. The addition of 0-5 % plasticiser caused the formulations to appear more flexible than that of the M.M.W. formulations. However, similarly to the M.M.W. formulations, the formulations containing 10-15 % plasticiser became overly soft and almost fluid in nature. As such, in terms of texture, it was apparent that 2-5 % plasticiser incorporated into L.M.W. chitosan dressings were the developed formulations. While these formulations were poorer in terms of antimicrobial activity using the modified disk diffusion method, they did perform in a statistically similar manner to that of the commercially available silver and iodine dressings tested as a positive control (Table 5.2). The disc diffusion method is an indicator of an initial, burst release of the antimicrobial and therefore, gives no indication of whether the compounds necessary for antimicrobial activity may have diffused slower in the formulation for a more sustained release of the antimicrobial. Considering the advantages of a burst release followed by sustained release of the antimicrobial in the wound dressing (254), the modified disk diffusion method may not be descriptive enough with only one time point. As such, a different method was used to determine the antimicrobial release profile which is described in section 5.3.6.

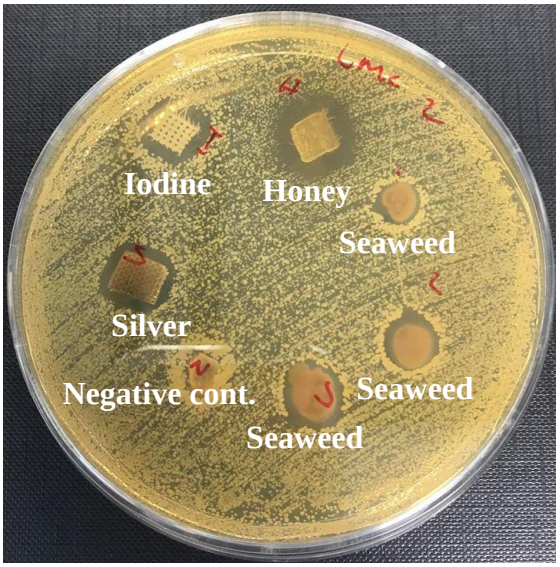

Figure 5.12: Antimicrobial evaluation of 1:1 % alginate:L.M.W. chitosan with 2 % propylene glycol and commercially available wound dressings against MRSA 676.

To further examine the textures of these formulations, the 1:1 % alginate:L.M.W. chitosan with 2 and 5 % propylene glycol were both prepared and cast into 5 mL petri dishes. This was to better demonstrate the improved texture by enlarging the gauze. As can be clearly visualised in Figure 5.13, the formulation containing 5 % propylene glycol was not suitable following this scale up. The resulting texture was more brittle than that of the smaller plugs. The formulation containing 2 % propylene glycol however, retained its desirable texture and therefore was considered as the developed formulation for further study and material characterisation.

Figure 5.13: Demonstration of flexibility of 1:1 alginate: L.M.W. chitosan containing 25 mg/mL of water extract from *F. vesiculosus* with 2 % and 5 % propylene glycol as plasticiser.

Of the commercially available dressings, honey outperformed silver and iodine, as well as all of the tested formulations Table 5.3. The negative controls for formulations with propylene glycol all exhibited come degree of antimicrobial activity. There are several possible reasons for this: Propylene glycol has a long since been established to have antimicrobial activity against various microbes (451,452) including *S. aureus* (447). Chitosan has also been established to have antimicrobial activity (453,454) which may have been enhanced with the addition of propylene glycol, as the plasticiser could potentially liberate chitosan by solubilising bonds trapping it within the formulation.

5.3.6 Antimicrobial release profile of developed alginate/chitosan dressings

The antimicrobial release profile of seaweed extract incorporated into wound dressings with commercially available dressings of different antimcirobial agents is displayed in Figures 5.14 and 5.15. Formulations made using L.M.W. chitosan had a greater antimicrobial activity on average over time than those of their M.M.W. counterparts. However, statistical analysis of this data using one-way ANOVA showed that only M.M.W. formulations containing 1 % or greater of plasticiser had a statistically poorer

drug release profile of the antimicrobial compounds than those containing L.M.W. chitosan.

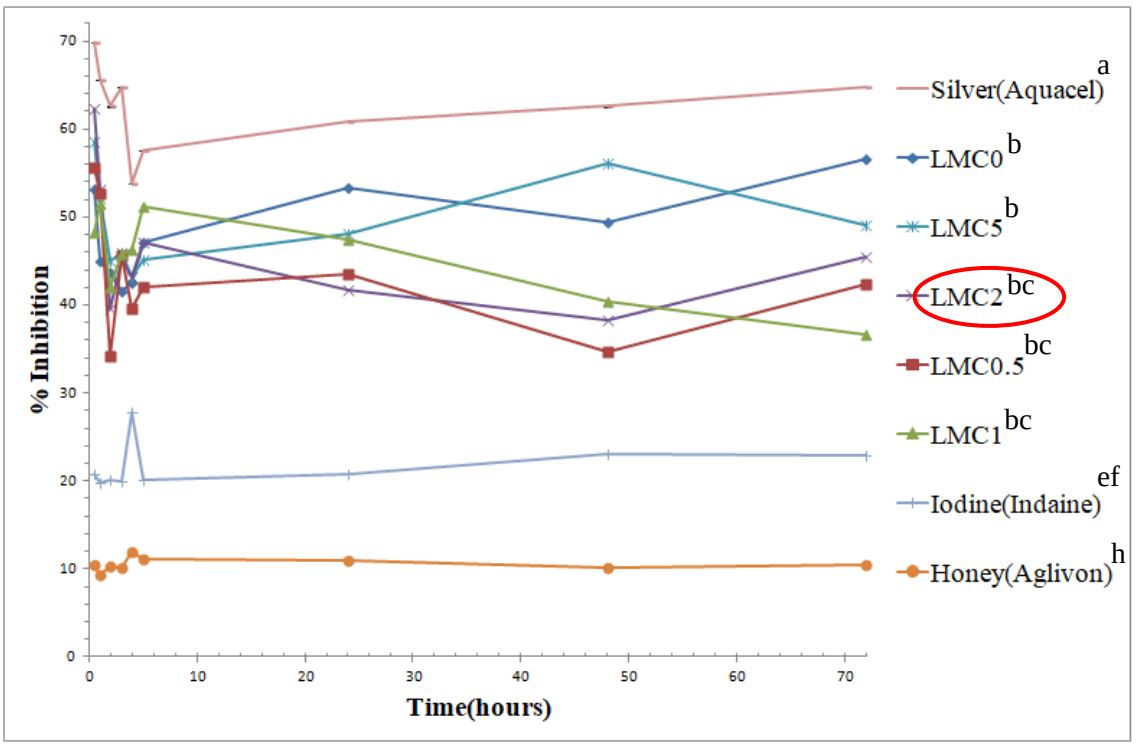

Figure 5.14: Antimicrobial release profiles for alginate:low molecular weight chitosan 5:5 dressings with various concentrations of propylene glycol as plasticiser. (n=3). LMC0 indicates 0 % propylene glycol, LMC05 indicates 0.5 %, :LMC1 indicates 1 %, LMC2 indicates 2 % and LMC5 indicates 5 % . Values with the same superscript letters indicate a P value of <0.05, these are linked with Figure 5.15.(n=3).

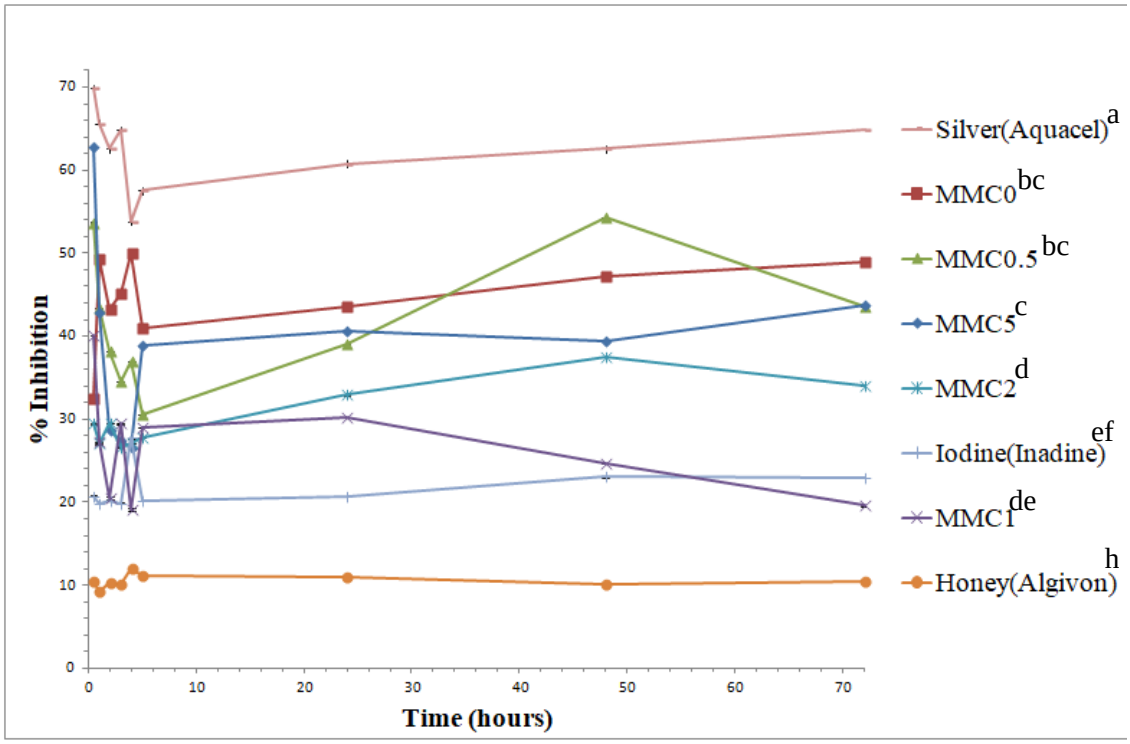

Figure 5.15: Antimicrobial release profiles for alginate:medium molecular weight chitosan 5:5 dressings with various concentrations of propylene glycol as plasticiser. (n=3). MMC0 indicates 0 % propylene glycol, MMC05 indicates 0.5 %, :MMC1 indicates 1 %, MMC2 indicates 2 % and MMC5 indicates 5 % . Values with the same superscript letters indicate a P value of <0.05, these are linked with Figure 5.14. (n=3).

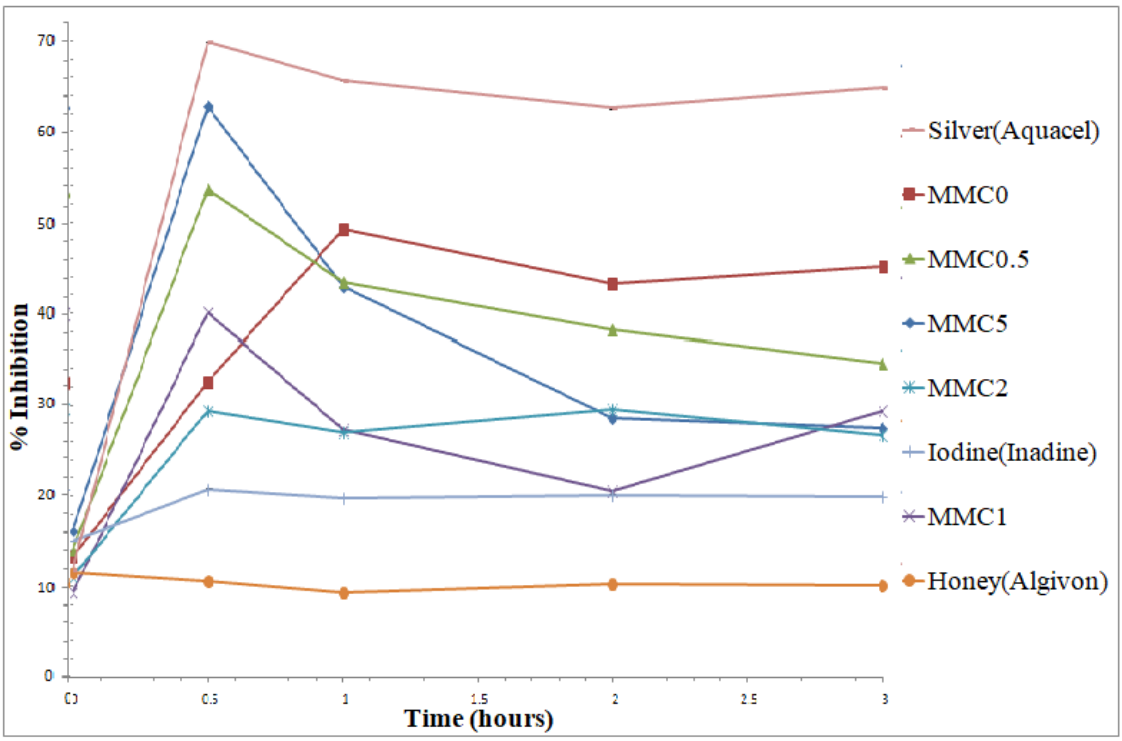

Figure 5.16: Antimicrobial release profile displayed in Figure 5.14, showing the 1 - 3 h time points.

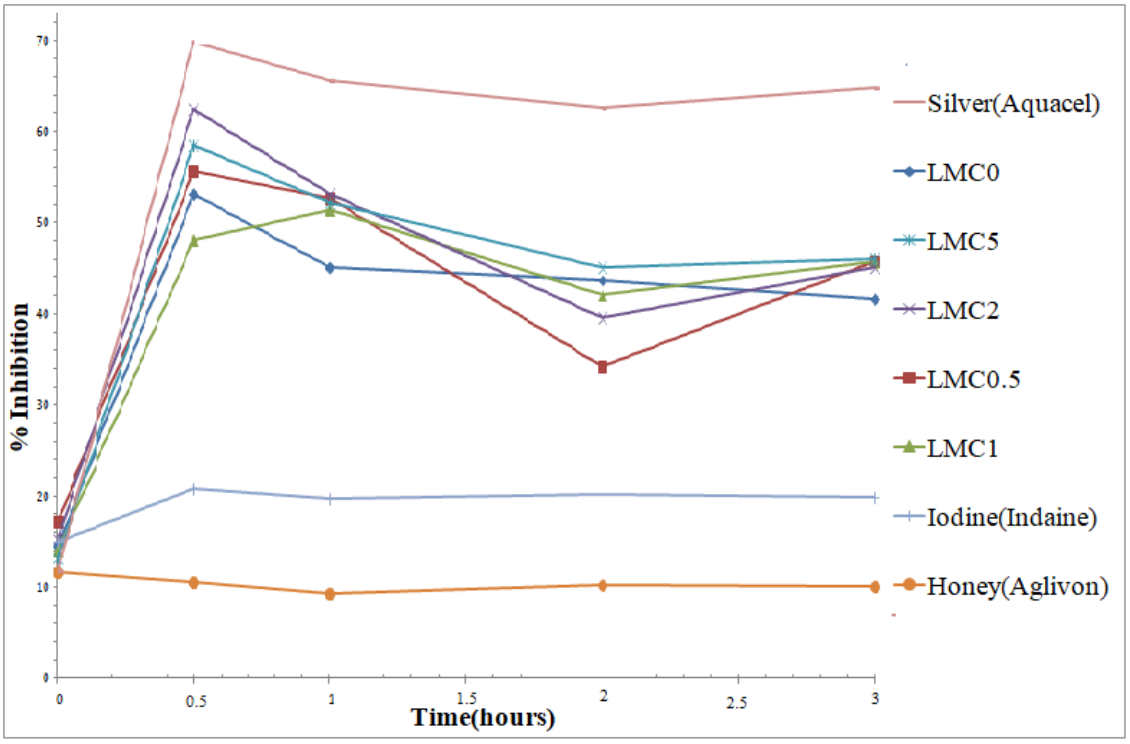

Figure 5.17: Antimicrobial release profile displayed in Figure 5.15, showing the 1 - 3 h time points.

1:1 % alginate:L.M.W. chitosan with 2-5 % propylene glycol were visually more flexable than other formulations tested; these formulations achieved an average of 46.2 % and 49.3 % bacterial inhibition over a 72 h period. As can be seen in Figure 5.14 and 5.15, the commercially available silver based wound dressing Aquacel® preformed statistically better than any of the tested wound dressing formulations for antimicrobial activity. This data, in conjunction with that presented in Figure 5.16, Figure 5.17 and Table 5.3 of section 5.3.5, would indicate that the silver wound dressings maintain a sustained release rather than a burst release of antimicrobial. This is confirmed by a study analysing the efficiency of Aquacel® containing silver as an active ingredient verses inactive Aquacel® as a control demonstrated that the silver dressing sustained antimicrobial activity against a range of wound pathogens including MRSA over a 14 day period using a similar method (455).

Contrary to this, Algivon, the honey based wound dressing statistically performed poorly compared to all other formulations tested, but was a superior formulation in the modified disc diffusion method carried out in section 5.3.5. This data demonstrates the reliance of Algivon on its initial burst release of antimicrobial, and suggested that these dressings do not function as well over time, or when diluted with exudate, and is contrary to the high antimicrobial activity typically reported for honey based dressings (446).

Considering Algivon was the only other natural product based antimicrobial used in this study, it was clear that an alternative natural antimicrobial may have its advantages in terms of sustained release of the antimicrobial. It was noted in a 2015 review that honey based wound dressings tend to dilute rapidly, decreasing the sustained release performance of the antimicrobial activity (456). This is due to the mode of action of honey requiring a high concentration to both remain antimicrobial and reduce biofilm formation (457). A prolonged release profile is essential to decrease the frequency of dressing changes that occur, thereby reducing trauma to the wound and increasing healing time. In particular, chronic wounds such as diabetic ulcers, require a sustained release of antimicrobial to discourage microbial invasion of these vulnerable sites (456).

The antimicrobial activity of iodine dressings using this method demonstrated that commercially available iodine functions approximately half as efficiently as the

developed formulation 1:1 % alginate:L.M.W. chitosan with 2-5 % propylene glycol. Similar to the results for the honey based Algivon, Inadine appeared to lack a prolonged release of its antimicrobial activity. Iodine has been noted to have sustained antimicrobial active however, this prolonged nature was defined as only over the 12 h duration (232). This is not in agreement with the results of section5.4.3.1, as iodine was found to sustain an antimicrobial activity over the duration of the study.

The development of wound dressing formulations incorporating antimicrobial seaweed extracts outlined in this work is in a preliminary stage, therefore, the emphasis was placed on maximising antimicrobial activity. Developments to the concentration of extract required to effectively eradicate wound infections while minimising potential adverse effects would be required to further characterise and formulate the wound dressing formulations for the purpose of commercialisation.

5.4 Conclusions

Wound dressing formulations were incorporated with the water extract from the seaweed *F. vesiculosus* and tested for their antimicrobial activity. While the alginate films did exhibit some antimicrobial activity, the method used to evaluate the antimicrobial activity was not appropriate due to the appearance of voids in the agar which inhibited the accurate evaluation of any zones of inhibition. Although these films maintained their shape, they were not stable enough to handle, which would result in a poor wound dressing. Further work could be carried out to improve the stability of the films by changing the formulation, possibly by adding a cross linker into the preparation, to improve its physical characteristics. The possibility of using antimicrobial films such as these as antimicrobial liners for wounds, as such, these films could be developed into a liner rather than a wound dressing.

The hydrogel dressings did not exhibit any antimicrobial benefits in addition to those of the dressing itself, as the negative control demonstrated antimicrobial activity on contact, which was similar to that of the seaweed incorporated plugs. Future work could possibly to control the degree of cross linkages in the hydrogel to encourage the diffusion of any larger antimicrobial compounds present in the seaweed extract from the hydrogel. Also, modifying the polarity of the hydrogel so that the antimicrobial is more weakly retained, encouraging diffusion.

The alginate/chitosan dressings displayed promising results in terms of physical robustness. The low molecular weight chitosan made more stable plugs with less effective antimicrobial activity, as medium molecular weight formulations tended to crumble on contact. Of the ten formulations tested, the 1:1 % ratio alginate: low molecular weight chitosan appeared to maintain its original shape while exhibiting good antimicrobial activity. These dressings were developed for flexibility with the addition of a plasticiser. It was found that an addition of 2 % propylene glycol benefited the flexibility of the formulation with no negative impact on activity.

Considering the beneficial ability observed for these dressings to provide a burst and then sustained release action for the seaweed derived antimicrobial agent contained within the formulation, there is promise for these wound preparations. Furthermore, the

lack of wound dressings in the literature containing seaweed derived antimicrobial compounds, emphasis the novelty of these dressings.

The incorporation of a purified and identified antimicrobial compound from the water extract of *F. vesiculosus* would greatly aid any chances of medical device approval. As seasonal variation is present and uncontrollable in natural products such as seaweed and their extracts, a pure, known and characterised antimicrobial compound would present less variation between dressings and therefore, greater reliability of a product.

Chapter 6: Development of separation, purification and identification of antimicrobial compounds methods for water extracts from *Fucus vesiculosus*

6.1 Introduction

It has long since been established that many natural sources have bioactive compounds which can be used to improve the lives of people everywhere. However, due to the complex, regulatory nature of the pharmaceutical industry, and the notorious variability of natural products, it is typically not feasible to use impure, crude extracts for pharmacological purposes. There are of course exceptions to this, for instance honey which has been described as a topical agent for wound infections elsewhere in this work (216,458,459). However, as concentrations of bioactives can be subject to environmental conditions and seasons (50,290,308), the objective of natural product chemistry typically strives to purify and identify the bioactive compounds found in natural sources. Purification and ultimately identification of compounds from natural products is an infamously difficult task owing to the complexity of the samples involved. The challenges associated with the isolation and purification of unknown bioactive compounds within a crude sample are often augmented by the often not disclosed nature of the rationale behind purification processes in published works (460).

The primary form of purification of the bioactive is the initial solvent extraction from the seaweed. Owing to the nature of solubility, the type of solvent and the conditions that the bioactive is soluble under will give some information on the polarity of the compound(s) of interest (460). For example, lipids, steroids and low polarity terpenoids will extract with non polar solvents such as hexane. Medium polarity solvents like chloroform and ethyl acetate can extract medium polarity compounds such as alkaloids and phenolics, whereas high polarity solvents such as aqueous systems can extract flavonoids and polysaccharides etc. (58). The method development of these extraction conditions to allow for the generation of sufficient material to purify is a critical step to the isolation and identification of unknown compound(s) from natural sources (58,460). Crude, primary extracts can contain hundreds of constituents, as such; there may be a very small percentage of the compound of interest in the mass of extract generated.

Sequential extraction can be utilised to aid the purification of crude extracts by separating components of the crude mixture that are more compatible with different solvents. This practise has been used to separate highly polar extracts from medium polar extracts in marine invertebrates (461), chlorophyll-α from algae using ethanol

(462) and is frequently used to remove lipid based molecules from extracts using dichloromethane (463).

Solvent extracts are frequently purified using chromatographic methods such as flash column chromatography, preparative TLC and preparative HPLC. Flash column chromatography involves the filling and packing of a stationary phase into a column and the subsequent elution and collection of fractions using a mobile phase. In normal phase chromatography, the stationary phase is typically a silica powder, whereas in reversed-phase the stationary phase comprises of hydrocarbons bound to a reverse-phase support particle (464). Silica column chromatography has been used in many cases to purify compounds from seaweeds, such as phlorotannins from *Cystoseria trinodis* (465), phytol from *Gracilaria edulis* (466) and fucoxanthin from *Sargassum sp.* (467). Similarly, preparative TLC has been used to purify fucoxanthin from *H. elongta* (468). Preparative TLC has the limitation of its loading capacity, whereby the mass that can be loaded is proportional to the square root of the thickness of the stationary phase (469). However, as developed bands in preparative TLC can be visualised using non-destructive techniques, such as under UV light, iodine and water vapour (470), and precisely cut out, fractions can be readily identified and isolated.

Preparative and semi preparative liquid chromatography can be used to collect purified fractions which can be tracked for compounds using TLC or analytical HPLC. Preparative HPLC can be used on a smaller scale than flash chromatography, as was displayed with the isolation of water soluble bioactive secondary metabolites from marine invertebrates, and this was achieved by reversed-phase preparative HPLC by Ebada *et. al.* (461). This method allows for more targeted recovery of compounds of interest, owing to the sample being passed through a non-destructive detector (UV-Vis, infrared (IR), PDA (photo-diode array)) which can identify peaks from eluting compounds. It often takes more than one purification method to achieve a pure or semi pure fraction.

Once sample purification is satisfactory, compound(s) of interest must be characterised and identified via structural elucidation. A variety of spectroscopic techniques can be used to elucidate the structure of isolated compounds, such as gas chomatography mass spectroscopy (GC-MS), liquid chomatography mass spectroscopy (LC-MS), IR and both solid and liquid phase nuclear magnetic resonance (NMR). The instrumentation

typically associated with these techniques is typically expensive, giving rise to limitations on the techniques available for this analysis. Liquid chromatography, diode array detection-electrospray ionisation, mass spectrometry, mass spectrometry (LC-DAD-ESI-MS/MS) was successfully used to identify phenolic compounds from *H. elongata* (471). IR analysis along with Liquid chromatography, diode array detection-electrospray ionisation, mass spectrometry (LC-ESI-MS) was used to identify phlorotannins from *Macrocytis pyifera* (472). LC-ESI-MS offers a soft ionisation method by the addition of a proton to the compound of interest which results in little fragmentation of thermally labile or large biomolecules (473). This is useful in discerning the molecular weight of a compound of interest. ^{1}H NMR, ^{13}C NMR and distortionless enhancement by polarisation transfer (DEPT) NMR were successfully used to elucidate the structure of kocumarin, a novel antimicrobial from *Kocuria marina,* a surface associated bacteria from brown seaweed. NMR is a highly useful method in terms of the structural elucidation of organic compounds. However, this technique is not appropriate for complex samples and, therefore, samples must be adequately purified first (474).

The studies completed in section 2 of this work indicated that the water extract from *F. vesiculosus* was the most promising in terms of bioactivity pertaining to the use of seaweed extracts as active ingredients in wound dressings for the topical management of wounds. This information was then used to formulate an antimicrobial wound dressing in section 5. The isolation and identification of the compound(s) responsible for antimicrobial activity in the water extract from *F. vesiculosus* would allow for the synthesis of the compound(s), removing the seasonal variations associated with natural products such as seaweed. However, this works describes the purification and identification of the antimicrobial compound(s) from the water extract from *F. vesiculosus.*

6.2 Experimental Procedure

6.2.1 Separation of crude water extract from F. vesiculosus using TLC

Subsequent to crude extraction using water, the extract was spotted onto reverse (Silica gel C_{18} - Merck Analytical Chromatography) and normal phase TLC plates and separated using various mobile phases detailed in Table 6.1. Extracts were made up to a concentration 1 mg/10 µL, spotted in 5 µL aliquots using a micropipette and dried until the specified concentration was achieved. The compounds separated using TLC were monitored visually. Activity was tracked using bioautography, the method of which is detailed in section 6.1.2. All organic solvents were disposed of in correct 'flammable waste' or chlorinated waste' containers for safe disposal.

Table 6.1: Mobile and stationary phases used during TLC.

Stationary phase	**Mobile phase (*v/v*)**	**No. of bands visualised**
Reverse (C_{18})		
	100 % H_2O	1
	H_2O+0.01 % TFA (trifloroacetic acid)	1
	H_2O+TEA (triethylamine)	1
	10:90 MeOH: H_2O	1
	10:90 MeOH: ACN	1 (broad)
	25:75MeOH:ACN	1 (broad)
	50:50 MeOH:ACN	1 (broad)
	90:10 ACN:MeOH	3
Normal Phase (Silica)		
	100 % Hexane	1
	1:1 Hexane:EtO:Ac	4
	100 % EtOAc	3
	1:1 EtOAc:$CHCl_3$	3
	100 % Chloroform ($CHCl_3$)	3
	100 % isopropyl alcohol (IPA)	2 (broad)
	100 % Toluene	1
	100 % DCM	2
	DCM 5 % MeOH **(utilised)**	7

6.2.2 Bioautography of TLC plates

TLC plates were aseptically placed in individual petri dishes and allowed to dry subsequent to being run in their respective mobile phases. Upon drying, 18 mL of MHA inoculated with 200 µL of MRSA 676 (grown overnight in BHI at 37 °C) was added to the plates aseptically. Once the dishes set, they were incubated overnight at 37 °C. A 2.5 mg/mL solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) was prepared and added to an aspiration bottle whereby it could be sprayed in a fine mist onto the bioautographic plates. These plates were then incubated for 1 h to allow for the dye to react and then visually examined for antimicrobial fractions. Inhibition zones were seen as a clear zone on a red background. All organic solvents were disposed of in correct 'flammable waste' or chlorinated waste' containers for safe disposal.

6.2.3 Specialised stain profiles of TLC separated crude water extract from F. vesiculosus

Several specialised stains were prepared for the visualisation and classification of the separated crude water extract from *F.vesiculosus,* according to Table 6.2. These stains were then sprayed onto developed TLC plates and allowed to dry before visual inspection. All organic solvents were disposed of in correct 'flammable waste' or chlorinated waste' containers for safe disposal.

Table 6.2: Stain formulations for the visualisation and characterisation of compounds in the water extract of *F. vesiculosus* separated using TLC (475). *stains most functional groups but does not distinguish between them.

Label	Stain	Compounds stained	Formula
Iodine	Iodine	Aromatic, unsaturated	Iodine crystals
CS	Cerium Sulphate	Alkaloids	10 % cerium(IV) sulphate, 15 % H_2SO_4
CAS	Ceric Ammonium Sulphate	Vinca alkaloids	1 % cerium(IV) ammonium sulphate, 50 % H_3PO_4
CA	σ-Phenylenediamine	Carboxylic acids	0.05 % p-phenylenediamine 10 % TFA + heat
PAS	p-Anisaldehyde	Terpenes, cineoles, withanolides, acronycine	0.9 % p-anisaldehyde, 0.09 % $HClO_4$, 18 % acetone, 72 % water
OAS	p-Anisaldehyde	Universal stain*	2.55 % p-anisaldehyde, 1 % glacial acetic acid, 3.44 % H_2SO_4 ,92 % EtOH
$KMnO_4$	Potassium permanganate	Antioxidant compounds	0.75 % $KMnO_4$, 0.0005 % K_2CO_3 in 10 % NaOH
FC	Ferric Chloride	Phenols	1 % ferric(III)chloride in 1:1 MeOH:H_2O
Nin	Ninhydrin	Amino acids	0.00015 % ninhydrin, 97 % n-butanol, 2.9 % acetic acid
Aromatic	Sodium fluorescein	Aromatic	0.05 % NaFl, 1:1 MeOH:H_2O
PMA	Phosphomolybdic acid	Universal stain*	10 % phosphomolybdic acid in EtOH
Van	Vanillin	Universal stain*	6 % vanillin in EtOH with 1 % H_2SO_4
BGS	Bromocresol Green	Carboxylic acids	0.04 % bromocresol green,
DPPH	2,2-Diphenyl-1-picrylhydrazyl	Antioxidant compounds	2.54 mM DPPH in MeOH

6.2.4 Purification by sequential solvent extraction

Sequential extraction was used on the water extract of *F. vesiculosus* to separate bioactive compound(s) in the crude extract from non-bioactive compound(s) into different fractions based on their different solubilities in solvents of varying polarity (methanol, acetonitrile and dichloromethane). 1g of dried crude water extract was agitated in their respective solvent at a 1:100 ratio for 1 h. The supernatant was then filtered by vacuum through a Buchner funnel using Whatman No. 1 filter paper (Whatman, Kent, UK.) from the precipitate and kept aside, while the precipitate was re-extracted in the chosen solvent for 15 min and filtered again. This was repeated one more time so that the insoluble segment of the sample was extracted three times in the chosen solvent.

Both the insoluble and soluble fractions were dried so that they could be dissolved in water and tested for antimicrobial activity against MRSA 676 using the well diffusion method described in section 3.3.2. Subsequent to well diffusion, the most promising fractions were separated using the method developed for normal phase TLC methods and visualised for activity using bioautography described in section 6.2.2. All organic solvents were disposed of in correct 'flammable waste' or chlorinated waste' containers for safe disposal.

6.2.5 Isolation of antimicrobial compound(s) by analytical and preparative high performance-liquid chromatography (HPLC)

The method for preparative HPLC was first developed on an analytical scale HPLC . An Agilent 1200 series HPLC, equipped with a binary pump and 1200 series G1316B SL temperature-controlled column oven, a micro vacuum degasser and a photodiode array (PDA) detector (Agilent Technologies, Cork, Ireland) were used and controlled using Chemstation software (Agilent Technologies, Cork, Ireland). HPLC method development was undertaken using a Phenomenex C_{18} column (250 x 4 mm dimensions with 5 µm particle size, 100 Å pore size) which was chosen due to it being the closest in dimensions and stationary phase to the C_{18} semi preparative column necessary for scale-up.

The water extract from *F. vesiculosus* was sequentially extracted using DCM. The insoluble components were then dried and re-dissolved in Milli-Q® water at a concentration of 1 mg/5 mL and filtered using 0.45 µm polytetrafluoroethylene (PTFE) filters (Chromafil® Xtra H-PTFE-45/25) which are compatible with aqueous samples. Mobile phases tested consisted of water and methanol as the sample was soluble in water and methanol up to a concentration of 40 % methanol. This was done to avoid the sample precipitating out of solution in the HPLC column. Once the method was developed subsequent to trying the conditions below in Table 6.3, the method was transferred to the semi-preparative HPLC system, a Varian Prostar 210 equipped with Prostar 210 pump (10 mL head), a 200 mL capacity loop and Prostar 325 UV/Vis detector (Agilent Technologies) controlled by Workstation software. The column used was a Phenomenex, Jupiter C_{18} column (250 x 10 mm dimensions with 10 µm particle size, 300 Å pore size). The method remained as described in Table 6.3, with the adjustment of a 7 mL/min flow rate and the introduction of 200 µL of a 100 mg/mL filtered sample. All organic solvents were disposed of in correct 'flammable waste' or chlorinated waste' containers for safe disposal.

Table 6.3: Chromatographic conditions used for the separation of DCM insoluble extracts of water extracts from *F. vesiculosus*

HPLC	Mobile phase (*v/v*)	Flow rate (mL/min)	Injection vol. (µL)
Agilent 1200*			
	40:60 MeOH:H_2O	1	10
	30:70 MeOH:H_2O	1	5
	30:70 MeOH:H_2O	1.5	5
	30:70 MeOH:H_2O	2	5
	10:90 MeOH:H_2O	1	5
	10:90 MeOH:H_2O	1.5	5
	10:90→40:60→10:90 MeOH: H_2O	1	5
	10:90→40:60→10:90 MeOH: H_2O	1	5
	5:95→20:80→40:60→5:95 MeOH: H_2O	1	5
	1:99→20:80→40:60→1:99 MeOH: H_2O	1	5
	1:99→20:80→40:60→1:99 MeOH: H_2O	1	10
Varian Prostar**			
	1:99→20:80→40:60→1:99 MeOH: H_2O (Utilised)	7	200

* Phenomenex C_{18} column (250 x 4 mm dimensions with 5 µm particle size, 100 Å pore size) **Phenomenex, Jupiter C_{18} column (250 x 10 mm dimensions with 10 µm particle size, 300 Å pore size).

6.2.6 Analysis of HPLC isolated fractions of water extract of *F. vesiculosus* using LC-ESI-MS

Subsequent to the isolation of a fraction using semi-preparative HPLC, analysis was performed on LC-ESI-MS using an Agilent 1200 series LC/MSD Trap XCT Ultra ion trap mass spectrometer (Agilent Technologies, Cork, Ireland). An Eclipse XDC-C_{18} column (Agilent 5 µm, 4.6 x 150 mm) was used to achieve separation at 25 °C with a mobile phase consisting of 95:5 *v/v* ACN:H_2O with 0.1 % *w/v* formic acid at 0.5 mL/min.. 5 µL of sample, prepared at a concentration of 1 mg/mL in Milli-Q® water and filtered through a 0.45 µm PTFE filter, was injected onto to the LC. ESI was used to generate positive ions and scans were taken at 200, 400 and 800 m/z to account for a range of possible molecular ions as the molecular weight of the compound is unknown. All organic solvents were disposed of in correct 'flammable waste' or chlorinated waste' containers for safe disposal.

6.2.7 Analysis of HPLC isolated fractions of water extract of *F. vesiculosus* using NMR

22 mg of semi purified extract from *F. vesiculosus* was dissolved in 1 mL of D_2O (>99.96 %, Sigma-Aldrich) and transferred into an NMR tube. The sample was then analysed using H^1NMR and C^{13}NMR spectroscopy with a Jeol ECX-400 MHz NMR system (Tokto, Japan). Tetramethylsilane (TMS) was not used as an internal standard in this procedure. H^1NMR was performed using 8, 50, 100 and 1000 scans and C^{13}NMR was performed using 1000 scans only. All organic solvents were disposed of in correct 'flammable waste' or chlorinated waste' containers for safe disposal.

6.2.8 Analysis of HPLC isolated fractions of water extract of *F. vesiculosus* using FTIR

5 mg of semi-pure extract from *F. vesiculosus* was ground in 50 mg of oven dried potassium bromide (KBr - Sigma-Aldrich). A disk of this mixture and a KBr only disk

was made using a hydraulic press (Atlas series autotouch automatic hydraulic press-Specac, USA).

FTIR analysis was performed on a Variant 600-IR FTIR (Agilent Technologies, Cork) using a total of 64 scans with a resolution of $4cm^{-1}$. A background scan of the KBr disk was taken and subtracted from the sample to negate the presence of IR absorbing gases in the air such as CO_2 and H_2O.

6.3 Results and discussion

6.3.1 Separation of the crude water extract from F. vesiculosus using reversed-phase TLC

Reversed-phase TLC was performed on the water extract of *F. vesiculosus* due to the polar nature of the sample. As the sample is 100 % soluble in water (polarity index = 10.2) (305), it should dissolve in polar solvents. Normal phase silica TLC plates can themselves be dissolved in aqueous solutions (476), and with this taken into consideration, reversed-phase TLC was performed so that a polar mobile phase could be used.

As a guide to which solvents systems should be chosen, a practical guide into TLC by Silicycle, a company which produces TLC plates, was referenced. It stated that in reversed-phase TLC, a solvent system of aqueous nature and buffers were typically used (477).

The first mobile phase selected was 100 % water. As the extract is water soluble, it was expected that the entire spot would solubilise and migrate from the baseline. However, this was not the case as can be seen in Figure 6.1, as the sample did not move from the baseline. This was then thought to be an issue relating to the pH of the mobile phase. As ionised compounds are always in solution, 0.01 % trifluroacetic acid was added to the 100 % water mobile phase to acidify it, giving unionised components in the extract access to H^+ ions, which may or may not protonate any weak bases the sample may contain, thus driving them into solution. As can also be seen in Figure 6.1, the extract was still yet to move from the baseline. With similar logic, 0.01 % triethylamine was added next to the 100 % water mobile phase with the intention of providing a proton sink (478) for weak acids that may be present in the sample. Again, this failed to encourage the sample to migrate from the baseline.

Mobile phases of decreasing polarity were then used including: 10:90 *v/v* MeOH:H_2O, 10:90 *v/v* MeOH:ACN, 25:75 *v/v* MeOH:ACN, 50:50 *v/v* ACN:MeOH and 90:10 *v/v* ACN:MeOH *v/v*, as can be seen in Figure 6.1. It was noted that none of these mobile phases succeeded in causing the sample to migrate from the baseline. However, a

decrease in polarity of the mobile phase caused a small number of broad unresolved bands are observed emerging from the sample.

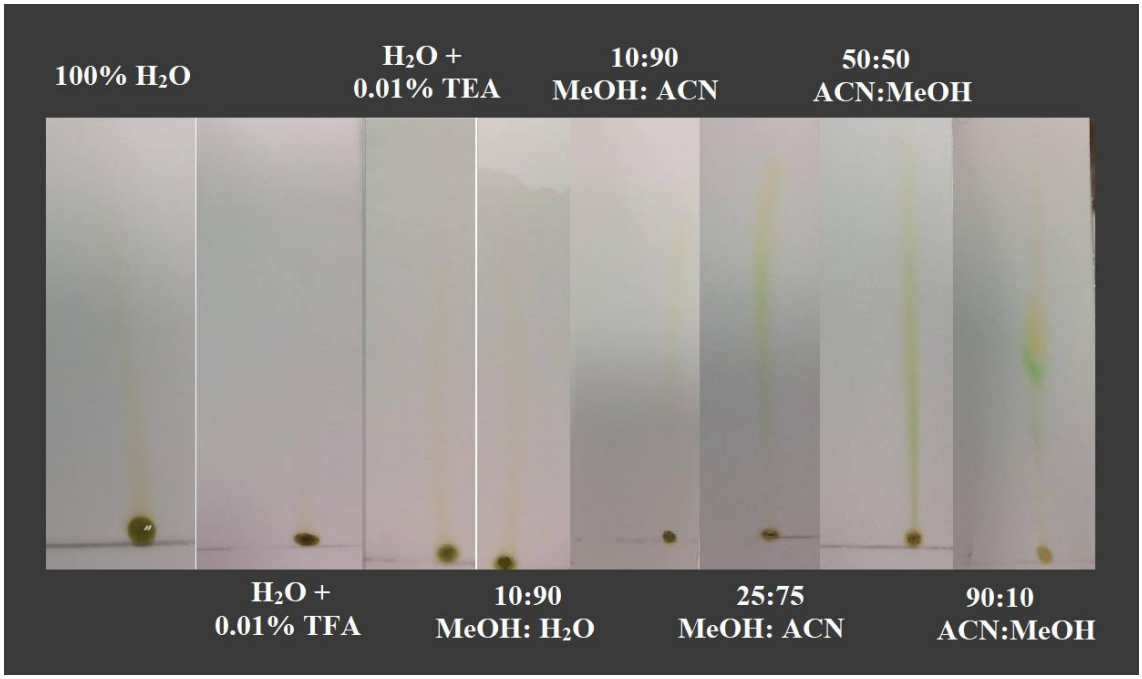

Figure 6.1: Reversed-phase TLC separations of the water extract from *F. vesiculosus* using different mobile phases. TFA = trifloroacetic acid, TEA = triethylamine. (n=3)

Owing to the inability of reversed phase TLC to satisfactorily separate the water sample from *F. vesiculosus* it was determined that normal phase TLC would be carried out.

6.3.2 Separation and bioautography of crude water extract from F. vesiculosus using normal phase TLC

For normal phase TLC non polar solvents are used, according to Silicycle, EtOAc:hexane *v/v* is a popular system. For polar compounds, it was recommended that solvent systems comprising of 100 % EtOAc or 5-10 % MeOH in DCM be used (477). Based on this a number of mobile phases for normal phase TLC were chosen which included; hexane, 1:1 *v/v* hexane:EtOAc, EtOAc, 1:1 *v/v* EtOAc:$CHCl_3$, $CHCl_3$, isopropyl alcohol, toluene, DCM and DCM with 5 % MeOH. The results of these runs can be seen in Figure 6.2. Although the baseline spot did not migrate, it was determined

that DCM with 5 % methanol was the most favourable mobile phase tested for the separation, as this mobile phase resulted in the most visible bands. While the exact reason of the failure of the baseline fraction to migrate is unknown, it could possibly be due to irreversible adsorption of solutes to the stationary phase (479). The possibility was noted in a previous study that a silica stationary phase could irreversibly adsorb nitrogen containing synthetic edible pigments in one 2019 study, resulting to the rejection of silica as a stationary phase for the separation (480).

Another explanation for the immobility of this fraction is that it was highly polar and, therefore, required a higher polarity solvent to encourage migration (477). However, a more polar mobile phase was not examined as a polar solvent will dissolve the stationary phase in normal phase TLC, resulting in poor chromatography which can be visualised in the IPA TLC plate is shown in Figure 6.2. As a similar phenomenon was observed using reversed-phase TLC for this sample, it was thought that the polarity of the mobile phase would not encourage the migration of this spot.

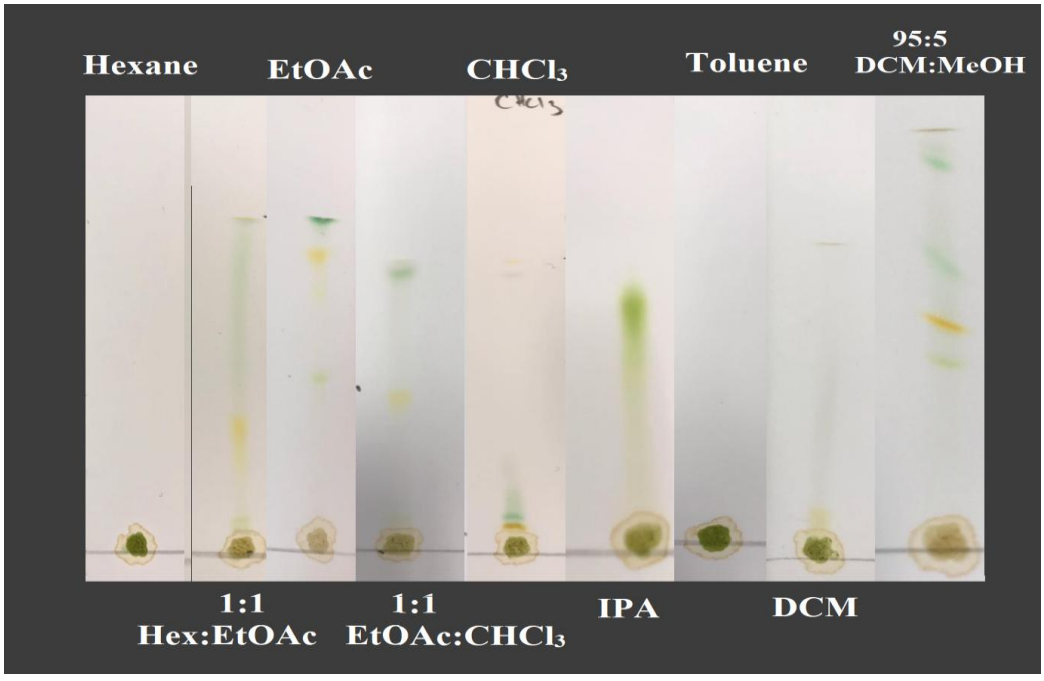

Figure 6.2: Normal phase TLC separations of the water extract from *F. vesiculosus* using different mobile phases. Hex = hexane, EtOAc = ethyl acetate, $CHCl_3$ = chloroform, IPA = isopropylalcohol, DCM = dichloromethane, MeOH = methanol. (n=3).

Bioautography was then run on 3 mg of the water extract from *F. vesiculosus* separated using 95:5 *v/v* DCM: methanol and then visualised using 1 mL of a 2.5 mg/mL solution of 2,3,5-triphenyltetrazolium chloride (TTC). Bioautography is a rapid technique for identifying antimicrobial activity in a sample based on the diffusion of fractions already separated using TLC into inoculated agar. After overnight incubation, an inhibition zone can be visualised with the aid of a stain like TTC. Bioautography has the advantage of being able to separate antagonistic compounds from one another, potentially uncovering further activity (113). Bioautography has successfully been used to screen for antimicrobial activity against *K. pneumoniae* from the plant *Justicia wynaadensis*. This study then isolated the active bands using preparative TLC for GC-MS analysis (480). As can be seen in Figure 6.3, antimicrobial activity was retained in the fraction at the baseline of the TLC plate. As this spot never migrated, compounds may not be separated from each other in this fraction.

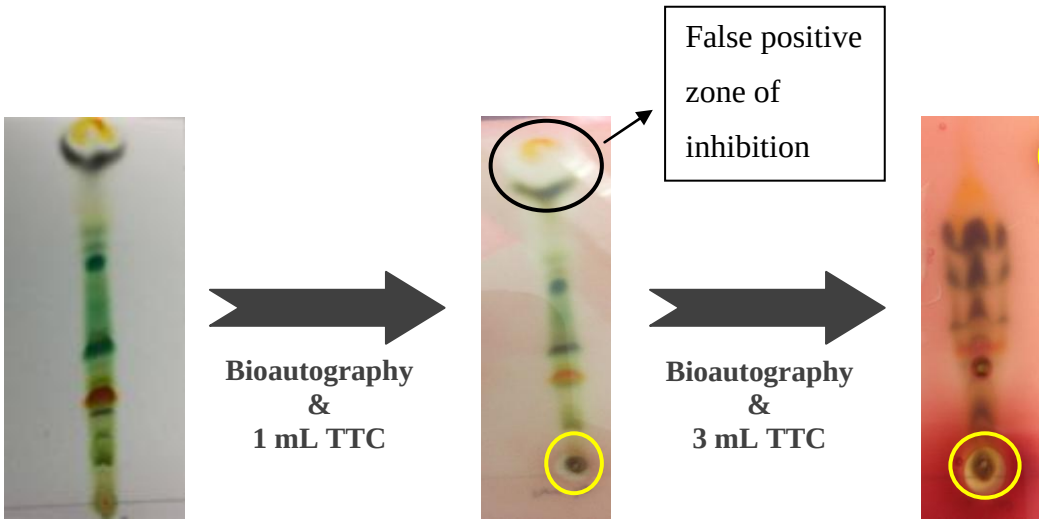

Figure 6.3: Bioautography of normal phase TLC of the water extract from *F. vesiuculosus*, with 95:5 *v/v* DCM:MeOH as mobile phase, against MRSA 676 dyed for visualisation using TTC.

The TLC separated extract was subsequently subjected to several specialised stains pictured in Figure 6.4. Aside from the general stains, one of the anisaldehyde stains (PAS, Figure 6.4) very clearly reacted with the antimicrobial active fraction of the

extract. This stain is specific for terpenes, cineoles, withanolides and acronycine. A search of the literature indicated that *F. vesiculosus* has not been reported to contain cineoles, withanolides or acronycine, which indicates that terpenes may be a prospective compound to be present in the baseline fraction. This is surprising, as terpenes are typically known for being non polar compounds (481).

Both the $KMnO_4$ and DPPH stains tested positive for antioxidant activity of the active fraction present on the baseline. This is significant as the mode of action for many antimicrobials, including iodine, revolves around proteins and DNA in bacteria being damaged due to oxidative stress (436). Many studies observed both antioxidant and antimicrobial activities in extracts from seaweed, such as Jiang *et al.* who reported these activities from petroleum ether and ethanol extracts of *Grateloupia livida* (482). Terpenes, which could potentially be present on the spot, as evidenced by the PAS stain, are also known to have antioxidant properties (483). However, as this spot is on the baseline of the TLC plate, it may contain more than one compound.

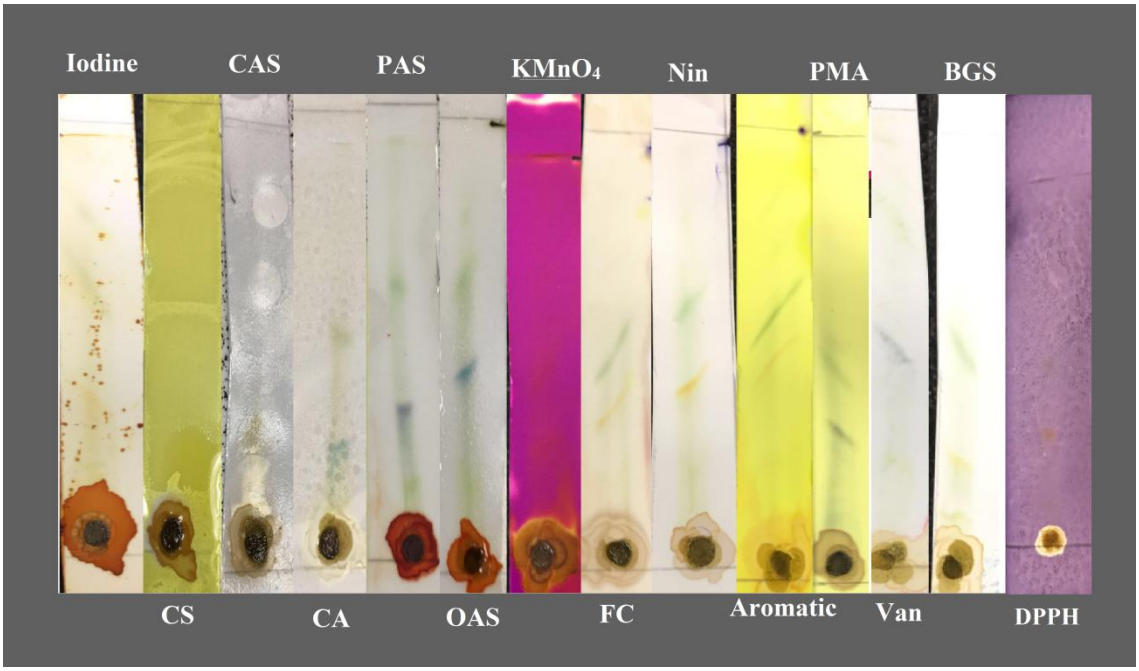

Figure 6.4: Stain formulations for the visualisation and characterisation of compounds from the water extract of *F. vesiculosus* separated using TLC (475).

As the area of activity resided in the fraction that would not migrate from the baseline of the TLC plate, the use of preparative TLC to purify this fraction was ruled out. It was reasoned that it would require a highly polar solvent to remove this band from the baseline, the use of which would also solvate the silica on the plate, causing contamination of the sample. Similarly, the results from both normal and reversed-phase TLC ruled out the use of flash chromatography as a technique for isolating the antimicrobial fraction, as it was thought that the fraction would follow the trend laid by TLC analysis and not move from the baseline.

The results of this experiment indicated the potential use of sequential solvent extraction followed by another method of purification such as semi preparative HPLC on this sample was required.

6.3.3 Purification by sequential solvent extraction

Due to the highly polar nature of the active band found using normal phase TLC, it was expected that purification using normal phase preparative chromatography would be ineffective as the active spot would not migrate from the baseline. The polarity of the active band also leads to the issue of separating active substances from the silica, as a polar solvent would dissolve the silica.

Due to the difficulty associated with TLC, other routes of purification were examined such as purification via the precipitation of inactive compounds in the autoclave, and solvent extraction. As the active compound was suspected to be polar due to its affinity for silica, two non polar solvents acetonitrile (ACN polarity index = 5.8) and dichloromethane (DCM, polarity index = 3.1 (484)) were chosen in a bid to 'wash' the extract of its less polar bands. Figure 6.5 shows a schematic diagram illustrating this extraction process. After extraction, both the filtrate and the precipitate were tested for activity against MRSA 676 using the well diffusion method as can be visualised in Figure 6.6.

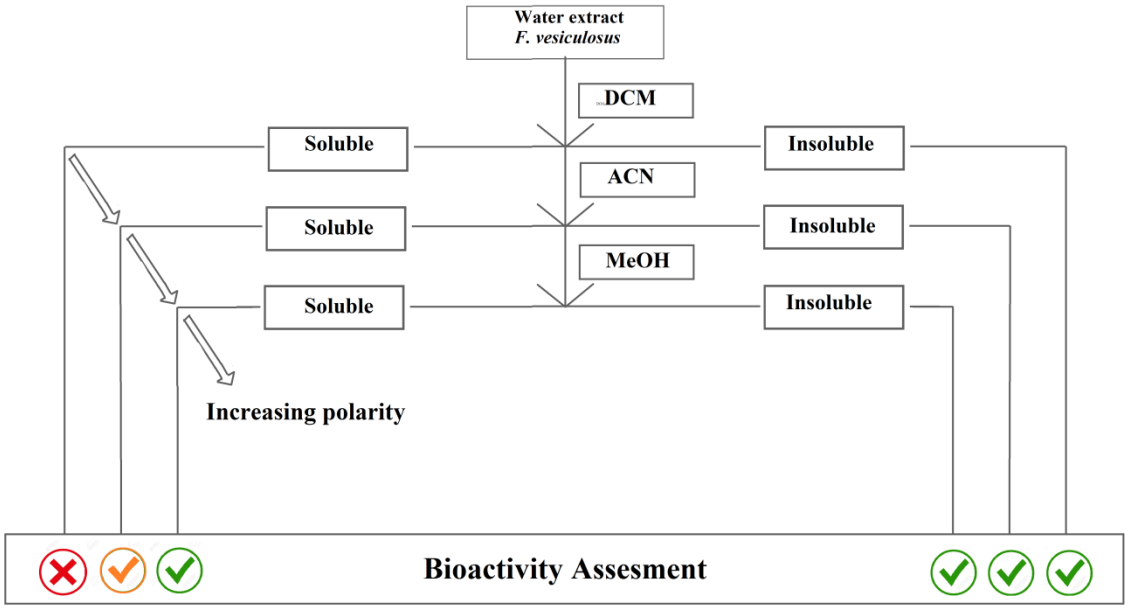

Figure 6.5: Schematic diagram depicting the sequential solvent extract of the water extract from *F. vesiculosus* using DCM, ACN and MeOH. Green tick indicates strong, , orange tick indicates weak and red 'x' indicates absence of antimicrobial activity against MRSA 676.

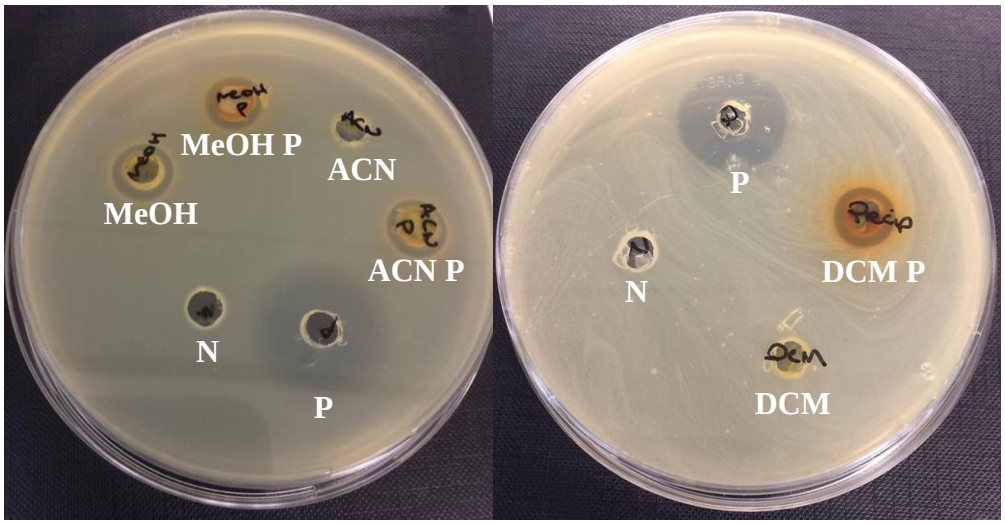

Figure 6.6: Well diffusion assay completed on solvent extracts of the water extract from *F. vesiculosus* against MRSA 676 (5 mg/well). MeOH = methanol soluble, MeOH P = methanol insoluble, ACN = acetonitrile soluble, ACN P = acetonitrile insoluble. DCM = dichloromethane soluble, DCM P = dichloromethane insoluble. P=positive control (10 µg chloramphenicol), N= negative control (sterile water).

The activity of both the soluble and insoluble methanol samples in Figure 6.6 indicates that methanol is not appropriate for sequential solvent extraction of the water extract from *F. vesiculosus*. This could be due to the polarity of methanol (polarity index of 5.1) being closer to that of water (polarity index of 10.2) than the other solvents (484). Figure 6.6 displays the activity of both the dichloromethane insoluble and acetonitrile insoluble samples along with the inactivity of their soluble counterparts. To further investigate the potential of using one or both of these solvents for sample clean up, normal phase TLC with the mobile phase of 95:5 *v/v* DCM:MeOH was used and followed with bioautography on the ACN, ACN P, DCM, and DCM P samples demonstrated in Figure 6.7.

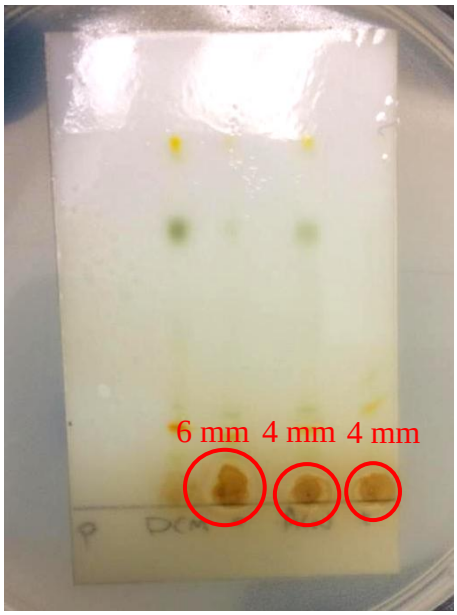

Figure 6.7: Bioautography of DCM, DCM P , ACN and ACN P samples using MRSA 676.

The results of the bioautography presented in Figure 6.7 illustrates that the acetonitrile insoluble compounds in the water extract of *F. vesiculosus* are slightly antimicrobial, which was contrary to the results of the initial well diffusion assay. This could be due to the TLC performed on the samples. As they are a mixture of compounds, activity

masking compounds may have been separated away from the antimicrobial band present on the baseline.

As such, DCM was selected as the solvent for sequential solvent extraction, as 92 % of the water extract from *F. vesiculosus* was insoluble in DCM but still antimicrobial, unlike the DCM soluble components. TLC comparing both the DCM soluble and insoluble samples can be seen in Figure 6.8, which shows the absence of the active, stationary band at the baseline of the plate.

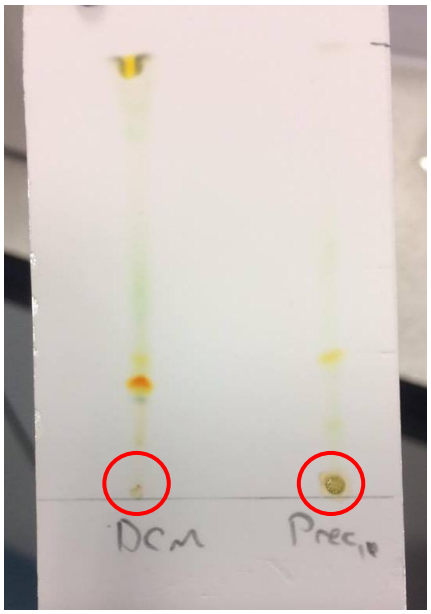

Figure 6.8: Normal phase TLC performed on DCM soluble and insoluble fractions of the water extract of *F. vesiculosus* using 95:5 *v/v* DCM:MeOH as mobile phase.

To ensure the suitability of this extract to be further purified using preparative HPLC, the DCM insoluble compounds were dissolved in water and filtered to ensure that the antimicrobial activity can survive the filtering process. The resulting solutions were then tested using well diffusion which is illustrated in Figure 6.9. As the sample retains activity subsequent to being filtered, it was determined that this extract was suitable for preperative HPLC, with the intention of isolating antimicrobial compounds.

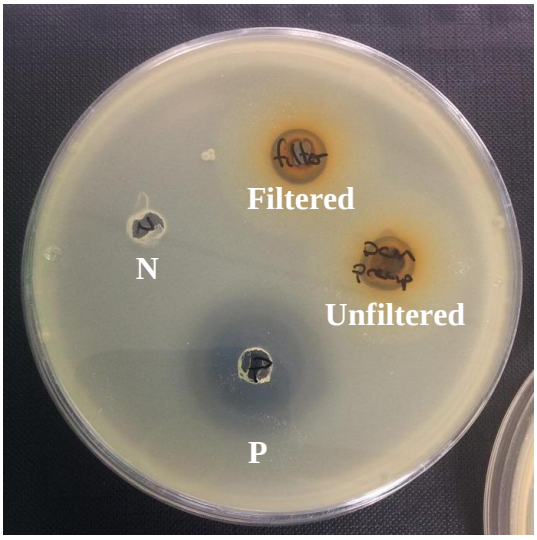

Figure 6.9: Antimicrobial activity of the DCM insoluble components in the water extract of *F. vesiculosus* samples unfiltered and filtered using a 0.45 µm PTFE syringe filter. P=positive control (10 µg chloramphenicol), N= negative control (sterile water).

6.3.4 Isolation of antimicrobial compound(s) by preparative high performance-liquid chromatography (HPLC)

The isolation of water soluble bioactive secondary metabolites from marine invertebrates was achieved using reversed-phase HPLC by Ebada *et. al.* (461). This study detailed the extraction of water extracts from invertebrates before sequential extraction using hexane, ethyl acetate and butanol, carried out on preparative HPLC. Similarly to this work, Ebada *et. al.* found that the most optimum mobile phase used for preparative HPLC analysis was a gradient method running from water to methanol for polar extracts (461).

The objective of first running the sample on an analytical column was to develop a method which could separate the greatest number of constituents in the sample in the lowest possible run time. A C_{18} column was chosen due to the literature reports using these stationary phases with the purification of extracts from seaweeds (485,486). As analytical HPLC requires less sample and mobile phase, developing a preliminary method using analytical HPLC was identified as a waste saving step. The method was developed based on the number of peaks that were separated in the sample. The developed gradient method of 1:99→20:80→40:60→1:99 *v/v* MeOH: H_2O separated 28

distinguishable peaks, which was higher than the other methods outlined in Table 6.3. The developed method which can be seen in Figure 6.10 was then transferred to semi preparative HPLC seen in Figure 6.11. Peaks were manually collected from the HPLC, in real time as they were detected and frozen as soon as possible to minimise sample degradation.

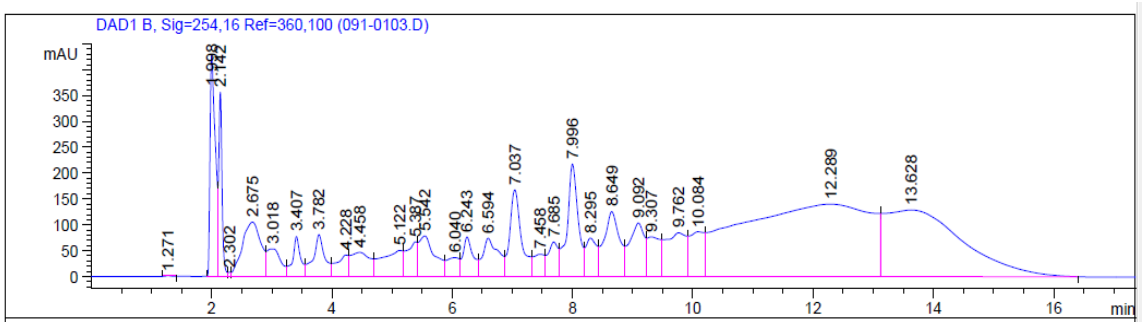

Figure 6.10: Developed gradient method on analytical HPLC for the crude water extract from *F. vesiculosus* using a C_{18} column with the gradient mobile phase 1:99→20:80→40:60→1:99 *v/v* MeOH: H_2O.

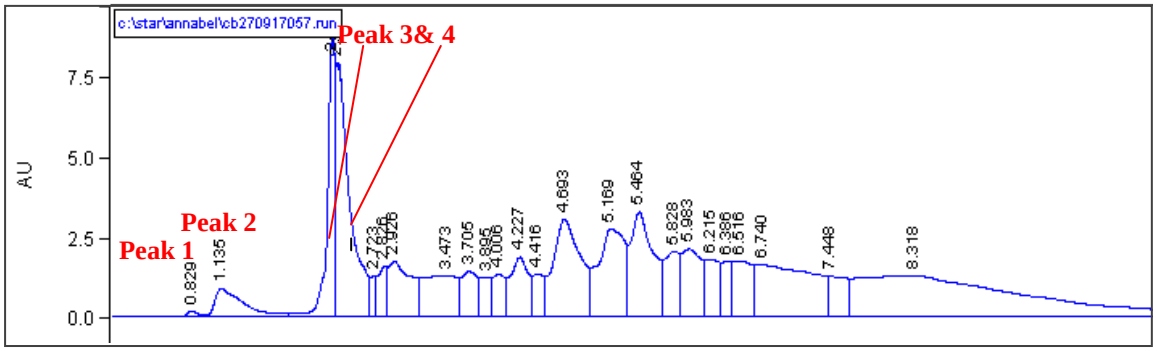

Figure 6.11: Method transfer to semi preparative HPLC for the crude water extract from F. vesiculosus using a C18 column with the gradient mobile phase 1:99→20:80→40:60→1:99 *v/v*, MeOH: H_2O.

The isolated fractions were dried and reconstituted in sterile water for the well diffusion assay against MRSA 676. This step was was used to check which peak(s) retained antimicrobial activity and, therefore, demonstrated which peak(s) should be further

isolated and characterised. As is presented in Figure 6.12, peaks 2 and 4 displayed antimicrobial activity, with peak 2 showing greater inhibition at the same concentration (25 mg/mL) when comapred to peak 4. Having uncovered the antimicrobial action of more than one extracted peak, it was speculated that the observed antimicrobial activity in the crude water exract of *F. vesiculosus* could potentially be due to more than one compound of interest, whih has been observed in previous studies (486–489). Five different bromophenols were structurally elucidated from methanol extracts of *Rhodomela confervoides* sequentially extracted using ethyl acetate, all of which were antimicrobial against *S. aureus* (486). An acetone extract of *Astragalus brachystachys* contained four different antimicrobial diterpenes which were active against *B. subtilis* (487). Antimicrobial activity against *Vibrio parahaemolyticus* by chloroform:methanol *v/v* extracts of *Ulva fasciata* was demonstrated to be due to two diffferent guaiane sesquiterpene structures (488). Similarly, a study in the Indian Peninsula also found extrats of *Ulva fasciata* to be antimicrobial against *Vibrio parahaemolyticus*. This study however attributed the bioactivity to two labdane diterpernoids (489).

This is particularly apparent in this study whereby, more than one peak from semi preparative HPLC was antimicrobial against MRSA 676. It would be expected that 5 mg of purfied antimicrobial would result in a much greater inhibition zone than that observed in Figure 6.12. However, if the activity is due to more than one antimicrobial compound in the crude extract as observed here, the purified extract may not produce a greater zone of inhibtion. It is also worth noting that peak 4 was not resolved in the chromatography and most likely contained impurities. As a result, peak 2 became the focus for the remainder of the study.

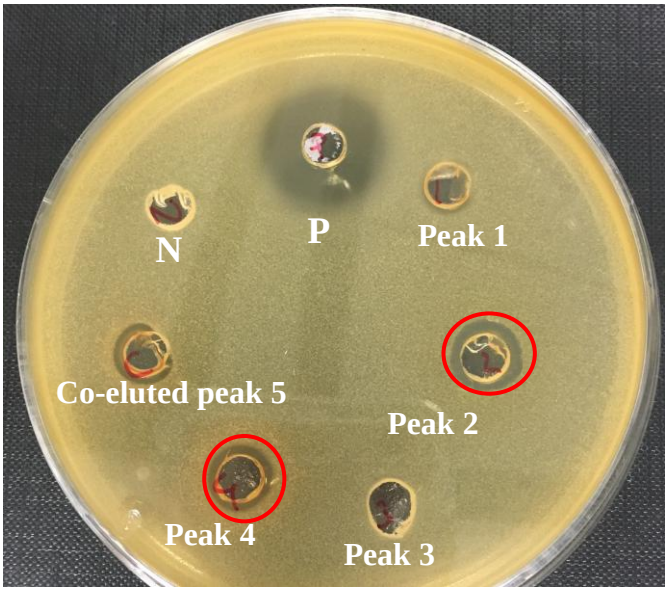

Figure 6.12: Antimicrobial testing of peaks from semi preparitve HPLC using the well diffusion method against MRSA 676. P=positive control (10 µg chloramphenicol), N= negative control (sterile water).

The compound(s) responsible for the antimicrobial activity in peak 2 were thought to be highly polar due to several reasons; the initial extract was water based, the compound(s) are insoluble in DCM and peak 2 elutes from the HPLC with a mobile phase of between 7-9 % MeOH in water at 1.35 min.

Having reviewed the bioactivity of peak 2, the analytical spectra from the HPLC was re-evaluated using the PDA detector, which are illustrated in Figure 6.13. The PDA detector was used to scan the sample accross the UV-Vis spectrum and subsequently compare the spectra to the spectra at the same retention time, over a range of wavelengths. Conflicting spectra will indicate the presence of any impurities present in the sample.

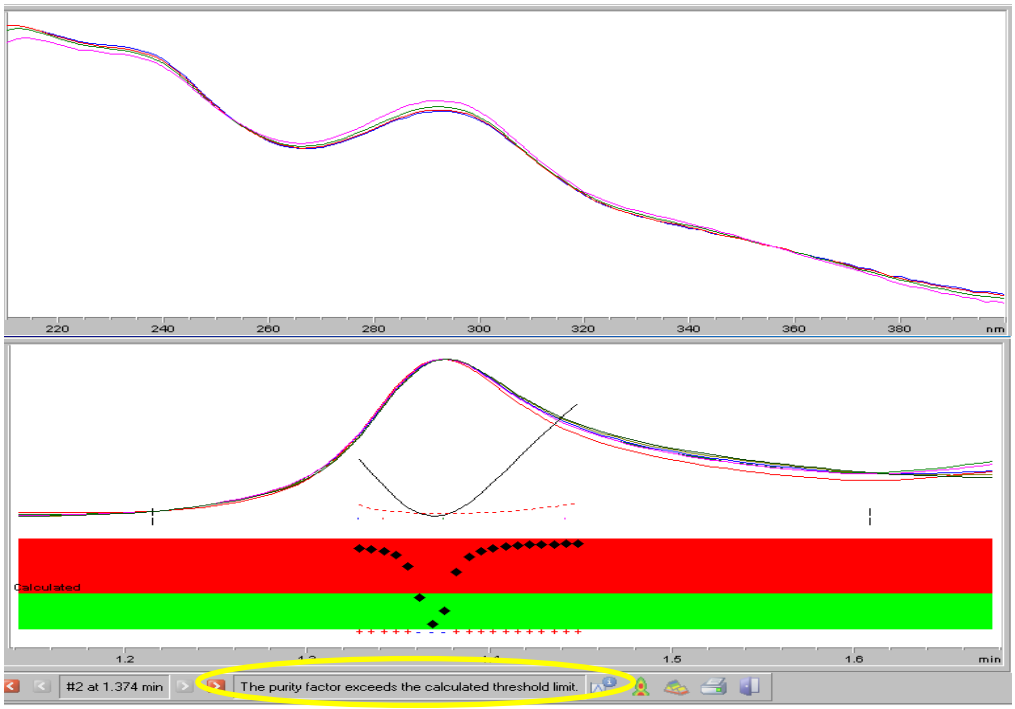

Figure 6.13: Photo diode array peak purity spectra from peak 2 depicting an impure sample, due to the puritiy factor exceeding the softwares threshold.

As shown in Figure 6.13, the UV-Vis spectra exceed the instruments purity factor threshold limit for peak 2 at 1.35 min meaning that the absorbtion spectra for the sample varied over a range of frequencies indicating that there were impurities in the sample. The λ_{max} for the sample was noted to be 285 nm.

As peaks 3 and 4 in Figure 6.11 are poorly resolved, the photo diode array detector data for both was analysed for purity and for their λ_{max} (Figure 6.14). Both the compounds responsible for these peask do absorb at 285 nm which was the λ_{max} of peak 2, however, neither of these peaks passed the threshold for the PDA detectors' peak purity, meaning that the co-eultion of two or more peaks were present in these fractions. The spectra for peak 3 closely resembles that of peak 2 where a λ_{max} of 285 nm is observed followed by an upward meandering peak around 200 nm. This information could imply that the main absorbing compounds present in both impure fractions could be related to each other via isomerisation or could contain the same chromophores, however, further analysis would be required to confirm these suggestions.

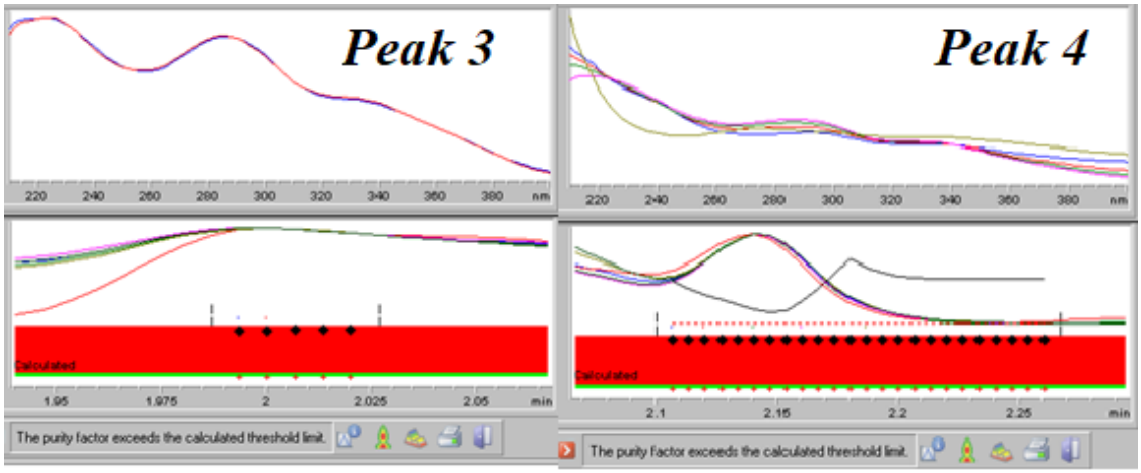

Figure 6.14: Photo diode array peak purity spectra from peak 3 and 4 depicting impure samples, due to the purity factor exceeding the softwares threshold.

6.3.5 Analysis of HPLC fractions of water extract of F. vesiculosus using FTIR, LC-ESI-MS and UV-Vis analysis

Following on from PDA data, analysis with a UV-Vis spectrophotometer for the HPLC isolated compound was undertaken to find the λ_{max} of the HPLC isolated extract. UV-Vis analysis cannot uncover the full structure of an unknown compound, however, the wavelength(s) that the compounds(s) absorb at can provide certain information about the types of bonds present or functional groups in the structure (490). The Woodward-Fieser rule predicts the wavelength of the $\pi \rightarrow \pi^*$ transition that occurs in unsaturated carbon compounds (491).

Figure 6.15 shows the UV-Vis spectrum of the HPLC isolated extract with a λ_{max} at 285 nm. There is a smaller peak at 564.1 nm and a shoulder on the peak corresponding to the λ_{max} at 341.1 nm. The peak at 564.1 nm has an absorbance of 0.003, indicating that the chromophore does not absorb strongly at this wavelength or that the compound responsible is perhaps an impurity present at low concentrations. The shoulder at 341.1 nm would suggest a highly conjugated system or the presence of substituent's which contain π electrons such as oxygen, nitrogen, sulphur or a halogen (120) as these atoms absorb light at lower energy wavelengths when compared to non-substituted alkanes.

Isoprene units (which form terpenes and terpenoids) absorb from 210 - 240 nm (492). However, the ample conjugation that occurs with the presence of greater numbers of isoprene units will drive the λ max to absorb light at higher wavelengths (492). For example, fucoxanthin, a terpene present in *F. vesiculosus* has a λ_{max} of 449 nm. Whereas, fucoidan presents as a broad peak around 250 nm (493) which is observed in the spectra. Highly conjugated organic compounds typically absorb light in the visible region, as such, it was speculated that the HPLC isolated extract was not highly conjugated as it presented as a white powder. The potential presence of isoprene units is interesting as the specialised stains performed as part of this chapter indicated the presence of terpenes/terpenoids in the active fraction of the extract. As isolated double bonds typically absorb under 200 nm (492). It is unknown if this functionality exists within the structure, owing to the downward limit of 200 nm.

n- π* transitions that typically occur in carbonyls under the influence of UV-Vis light generally occur at a wavelength of 270 - 300 nm for saturated carbonyls and 300 - 350 nm in conjugated systems (120). The inclusion of such a peak indicates that carbonyl containing compound(s) may be present in this compound. The addition of alcohol or halogen groups to a structure can act to lower the energy difference in the n→π*, resulting in the adsorption at shorter wavelengths of light (120), which opens the possibility for alkyl halide and alcohol functionality.

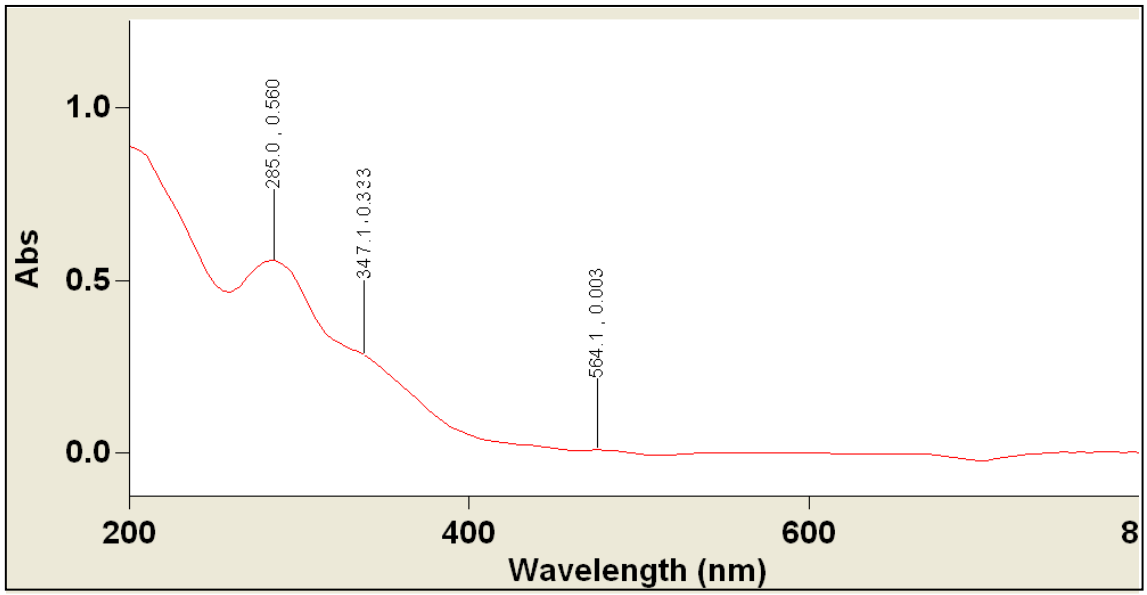

Figure 6.15: UV-Vis spectrum of peak 2 of the semi-purified extract dissolved in water and using water to blank the instrument before use.

Also noted in the UV-Vis data generated, fucoidan is noted to have a broad peak around 250 nm in the literature (493). The UV-Vis spectra generated for this sample does indeed include a broad peak around 250 nm, however, it also has a shoulder with a λ max of 285 nm. Water soluble polysaccharides like fucoidan, have been noted in the literature to have a similar UV-Vis profile as the one generated from this extract, examples of which can be observed in Figure 6.16. FTIR was performed on the solid sample with the use of KBr disks. The resulting spectra is can be seen in Figure 6.17.

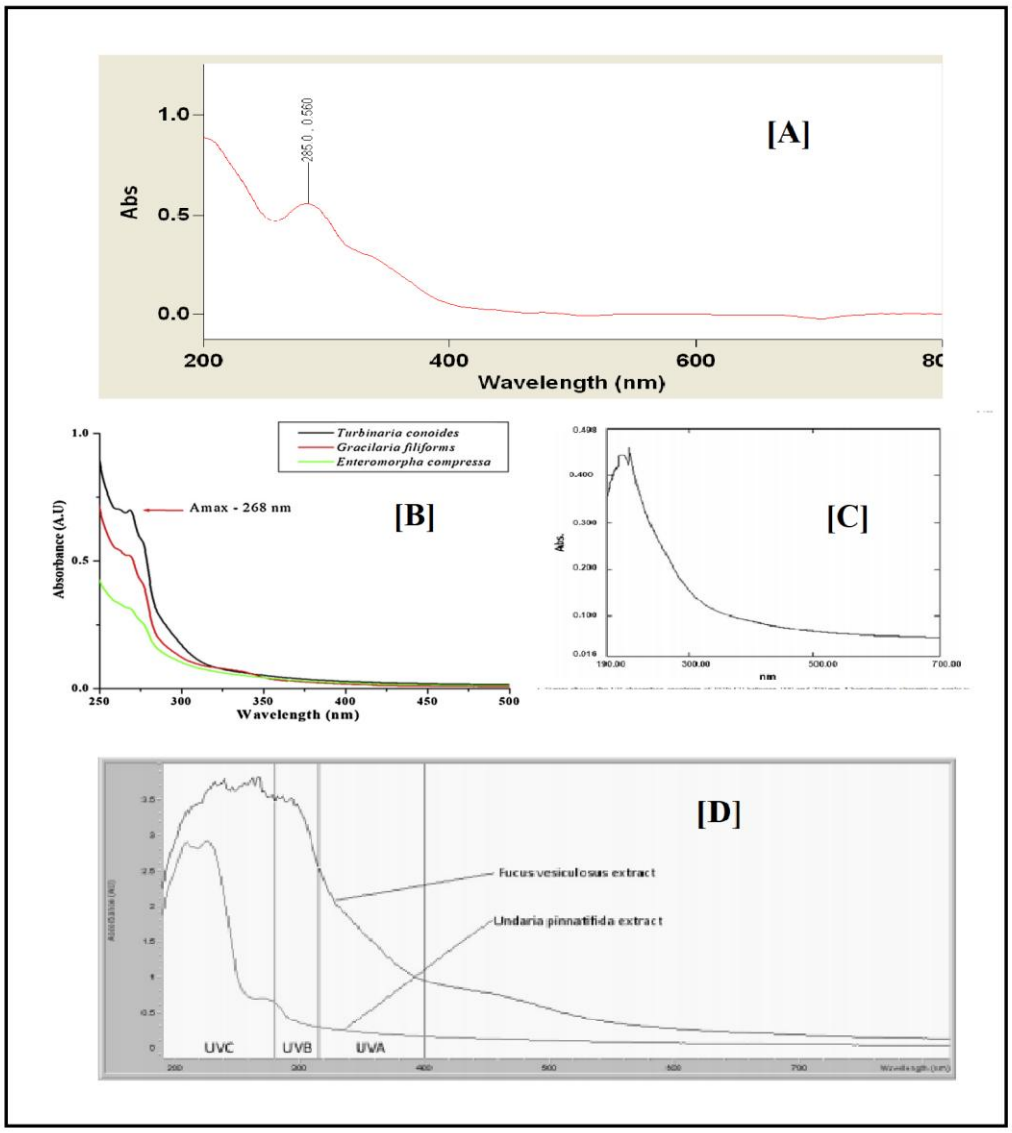

Figure 6.16: UV-Vis spectra generated from; [A] this work, [B] fractionated sulphated polysaccharides, λ_{max} = 268 nm (494), [C] column purified sulphated polysaccharides, λ_{max} = 215 nm (495) and [D] fucoidan from *F. vesiculosus*, λ_{max} fucoidan from *Undaria pinnatifida* = 273 nm (496).

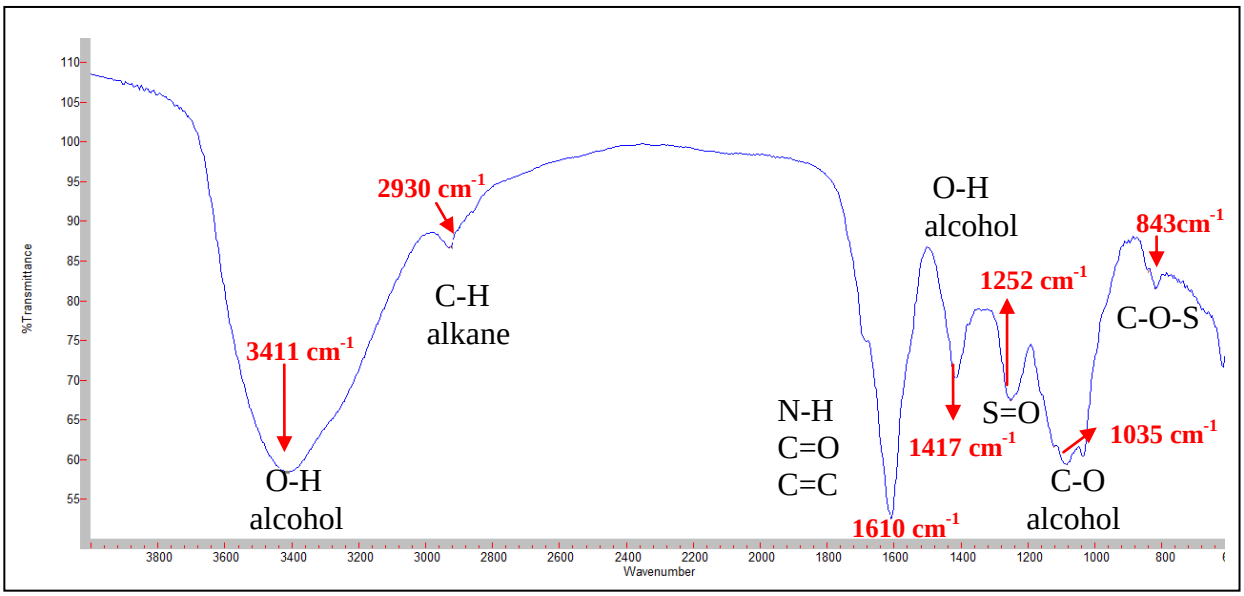

Figure 6.17: FTIR analysis of of peak 2 of the semi-purified extract dissolved in water and using water to blank the instrument before use.

There was a large peak at 3411 cm^{-1} corresponding to an OH stretch, this could be due to O-H groups or water present in the molecule. A C-H stretch can also be observed at 2930 cm^{-1}, indicating the presence of unsaturated hydrocarbons in the structure. A strong signal was observed at 1610 cm^{-1}, it was speculated that this stretch could correspond to a carbonyl, low wavenumber carbonyl stretches typically correspond to carboxylate ions (497). However, this stretch could also correspond to a C=C or amine N-H stretch within the molecule. A signal at 1417cm^{-1} could correspond to an O-H bend which would correlate with the signal at 3441 cm^{-1}. The signal at 1252 and 1035 cm^{-1} was thought to be due to a S=O asymmetric stretching bands associated with C-O-SO_3 (498).

Owing to the presence of an S=O bond in the FTIR spectra, a literature search of the IR spectra for water soluble sulphated molecules from *F. vesiculosus* was undertaken. It was found that many fucan based, water soluble sulphated polysaccharides (such as fucoidan) found in brown seaweeds such as *F. vesiculosus* share a very similar FTIR spectrum, as can be seen in Figure 6.19.

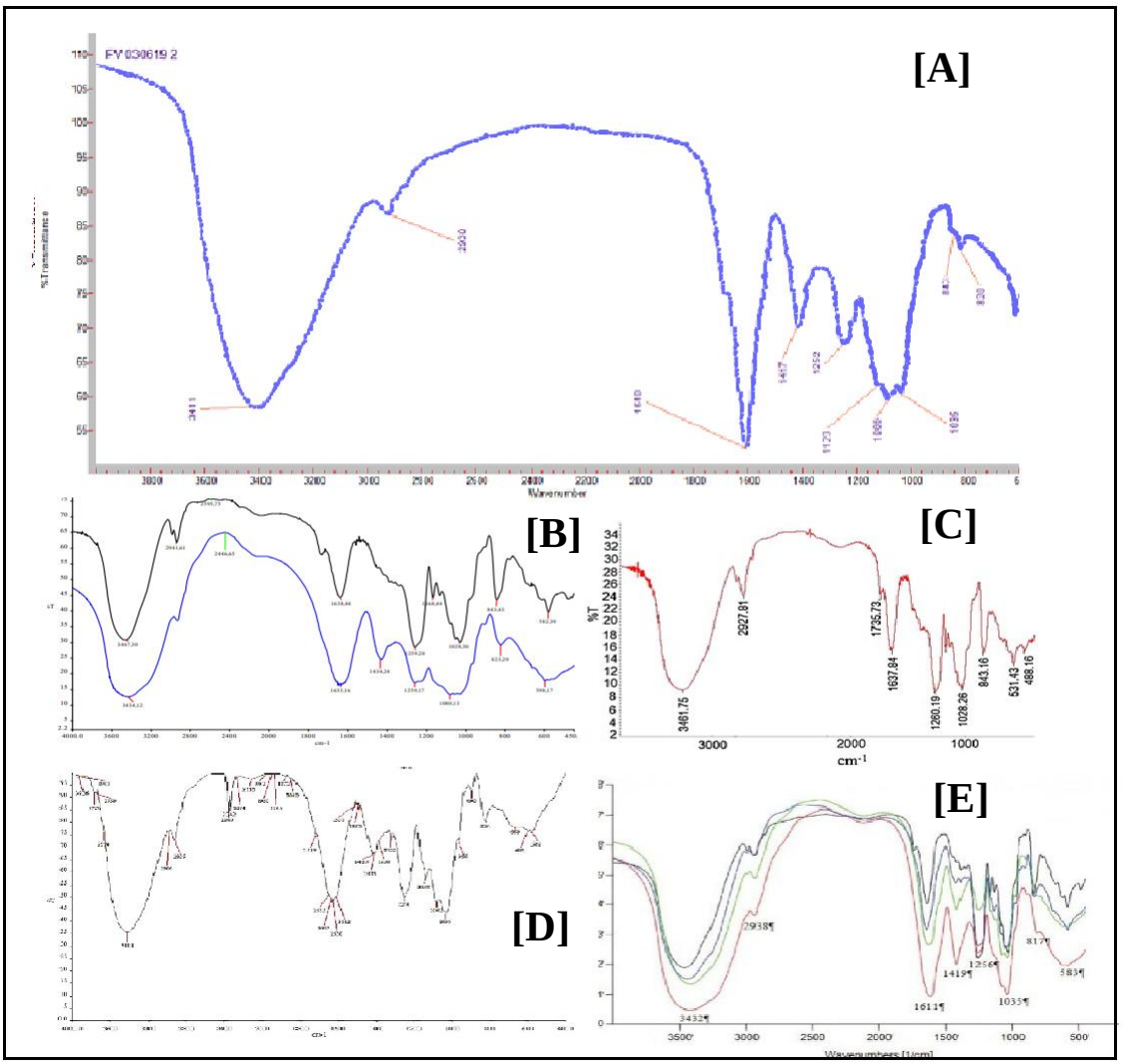

Figure 6.18: FTIR spectra for the sulphated polysaccharide fucoidan. [A] peak 2 of the water extract from *F. vesiculosus*, [B] (499), [C] (500) and [D] (326).

Owing to the extreme simularity between these spectra, it was concluded that a sulphated polysaccharide such as fucoidan, may be responsible for antimicrobial activity in the extract generated as part of this work.

As the sample is water soluble, GC-MS is not an appropriate method for the analysis and characterisation of the HPLC isolated fraction. As such, LC-MS was used on the sample. The LC-ESI-MS spectra displayed in Figure 6.18 shows a peak at 2.9 min. The ion at *m/z* 189 appears to be an adduct of *m/z* 223 which would correspond to a M+CH_3OH+H adduct. None of the more common signals such as M+Na or M+K appeared on the chromatogram. As such, there is some evidence that 190 Da could

potentially be the molecular mass of the compound in question. However it is unlikely owing to no other notable similarities. These ions could be both due to the fragmentation of a larger molecule, as such the results from LC-MS were inconclusive. If the antimicrobial activity from the sample is a large sulphated polysacharide such as fucoidan, it could be expected that excessive fragmentation would occur even with soft ionisation techniques. Considering this and the data generated via FTIR, a comparison between the literature spectra of fucoidan was made to this sample detailed in Figure 6.20.

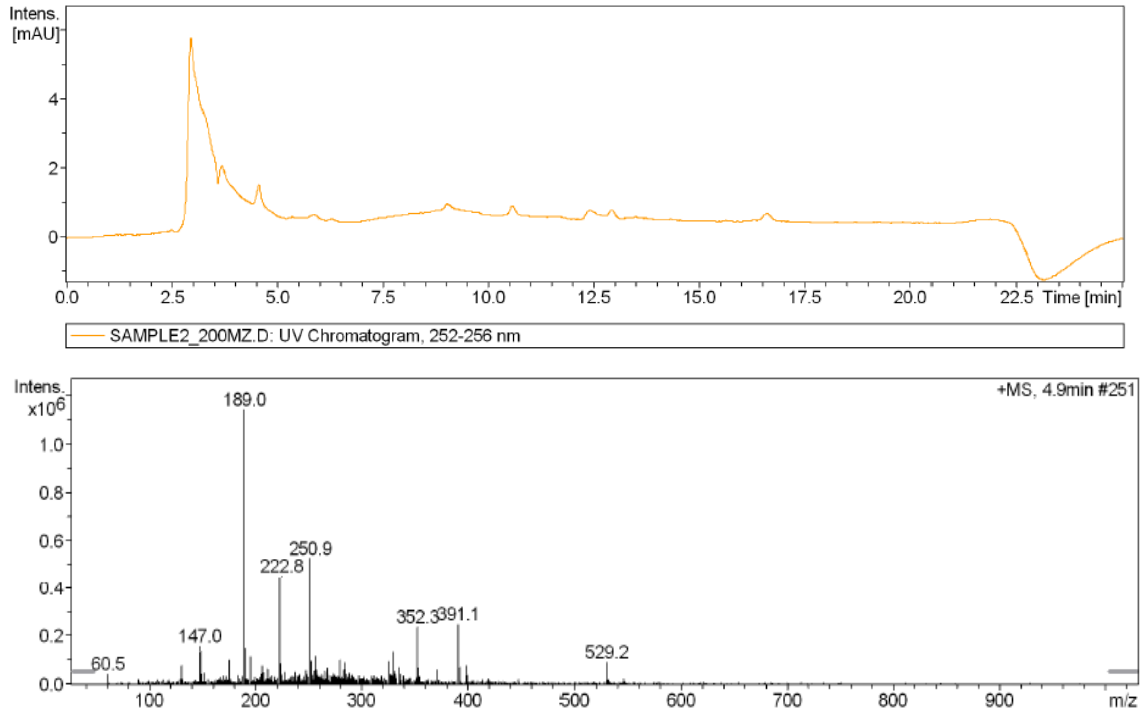

Figure 6.19: LC-ESI-MS data for HPLC isolated fraction of water extract from *F. vesiculosus*

A study investigating the seasonal variation of fucoidan in three brown seaweeds; *F. vesiculosus, F. serratus and A. nodosum* were tested using LC-MS (501). This spectra was compared to that of the extract generated as part of this study. While it was noted that there were many different ions present on this spectra, an ion at *m/z* 147 presented on both spectra.

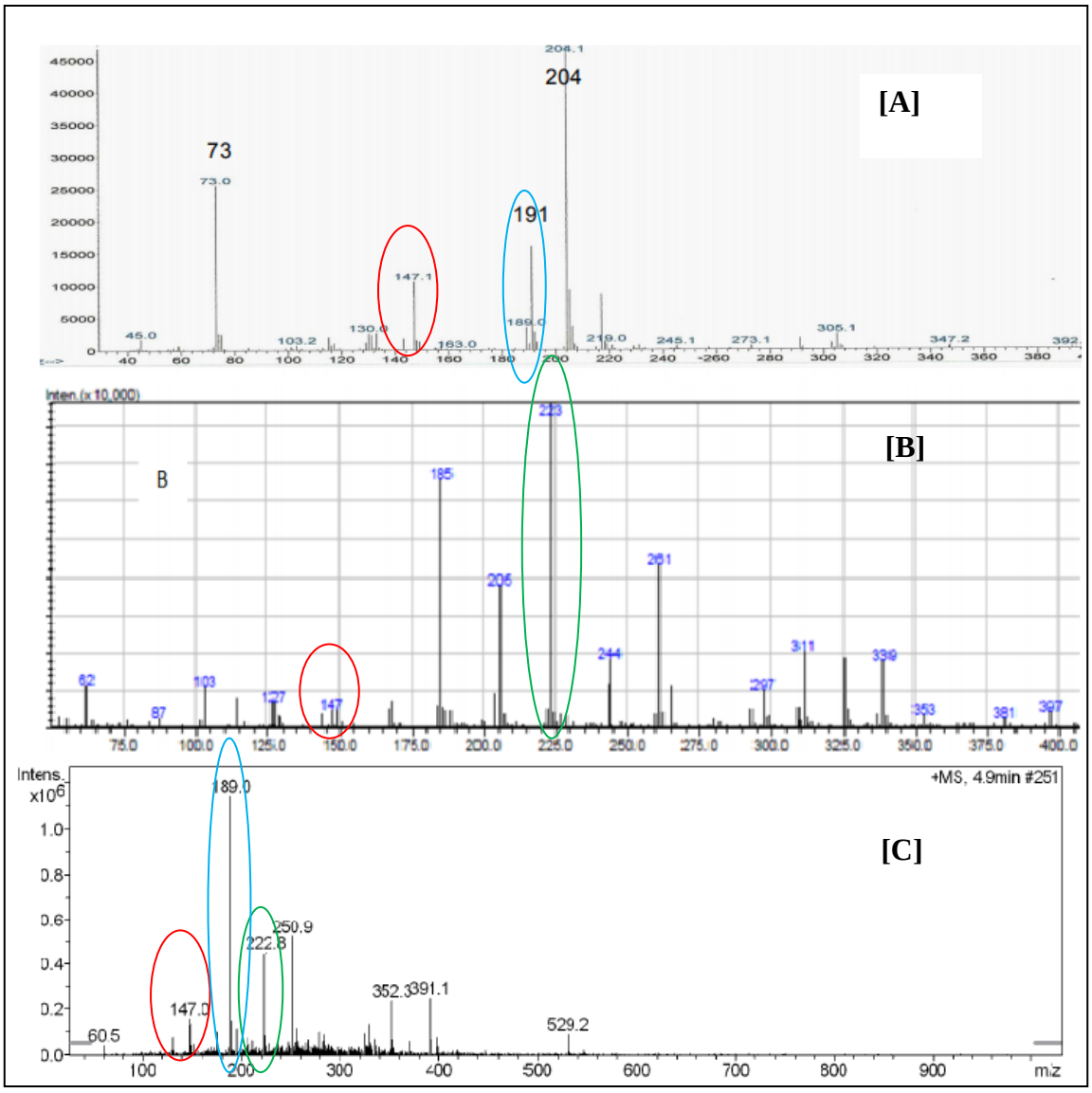

Figure 6.20: LC-MS data for fucoidan from [A] *F. vesiculosus* (501), *[B] D. polypodioides* (502) and [C] peak two semi purified extract from F. *vesiculosus* showing ions at *m/z* 147, 223 and 189.

As with FTIR analysis, this was taken as a suggestion of the presence of fucoidan in the sample. However, further analysis would be required to definitively confirm the identity of the sample.

Moreover, fucoidan is water soluble and has been noted to have antimicrobial activity (311,334,493), prevent and disrupt biofilm formation (312), and be cytotoxic to cancer cells (326,330,372). The overlap in literature data for bioactivity and structural characteristics for fucoidan with the data generated as part of this study suggests that fucoidan is a bioactive compound present in the water extract from *F. vesiculosus.*

6.4 Conclusions

The water extract from *F. vesiculosus* was separated using normal and reverse phase TLC analysis. In both instances, the active fraction did not migrate from the baseline of the plate. This contributed to the reasoning that flash column and preparative TLC would be inappropriate methods for sample purification. Subsequently, sequential solvent extraction was chosen as an initial purification step. It was found that by using DCM as a secondary extraction on the water extract of *F. vesiculosus*, antimicrobial activity was retained in the DCM insoluble constitutes of the sample and not in the DCM soluble components. This resulted in an 8 % loss in mass due to non-antimicrobial compounds.

Further purification was undertaken using a semi-preparitive HPLC system. A gradient method of 1:99→20:80→40:60→1:99 *v/v* MeOH: H_2O was developed over a 15 min run time. This method was sucessfully transfered onto a semi- preparative system whereby peaks were isolated into different fractions. Peaks 2 and 4 displayed antimicrobial activity when reconstituted in water and tested against MRSA 676 using the well diffusion method. As peak 2 displayed the greater activity of the two, this fraction was elected to go on to further characterisation and analysis. The PDAD analysis indicated that peak two contains impurities, and therefore would be unsuitable for analysis. The results from the NMR were inconclusive however, as some ions were common between the HPLC isolated fraction and literature spectra for fucoidan, it was thought that this data leanded some support for the presence of fucoidan.

The results for FTIR analysis gave a promising indication that the antimicrobial present in the extract was a sulphated polysaccaharide such as fucoidan. LC-ESI-MS data gave further indication of the presence of fucoidan in the sample as a number of ions coincided with ions noted in the literature. However, owing to the non-specific nature of FTIR, further analyis would have to be carried out to confirm this concept. Similarly, the UV-Vis data for peak two of the semi-purified extract from *F. vesiculosus* had a general profile displayed by fucoian in the literature, however, no spectra were exactly alike. This evidence for the presence of fucoidan as a bioactive in the water extract of *F.vesiculosus* is further bolstered by the bioactivity that fuciodan has displayed in the literature. As fucoidan has been noted to be antimicrobial, anti-biofilm and cytotoxic, all of which allign with the results of this work, giving further indication of fucoidan.

Chapter 7: Conclusions & Future Work

7.1 Conclusions

To investigate the antimicrobial activity of two different seaweeds, *Fucus serratus* and *Fucus vesiculosus* were extracted via solvent extract using the solvents; acetone, ethyl acetate, methanol and water at a 1:100 *w/v* ratio. A broad range of antimicrobial activity was demonstrated by all extracts, indicating the presence of antimicrobial compounds in these seaweeds. Aqueous extracts from *F. vesiculosus* in particular showed potential in terms of its inhibition of the growth of various strains of pathogens, and the extent of its antimicrobial activity against clinically isolated MRSA strains obtained via collaboration with University Hospital Waterford was determined. Due to the demonstrated antimicrobial activity, the aqueous extract generated from *F. vesiculosus* was pursued in further studies. A concentration of 3.125 mg/mL of the aqueous extract from *F. vesiculosus* was determined to be the MIC_{80} required to inhibit the growth of MRSA676, a clinically isolated strain of bacteria and was used as an indicator for this analysis. The MBC of the extract was also carried out; a concentration of 25 mg/mL of the aqueous extract from *F. vesiculosus* was determined to have a bactericidal effect against MRSA 676.

The aqueous extract generated from *F. vesiculosus,* underwent antibiofilm and cytotoxicity analysis. This extract disrupted biofilms formed by MRSA 676 at a concentration of 25 mg/mL and prevented the formation of the biofilm at 6.125 mg/mL. This result supported the use of the extract as part of a wound dressing formulation, as biofilms have been confirmed as a major hindrance for the management of wound infections. However, the crude aqueous extract from *F. vesiculosus* demonstrated cytotoxicity using the MTT assay against THP-1, HaCaT and HepG2 cells at a concentration of 0.3906, 0.1953 and 0.195 mg/mL respectively. As these concentrations are lower than the MIC (0 3.925 mg/mL) of the extract, it was thought that the crude extract may not be suitable for a topical formulation. As it was a crude extract tested for cytotoxicity, it was speculated that the antimicrobial and cytotoxic components of this extract could be different and, therefore, could be separated from each other on purification of the extract. Also, the extract could be solely cytotoxic to cancerous cells. A search of the literature concluded that there are compounds (such as fucoidan) found in the water extract of *F. vesiculosus* that are anti-cancer, and therefore are toxic to fast growing cells.

Microwave assisted extraction was used to develop the method of extraction for the water extract of *F. vesiculosus.* This established that the microwave did not affect the antimicrobial activity of the extract and, as such, the method development was carried out to improve the yields generated from the dry mass of seaweed, and also to make the method of extraction shorter in duration, and therefore, more cost effective. The yields generated using developed microwave conditions resulted in a 224 % higher yield than that of a 2 h solvent extraction in 89 % less time.

A preliminary study comparing inert dressings incorporating aqueous extracts from *F. vesiculosus* were tested for antimicrobial activity and compared to three commercially available antimicrobial wound dressings; Inadine (iodine), Algivon (honey) and Aquacel (silver). It was found that all four dressings preformed statistically the same in the first hour of this study. However, only the seaweed incorporated dressings retained activity for the duration of the assay (72 h) and this was second only to the silver based wound dressing which lost activity after 6 h during this analysis. This was treated as an in indication that the water extract from *F. vesiculosus* could be an appropriate antimicrobial in a wound dressing formulation, which could be significant in terms of wound management due to the established antimicrobial and anti-biofilm nature of the extract.

Several wound dressing formulations were incorporated with the water extract from *F. vesiculosus* and tested for antimicrobial activity. A hydrogel formulation was prepared but was found to not exhibit any antimicrobial activity beyond that of the negative controls. Antimicrobial film wound dressings based on alginate were also formulated; however, they had poor structural ability to retain their intended shape and the antimicrobial activity of these dressings were poor. Solid, gauze like alginate dressings were prepared using M.M.W. chitosan and L.M.W. chitosan in a 1 % acetic acid solution. These dressings showed promise in terms of antimicrobial activity, but lacked flexibility and retained a strong smell from the acetic acid which was used in the formulation. Due to these reasons these dressings were developed with the use of propylene glycol as a plasticiser and lactic acid in the place of acetic acid as a biocompatible proton donor for the solubilisation of chitosan. These wound dressings displayed antimicrobial activity over a 72 h period.

The concept of producing an antimicrobial dressing for burn wounds which can remain stable at varying environmental temperatures, such as those in third world countries which may lack refrigeration facilities, will be focused on as part of future work, as further analysis would be required to determine the thermal stability and therefore shelf life of the dressings. This is due to a commercial financial feasibility study which was carried out for WIT in 2013 indicating that stable burn wound dressings have a high level of commercial interest (18).

The extract was purified using TLC, bioautography and semi-preparative HPLC. The resulting fraction was found to be HPLC isolated using PDA analysis. Identification techniques were used for the purpose of structurally elucidating the compound(s) responsible for the antimicrobial activity. NMR data was found to be inconclusive due to a concentration issue and LC-MS data did not show a molecular ion. However, when the FTIR spectra were compared to literature results, it appeared similar to that of fucoidan, a water soluble sulphated polysaccharide present in *F. vesiculosus*. The LC-MS data was re-evaluated and presented ions that corresponded to fragments found in the LC-MS spectra of fucoidan reported in literature. Whilst this is not a confirmation that the antimicrobial is fucoidan, it was taken as a possibility, but further analysis would need to be carried out to confirm this data. The antimicrobial, antibiofilm and cytotoxic activity of fucoidan have been noted previously in the literature, supporting the identity of fucoidan and the antimicrobial agent isolated from the aqueous extract of *F. vesiculosus*.

7.2 Future Work

There are a number of areas in relation to this work where more analysis could be done to continue on with the extraction, purification and identification of extracts from *F. vesiculosus* and *F. serratus* for their incorporation into wound dressings.

- Further purification could be carried out on the HPLC isolated fraction of the aqueous extract from *F. vesiculosus* via running this extract on the semi-preparative HPLC with a method developed to separate analytes.

- As the MIC, MBC, antibiofilm and cytotoxicity of the water extract from *F. vesiculosus* was undertaken on a crude extract, these assays could be performed on the HPLC isolated extract generated in section 6 of this study or a pure sample of fucoidan. This analysis would offer more information on the suitability of fucoidan as an antimicrobial agent used for the treatment on wound infections.

- In addition to this, the crude and HPLC isolated extract could be analysed for cytotoxicity against primary, cell lines to examine the potential benefits of the extracts against rapidly dividing cells.

- Other compounds of interest could be isolated from the aqueous extract of *F vesiculosus*, such as 'peak 4'. These compounds could subsequently be characterised and identified using: H^1NMR, C^{13} NMR, FTIR, UV-Vis and LC-MS analysis.

- The water extract from *F. vesiculosus* added with commercially available fucoidan from *F. vesiculosus* could be analysed using HPLC to determine if the elution time of the antimicrobial peak and the fucoidan coincide.

- Upon identification, a synthetic route to the bioactive compound(s) could be devised to remove the variation associated with natural products.

- A mode of antimicrobial action could be studied in order to understand the mechanism responsible and potentially build other antimicrobials based on the structure and/mechanism.

- Other antimicrobial extracts found as part of section 2 of this work could be isolated and developed in a similar manner to that of the water extract of *F. vesiculosus*, such as the acetone extracts of *F. vesiculosus* and *F. serratus.*

- The HPLC isolated extract could be incorporated into the developed wound dressing formulation (50:50 % alginate:L.M.W. chitosan in a 1 % lactic acid solution with 2 % proplyene glycol) and tested for antimicrobial activity and dressing characteristics.

- Further materials characterisation could potentially be performed on the developed wound dressing along with *in vivo* and *in vitro* cytotoxicity studies, with the aim of bringing this dressing to the market.

- The developed wound dressing could be further studied to assess any pro-wound healing activity that the dressing may have due to the presence of chitosan, alginate and seaweed derived antimicrobial compounds.

- Different methods of sterilisation for the wound dressings formulated (such as gamma or UV irradiation) could be analysed to ensure that antimicrobial and antibiofilm activity remained the same pre and post sterilisation.

- Other classes of wound dressings could be examined, formulated and developed with the incorporation of seaweed derived antimicrobial compounds, such as antimicrobial liners or different hydrogel formulation.

LIST OF OUTPUTS

Papers under review:

Annabel Higgins Hoare, Peter McLoughlin, Shiau Pin Tan and Helen Hughes. (2019) 'The screening and evaluation of solvent extracted seaweeds against current strains of MRSA isolated from a clinical hospital setting', **Accepted with corrections** *In Scientific Reports*

Published abstracts:

Poster presentations:

Annabel Higgins Hoare, Peter McLoughlin, Shiau Pin Tan and Helen Hughes. (2016) 'The screening and evaluation of solvent extracted seaweeds against wound pathogens', *In Waterford Institute of Technology Research Day,* 4th May 2016, Waterford, Ireland.

Annabel Higgins Hoare, Peter McLoughlin, Shiau Pin Tan and Helen Hughes. (2017) 'The screening and evaluation of solvent extracted seaweeds against wound pathogens', *In Waterford Institute of Technology Research Day,* 18th May 2017, Waterford, Ireland.

Annabel Higgins Hoare, Peter McLoughlin, Shiau Pin Tan and Helen Hughes. (2017) 'The screening and evaluation of solvent extracted seaweeds against wound pathogens', *In the International Phytotechnological Conference,* 25-29th September 2017, Montréal, Canada.

Oral presentations:

Annabel Higgins Hoare, Peter McLoughlin, Shiau Pin Tan and Helen Hughes. (2018) 'The development of novel heat-stable wound dressings using seaweed derived antimicrobial compounds', *The 70th Irish Universities Chemistry Research Colloquium,* 21-22nd June 2018, Belfast, Northern Ireland.

Annabel Higgins Hoare, Peter McLoughlin, Shiau Pin Tan and Helen Hughes. (2017) 'The development of novel heat-stable wound dressings using seaweed derived

antimicrobial compounds", *In the 67th annual British Phycological Society Meeting*, 7-10th January 2019, Oban, Edinburgh.

Online:

Blog on the Irish Research Council website at the request of the IRC available at: *http://www.research.ie/event/2016-11-11/loveirishresearch-blog-irish-seaweed-wound-dressing*

Promotion of IRC programme via newspapers available: *http://www.irishtimes.com/news/science/irish-research-council-awards-16-2m-to-postgraduates-1.2415710*

http://www.thejournal.ie/research-scholarships-awards-2424004-Nov2015/

https://www.siliconrepublic.com/discovery/213-postgrad-researchers-awarded-e16-2m-in-funding-for-next-gen-research

The Irish Research Council's Science Week campaign, available at:

http://research.ie/what-we-do/loveirishresearch/blog/irish-research-council-shines-spotlight-on-topical-questions-this-science-week/

REFERENCES

1. Hassan MR, Chakrabarti R. Use of algae and aquatic macrophytes as feed in small-scale aquaculture [Internet]. Vol. 531, FAO Fisheries and Aquaculture Technical Paper. 2009. 135 p. Available from: http://www.lavoisier.fr/notice/fr286371.html

2. Harley CDG, Anderson KM, Demes KW, Jorve JP, Kordas RL, Coyle T a., et al. Effects Of Climate Change On Global Seaweed Communities. J Phycol. 2012;48(5):1064–78.

3. Rosell KG, Srivastava LM. Fatty acids as antimicrobial substances in brown algae. Hydrobiologia. 1987;151–152:471–5.

4. Sridhar KR, Vidyavathi N. Antimicrobial Activity of Seaweeds. Acta Hydrochim Hydrobiol [Internet]. 1991;19(5):455–96. Available from: http://doi.wiley.com/10.1002/aheh.19910190502

5. Sims JJ, Donnell MS, Leary J V, Lacy GH. Antimicrobial agents from marine algae. Antimicrob Agents Chemother [Internet]. 1975;7(3):320–1. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=429132&tool=pmcentrez&rendertype=abstract

6. Puglisi MP, Engel S, Jensen PR, Fenical W. Antimicrobial activities of extracts from Indo-Pacific marine plants against marine pathogens and saprophytes. Mar Biol [Internet]. 2006;150(4):531–40. Available from: http://link.springer.com/10.1007/s00227-006-0376-3

7. Berna Kılınç, Semra Cirik GT, Koru HT and E. Seaweeds for Food and Industrial Applications. In: Muzzalupo I, editor. Food Industry [Internet]. InTech; 2013. Available from: http://www.intechopen.com/books/food-industry/seaweeds-for-food-and-industrial-applications

8. Bocanegra A, Bastida S, Benedí J, Ródenas S, Sánchez-Muniz FJ. Characteristics and nutritional and cardiovascular-health properties of seaweeds. J Med Food.

2009;12(2):236–58.

9. Bedoux G, Hardouin K, Burlot AS, Bourgougnon N. Bioactive components from seaweeds: Cosmetic applications and future development [Internet]. Vol. 71, Advances in Botanical Research. Elsevier; 2014. 345–378 p. Available from: http://dx.doi.org/10.1016/B978-0-12-408062-1.00012-3

10. Lee TF. The seaweed Handbook: An Illustrated Guide to Seweeds from North CArolina to the Arctic. 1986. 1–35 p.

11. Hester J. Green Reefkeeping: Chaetomorpha Algae in the Reef Aquarium [Internet]. 2012 [cited 2015 Sep 22]. Available from: http://www.reefnation.com/green-reefkeeping-chaetomorpha-algae-in-the-reef-aquarium/

12. Simply Science. Algae Vs. Plants [Internet]. Explaining Environmental Issues. 2019 [cited 2019 Sep 25]. Available from: http://simply-science-nbep.blogspot.com/2011/06/algae-vs-plants.html

13. Bunker FSD, Brodie J, Maggs C., Bunker A. Guide to Seaweeds of Britan and Ireland. Marine Conservarion Society, editor. Ross-on-Wye; 2010. 8–21 p.

14. Kraan S, Verges Tramullas A, Guiry MD. The edible brown seaweed Alaria esculenta (Phaeophyceae, Laminariales): Hybridization, growth and genetic comparisons of six Irish populations. J Appl Phycol. 2000;12(6):577–83.

15. Diaz-Pulido, G., McCook LJ. Environmental Status : Macroalgae (Seaweeds). Science (80-). 2008;(July):1–47.

16. Gupta S, Abu-Ghannam N. Bioactive potential and possible health effects of edible brown seaweeds. Trends Food Sci Technol. 2011;22(6):315–26.

17. The Department of Housing Planning and Local Government. National Marine Planning Framework Baseline Report. Dublin; 2018.

18. Rd C, Laoghaire D. Novel Antibacterial Dressing Commercial Case Feasibility Study. 2013;(November):1–61.

19. Hollmann J, Themeier H, Neese U, Lindhauer MG. Dietary fibre fractions in

cereal foods measured by a new integrated AOAC method. Food Chem [Internet]. 2013;140(3):586–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23601411

20. Fleurence È, Physico-chimie Â, Qualite L. Seaweed proteins : biochemical , nutritional aspects and potential uses. 1999;10:26–9.

21. Dhargalkar VK, Pereira N. Seaweed : Promising Plant of the Millennium. Source [Internet]. 2005;71(Table 2):60–6. Available from: http://drs.nio.org/drs/handle/2264/489

22. Guiry M. Phaeophyceae: Brown Algae [Internet]. 2015 [cited 2015 Sep 21]. Available from: http://www.seaweed.ie/algae/phaeophyta.php

23. Michael Guiry. Rhodophyta: Red algae [Internet]. 2015. Available from: http://www.seaweed.ie/algae/rhodophyta.php

24. Moroney NC, O'Grady MN, Robertson RC, Stanton C, O'Doherty JV, Kerry JP. Influence of level and duration of feeding polysaccharide (laminarin and fucoidan) extracts from brown seaweed (Laminaria digitata) on quality indices of fresh pork. Meat Sci [Internet]. 2015;99:132–41. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0309174014002812

25. Muradian K, Vaiserman a., Min K-J, Fraifeld VE. Fucoxanthin and lipid metabolism: A minireview. Nutr Metab Cardiovasc Dis [Internet]. 2015;25(10):891–7. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0939475315001404

26. Sadler MJ. Authorised EU health claims for vitamins and minerals [Internet]. Foods, Nutrients and Food Ingredients with Authorised Eu Health Claims. Woodhead Publishing Limited; 2014. 274–297 p. Available from: http://linkinghub.elsevier.com/retrieve/pii/B9780857098429500133

27. Chapman VJ. Seaweeds and their Uses. 3rd ed. Igarss 2014. Chapman and Hall; 1980. 1–345 p.

28. Valente LMP, Rema P, Ferraro V, Pintado M, Sousa-Pinto I, Cunha LM, et al. Iodine enrichment of rainbow trout flesh by dietary supplementation with the red

seaweed Gracilaria vermiculophylla. Aquaculture [Internet]. 2015;446:132–9. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0044848615002604

29. Strand A, Herstad O, Liaaen-Jensen S. Fucoxanthin metabolites in egg yolks of laying hens. Comp Biochem Physiol - A Mol Integr Physiol. 1998;119(4):963–74.

30. Fuge R, Johnson CC. Iodine and human health, the role of environmental geochemistry and diet, a review. Appl Geochemistry [Internet]. 2015; Available from: http://linkinghub.elsevier.com/retrieve/pii/S0883292715300470

31. López-Mosquera ME, Fernández-Lema E, Villares R, Corral R, Alonso B, Blanco C. Composting fish waste and seaweed to produce a fertilizer for use in organic agriculture. Procedia Environ Sci [Internet]. 2011;9:113–7. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1878029611007808

32. Wang R, Paul VJ, Luesch H. Seaweed extracts and unsaturated fatty acid constituents from the green alga Ulva lactuca as activators of the cytoprotective Nrf2-ARE pathway. Free Radic Biol Med [Internet]. 2013;57:141–53. Available from: http://dx.doi.org/10.1016/j.freeradbiomed.2012.12.019

33. Peng J, Yuan JP, Wu CF, Wang JH. Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: Metabolism and bioactivities relevant to human health. Mar Drugs. 2011;9(10):1806–28.

34. Charoensiddhi S, Franco C, Su P, Zhang W. Improved antioxidant activities of brown seaweed Ecklonia radiata extracts prepared by microwave-assisted enzymatic extraction. J Appl Phycol. 2015;27(5):2049–58.

35. Kirke DA, Smyth TJ, Rai DK, Kenny O, Stengel DB. The chemical and antioxidant stability of isolated low molecular weight phlorotannins. Food Chem [Internet]. 2017;221(December 2017):1104–12. Available from: http://dx.doi.org/10.1016/j.foodchem.2016.11.050

36. Zhang Q, Li N, Liu X, Zhao Z, Li Z, Xu Z. The structure of a sulfated galactan from Porphyra haitanensis and its in vivo antioxidant activity. Carbohydr Res. 2004;339(1):105–11.

37. Fletcher HR, Biller P, Ross AB, Adams JMM. The seasonal variation of fucoidan within three species of brown macroalgae. Algal Res [Internet]. 2017;22:79–86. Available from: http://dx.doi.org/10.1016/j.algal.2016.10.015

38. Sai K P, Babu M. Collagen based dressings: a review. Burns [Internet]. 1999;26:54–62. Available from: https://books.google.it/books?id=I4aoNwAACAAJ

39. Hu JF, Gen MY, Zhang JT, Jiang HD. An in vitro study of the structure-activity relationships of sulfated polysaccharide from brown algae to its antioxidant effect. J Asian Nat Prod Res. 2001;3(4):353–8.

40. Awad NE. Biologically active steroid from the green alga Ulva lactuca. Phyther Res. 2000;14:641–3.

41. Wijesinghe W a. JP, Kim E-A, Kang M-C, Lee W-W, Lee H-S, Vairappan CS, et al. Assessment of anti-inflammatory effect of 5β-hydroxypalisadin B isolated from red seaweed Laurencia snackeyi in zebrafish embryo in vivo model. Environ Toxicol Pharmacol [Internet]. 2014;37(1):110–7. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1382668913002366

42. Thuy TTT, Ly BM, Van TTT, Van Quang N, Tu HC, Zheng Y, et al. Anti-HIV activity of fucoidans from three brown seaweed species. Carbohydr Polym [Internet]. 2015;115:122–8. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0144861714008443

43. Daniela Montanari MG. Cosmetic composition with a lifting effect for sustaining relaxed skin tissues. WO2008146116 A2, 2007.

44. Park H-J, Kurokawa M, Shiraki K, Nakamura N, Choi J-S, Hattori M. Antiviral activity of the marine alga Symphyocladia latiuscula against herpes simplex virus (HSV-1) in vitro and its therapeutic efficacy against HSV-1 infection in mice. Biol Pharm Bull. 2005;28(12):2258–62.

45. European Commission. Regulation (EC) No. 1223/2009 of the European Parlament and of the Council. Official Journal of the European Union. 2009.

46. Antunes EM, Afolayan AF, Chiwakata MT, Fakee J, Knott MG, Whibley CE, et

al. Identification and in vitro anti-esophageal cancer activity of a series of halogenated monoterpenes isolated from the South African seaweeds Plocamium suhrii and Plocamium cornutum. Phytochemistry [Internet]. 2011;72(8):769–72. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0031942211000720

47. Rupérez P, Ahrazem O, Leal a J. Potential antioxidant capacity of sulfated polysaccharides from the edible brown seaweed Fucus vesiculosus. J Agric Food Chem [Internet]. 2002;50(4):840–5. Available from: http://pubs.acs.org/cgi-bin/article.cgi/jafcau/2002/50/i04/pdf/jf010908o.pdf

48. International Energy Agency. State of Technology Review – Algae Bioenergy [Internet]. McMillan J, Laurens L, editors. 2017. Available from: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52017DC0003&from=EN

49. Subitec. Products [Internet]. [cited 2017 Jul 20]. Available from: http://subitec.com/en/products-gained-from-microalgae-algaebiomass

50. Tan SP, O'Sullivan L, Prieto ML, Gardiner GE, Lawlor PG, Leonard F, et al. Extraction and bioautographic-guided separation of antibacterial compounds from Ulva lactuca. J Appl Phycol [Internet]. 2012;24(3):513–23. Available from: http://link.springer.com/10.1007/s10811-011-9747-3

51. Cox S, Abu-Ghannam N, Gupta S. An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds. Int Food Res J. 2010;17(1):205–20.

52. Mtolera MSP, Semesi AK. Antimicrobial Activity of Extracts from Six Green Algae from Tanzania. Curr trends Mar Bot Res East African Reg. 1988;(1996):211–7.

53. Sandsdalen E, Haug T, Stensvåg K, Styrvold OB. The antibacterial effect of a polyhydroxylated fucophlorethol from the marine brown alga, Fucus vesiculosus. World J Microbiol Biotechnol. 2003;19(8):777–82.

54. Nafiu MO, Hamid AA, Muritala HF, Adeyemi SB. Quality Control of Medicinal Plants in Africa [Internet]. Medicinal Spices and Vegetables from Africa. Elsevier Inc.; 2017. 171–204 p. Available from: http://dx.doi.org/10.1016/B978-

0-12-809286-6/00007-8

55. Luque, M.D. and Ayuso LE. Encyclopedia of Separation Science. Environ Appl | Soxhlet Extr. 2000;(1995):2701–9.

56. Sarker SD, Dinan L, Šik V, Rees HH. Moschatine: An unusual steroidal glycoside from Centaurea moschata. Phytochemistry. 1998;48(6):1039–43.

57. Christen P, Kaufmann B. Recent Extraction Techniques for Natural Products : Microwave-assisted Extraction and Pressurised Solvent Extraction. Phytochem Anal [Internet]. 2002;113(Phytochem. Anal. 13):105–13. Available from: University of Geneva, School of Pharmacy, Laboratory of Pharmaceutical Analytical Chemistry, 20 bd d'Yvoy, CH-1211 Geneva 4, Switzerland

58. Bohlin L. Natural Products Isolation. Drug Discov Today. 1998;3(12):536–7.

59. Delazar A, Nahar L, Hamedeyazdan S, Sarker SD. Microwave-Assisted Extraction in Natural Products Isolation. In: Nahar L, Sarker SD, editors. Natural Products Isolation, Methods in Molecular Biology [Internet]. 2012. p. 536–7. Available from: https://www.globenewswire.com/news-release/2019/05/16/1826492/0/en/Bioactive-Wound-Care-Market-To-Reach-USD-13-50-Billion-By-2026-Reports-And-Data.html

60. Pitchai K. Electromagnetic and Heat Transfer Modeling of Microwave Heating in Domestic Ovens. University of Nebraska; 2011.

61. Veggi PC, Martinez J, Meireles MAA. Chapter 2: Fundamentals of Microwave Extraction. In: Chemat F, Cravotto G, editors. Microwave-assisted Extraction for Bioactive Compounds [Internet]. New York: Springer; 2013. p. 15–53. Available from: http://link.springer.com/10.1007/978-1-4614-4830-3

62. Hahn T, Lang S, Ulber R, Muffler K. Novel procedures for the extraction of fucoidan from brown algae. Process Biochem [Internet]. 2012;47(12):1691–8. Available from: http://dx.doi.org/10.1016/j.procbio.2012.06.016

63. Chemat S, Aït-Amar H, Lagha A, Esveld DC. Microwave-assisted extraction kinetics of terpenes from caraway seeds. Chem Eng Process Process Intensif. 2005;44(12):1320–6.

64. Xiao XH, Yuan ZQ, Li GK. Preparation of phytosterols and phytol from edible marine algae by microwave-assisted extraction and high-speed counter-current chromatography. Sep Purif Technol [Internet]. 2013;104:284–9. Available from: http://dx.doi.org/10.1016/j.seppur.2012.11.032

65. Magnusson M, Yuen AKL, Zhang R, Wright JT, Taylor RB, Maschmeyer T, et al. A comparative assessment of microwave assisted (MAE) and conventional solid-liquid (SLE) techniques for the extraction of phloroglucinol from brown seaweed. Algal Res [Internet]. 2017;23:28–36. Available from: http://dx.doi.org/10.1016/j.algal.2017.01.002

66. Señoráns FJ, Luna P. Sample preparation techniques for the determination of fats in food. Compr Sampl Sample Prep. 2012;4:203–11.

67. Veggi PC, Martinez J, Meireles MAA. From Laboratory to Industry: Scale-Up, Quality, and Safety Consideration for Microwave-Assisted Extraction. In: Microwave-assisted Extraction for BioactiveCompounds: Theory and Practice, Food Engineering Series 4,. Avignon , France e; 2013. p. 72.

68. Pasquet V, Chérouvrier JR, Farhat F, Thiéry V, Piot JM, Bérard JB, et al. Study on the microalgal pigments extraction process: Performance of microwave assisted extraction. Process Biochem [Internet]. 2011;46(1):59–67. Available from: http://dx.doi.org/10.1016/j.procbio.2010.07.009

69. Rodriguez-Jasso RM, Mussatto SI, Pastrana L, Aguilar CN, Teixeira J a. Microwave-assisted extraction of sulfated polysaccharides (fucoidan) from brown seaweed. Carbohydr Polym [Internet]. 2011;86(3):1137–44. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0144861711004528

70. Romarís-Hortas V, Moreda-Piñeiro A, Bermejo-Barrera P. Microwave assisted extraction of iodine and bromine from edible seaweed for inductively coupled plasma-mass spectrometry determination. Talanta. 2009;79(3):947–52.

71. Yuan Y, Macquarrie D. Microwave assisted extraction of sulfated polysaccharides (fucoidan) from Ascophyllum nodosum and its antioxidant activity. Carbohydr Polym [Internet]. 2015;129:101–7. Available from: http://dx.doi.org/10.1016/j.carbpol.2015.04.057

72. Qv XY, Zhou QF, Jiang JG. Ultrasound-enhanced and microwave-assisted extraction of lipid from Dunaliella tertiolecta and fatty acid profile analysis. J Sep Sci. 2014;37(20):2991–9.

73. Jaime L, Rodríguez-Meizoso I, Cifuentes A, Santoyo S, Suarez S, Ibáñez E, et al. Pressurized liquids as an alternative process to antioxidant carotenoids' extraction from Haematococcus pluvialis microalgae. LWT - Food Sci Technol. 2010;43(1):105–12.

74. Wang B, Tong GZ, Qu Y Le, Li L. Microwave-Assisted Extraction and *In Vitro* Antioxidant Evaluation of Polysaccharides from *Enteromorpha prolifera*. Appl Mech Mater. 2011;79:204–9.

75. Li Z, Wang B, Zhang Q, Qu Y, Xu H, Li G. Preparation and antioxidant property of extract and semipurified fractions of Caulerpa racemosa. J Appl Phycol. 2012;24(6):1527–36.

76. Zhang R, Yuen AKL, Magnusson M, Wright JT, de Nys R, Masters AF, et al. A comparative assessment of the activity and structure of phlorotannins from the brown seaweed Carpophyllum flexuosum. Algal Res. 2018;29(August 2017):130–41.

77. Poole J, Diop A, Rainville L-C, Barnabé S. Bioextracting Polyphenols from the Brown Seaweed *Ascophyllum nodosum* from Québec's North Shore Coastline. Ind Biotechnol [Internet]. 2019;15(3):212–8. Available from: https://www.liebertpub.com/doi/10.1089/ind.2019.0008

78. Herrero M, Ibáñez E. Green processes and sustainability: An overview on the extraction of high added-value products from seaweeds and microalgae. J Supercrit Fluids [Internet]. 2015;96:211–6. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0896844614002812

79. Herrero M, Sánchez-Camargo ADP, Cifuentes A, Ibáñez E. Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction. TrAC Trends Anal Chem [Internet]. 2015;71:26–38. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0165993615001326

80. Marsili R. Comparison of a Liquid Solvent Extraction Technique and Supercritical Fluid Extraction for the Determination of a- and β-Carotene in Vegetables. 1993;31(October):422–8.

81. Iheozor-Ejiofor P, Dey ES. Extraction of rosavin from Rhodiola rosea root using supercritical carbon dioxide with water. J Supercrit Fluids. 2009;50:29–32.

82. Langa E, Cacho J, Palavra a. MF, Burillo J, Mainar a. M, Urieta JS. The evolution of hyssop oil composition in the supercritical extraction curve. J Supercrit Fluids [Internet]. 2009;49(1):37–44. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0896844608003884

83. Ren Q, Xing H, Bao Z, Su B, Yang Q, Yang Y, et al. Recent advances in separation of bioactive natural products. Chinese J Chem Eng [Internet]. 2013;21(9):937–52. Available from: http://dx.doi.org/10.1016/S1004-9541(13)60560-1

84. Zarena a. S, Udaya Sankar K. Supercritical carbon dioxide extraction of xanthones with antioxidant activity from Garcinia mangostana: Characterization by HPLC/LC-ESI-MS. J Supercrit Fluids. 2009;49:330–7.

85. Li J, Zhang M, Zheng T. The in vitro antioxidant activity of lotus germ oil from supercritical fluid carbon dioxide extraction. Food Chem [Internet]. 2009;115(3):939–44. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0308814609000363

86. Yesil-Celiktas O, Otto F, Parlar H. A comparative study of flavonoid contents and antioxidant activities of supercritical CO2 extracted pine barks grown in different regions of Turkey and Germany. Eur Food Res Technol [Internet]. 2009;229(4):671–7. Available from: http://link.springer.com/10.1007/s00217-009-1101-5

87. Tzeng TC, Lin YL, Jong TT, Chang CMJ. Ethanol modified supercritical fluids extraction of scopoletin and artemisinin from Artemisia annua L. Sep Purif Technol. 2007;56(1):18–24.

88. Park HS, Im NG, Kim KH. Extraction behaviors of caffeine and chlorophylls in supercritical decaffeination of green tea leaves. LWT - Food Sci Technol

[Internet]. 2012;45(1):73–8. Available from: http://dx.doi.org/10.1016/j.lwt.2011.07.023

89. Upadhyay NK, Kumar R, Mandotra SK, Meena RN, Siddiqui MS, Sawhney RC, et al. Safety and healing efficacy of Sea buckthorn (Hippophae rhamnoides L.) seed oil on burn wounds in rats. Food Chem Toxicol [Internet]. 2009;47(6):1146–53. Available from: http://dx.doi.org/10.1016/j.fct.2009.02.002

90. Chan KW, Ismail M. Supercritical carbon dioxide fluid extraction of Hibiscus cannabinus L. seed oil: A potential solvent-free and high antioxidative edible oil. Food Chem [Internet]. 2009;114(3):970–5. Available from: http://dx.doi.org/10.1016/j.foodchem.2008.10.055

91. Kim SM, Jung YJ, Kwon ON, Cha KH, Um BH, Chung D, et al. A potential commercial source of fucoxanthin extracted from the microalga Phaeodactylum tricornutum. Appl Biochem Biotechnol. 2012;166(7):1843–55.

92. Shang YF, Kim SM, Lee WJ, Um B-H. Pressurized liquid method for fucoxanthin extraction from Eisenia bicyclis (Kjellman) Setchell. J Biosci Bioeng [Internet]. 2011;111(2):237–41. Available from: http://www.sciencedirect.com/science/article/pii/S1389172310003567

93. Koo SY, Cha KH, Song D-G, Chung D, Pan C-H. Optimization of pressurized liquid extraction of zeaxanthin from Chlorella ellipsoidea. J Appl Phycol [Internet]. 2011;24(4):725–30. Available from: http://link.springer.com/10.1007/s10811-011-9691-2

94. Plaza M, Santoyo S, Jaime L, Avalo B, Cifuentes A, Reglero G, et al. Comprehensive characterization of the functional activities of pressurized liquid and ultrasound-assisted extracts from Chlorella vulgaris. LWT - Food Sci Technol [Internet]. 2012;46(1):245–53. Available from: http://dx.doi.org/10.1016/j.lwt.2011.09.024

95. Herrero M, Jaime L, Martín-Álvarez PJ, Cifuentes A, Ibáñez E. Optimization of the extraction of antioxidants from Dunaliella salina microalga by pressurized liquids. J Agric Food Chem. 2006;54(15):5597–603.

96. Castro-Puyana M, Herrero M, Urreta I, Mendiola J a., Cifuentes A, Ibáñez E, et

al. Optimization of clean extraction methods to isolate carotenoids from the microalga Neochloris oleoabundans and subsequent chemical characterization using liquid chromatography tandem mass spectrometry. Anal Bioanal Chem [Internet]. 2013;405(13):4607–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23314588

97. Mulbry W, Kondrad S, Buyer J, Luthria DL. Optimization of an Oil Extraction Process for Algae from the Treatment of Manure Effluent. J Am Oil Chem Soc [Internet]. 2009;86(9):909–15. Available from: http://link.springer.com/10.1007/s11746-009-1432-1

98. Heffernan N, Smyth TJ, Fitzgerald RJ, Soler-Vila A, Brunton N. Antioxidant activity and phenolic content of pressurised liquid and solid-liquid extracts from four Irish origin macroalgae. Int J Food Sci Technol. 2014;1–8.

99. Onofrejová L, Vašíčková J, Klejdus B, Stratil P, Mišurcová L, Kráčmar S, et al. Bioactive phenols in algae: The application of pressurized-liquid and solid-phase extraction techniques. J Pharm Biomed Anal [Internet]. 2010;51(2):464–70. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0731708509002088

100. Anaëlle T, Serrano Leon E, Laurent V, Elena I, Mendiola J a., Stéphane C, et al. Green improved processes to extract bioactive phenolic compounds from brown macroalgae using Sargassum muticum as model. Talanta [Internet]. 2013;104:44–52. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0039914012009319

101. O'Sullivan a. M, O'Callaghan YC, O'Grady MN, Hayes M, Kerry JP, O'Brien NM. The effect of solvents on the antioxidant activity in Caco-2 cells of Irish brown seaweed extracts prepared using accelerated solvent extraction (ASE??). J Funct Foods. 2013;5(2):940–8.

102. Santoyo S, Rodríguez-Meizoso I, Cifuentes a., Jaime L, García-Blairsy Reina G, Señorans FJ, et al. Green processes based on the extraction with pressurized fluids to obtain potent antimicrobials from Haematococcus pluvialis microalgae. LWT - Food Sci Technol [Internet]. 2009;42(7):1213–8. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0023643809000279

103. Plaza M, Santoyo S, Jaime L, García-Blairsy Reina G, Herrero M, Señoráns FJ, et al. Screening for bioactive compounds from algae. J Pharm Biomed Anal. 2010;51(2):450–5.

104. Picó Y. Ultrasound-assisted extraction for food and environmental samples. TrAC - Trends Anal Chem. 2013;43:84–99.

105. Kadam SU, Donnell CPO, Rai DK, Hossain MB, Burgess CM, Walsh D, et al. Laminarin from Irish brown seaweeds *Ascophyllum nodosum* and *Laminaria hyperborea*: ultrasound assisted extraction, characterization and bioactivity. Mar Drugs. 2015;13:4270–80.

106. Dhanani T, Shah S, Gajbhiye NA, Kumar S. Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera. Arab J Chem [Internet]. 2017;10:S1193–9. Available from: http://dx.doi.org/10.1016/j.arabjc.2013.02.015

107. Vilkhu K, Mawson R, Simons L, Bates D. Applications and opportunities for ultrasound assisted extraction in the food industry - A review. Innov Food Sci Emerg Technol. 2008;9(2):161–9.

108. Zhu J-S, Halpern GM, Jones K. The Scientific Rediscovery of an Ancient Chinese Herbal Medicine:Cordyceps sinensis. J Altern Complement Med. 1998;4(3):289–303.

109. Kenny C, Furey A, Lucey B. A post-antibiotic era looms : can plant natural product research fill the void ? Br J Biomed Sci. 2015;4(32).

110. Institute C and LS. M02-A12: Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Twelfth Edition. Clin Lab Stand Inst. 2015;35(M02-A12):73.

111. Cleidson Valgas;, Simone Machado de Souza;, Elza F A Smânia;, Artur Smânia Jr. Screening methods to determine antibacterial activity of natural products. Brazilian J Microbiol [Internet]. 2007;38(2):369–80. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1517-83822007000200034

112. Narasinh C, Kumar S, Sri P, Murthy R. An optimized triphenyltetrazolium chloride method for identification of cerebral infarcts. 2004;13:11–7.

113. Tan SP, O'Sullivan L, Luz Prieto M, McLoughlin P, Lawlor PG, Hughes H, et al. Seaweed Antimicrobials: Isolation Characterisation and Potential Use in Functional Foods. In: Hernandez-Ledesma B, Herrero M, editors. Bioactive Compounds from Marine Foods. 1 st. West Sussex: John Wiley & Sons Ltd.; 2014. p. 295.

114. Jr DLV, Puzon JJM, Cabrera EC, Rivera WL. Thin Layer Chromatography-Bioautography and Gas Chromatography-Mass Spectrometry of Antimicrobial Leaf Extracts from Philippine Piper betle L . against Multidrug-Resistant Bacteria. Evidence-Based Complement Altern Med. 2016;2016.

115. Patridge E, Gareiss P, Kinch MS, Hoyer D. An analysis of FDA-approved drugs: Natural products and their derivatives. Drug Discov Today [Internet]. 2016;21(2):204–7. Available from: http://dx.doi.org/10.1016/j.drudis.2015.01.009

116. Wang, Mingxun. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. Nat Biotechnol. 2019;34(8):828–37.

117. U.S. Department of Commerce. National Instute of Standards and Technology [Internet]. NIST. 2019. Available from: https://www.nist.gov/

118. ThermoFisher scientific. Basic UV-Vis Theory , Concepts and Applications Basic UV-Vis Theory , Concepts and Applications. Thermo Spectronic. 1995;1–28.

119. Slater LB. Instruments and rules : R.B. Woodward and the tools of twentieth-century organic chemistry. Stud Hist Philos Sci. 2002;33:1–33.

120. Douglas A, Skoog F, Holler J, Nieman TA. Ultraviolet / Visible Molecular Absorption Spectrometry I. In: The Principles of Instrumental Analysis. 6th ed. Philadelphia: Saunders College Pub.; 2007. p. 13–5.

121. Hoysecroft CE, Constable EC. Mass Spectroscopy. In: Peason, editor. Chemistry.

2nd ed. Essex: Prentice Hil; 2002. p. 293–306.

122. Li Y, Fu X, Duan D, Liu X, Xu J, Gao X. Extraction and Identification of Phlorotannins from the Brown Alga, Sargassum fusiforme (Harvey) Setchell. Mar Drugs. 2017;15(2).

123. Ghribi L, Waffo-Téguo P, Cluzet S, Marchal A, Marques J, Mérillon JM, et al. Isolation and structure elucidation of bioactive compounds from the roots of the Tunisian Ononis angustissima L. Bioorganic Med Chem Lett [Internet]. 2015;25(18):3825–30. Available from: http://dx.doi.org/10.1016/j.bmcl.2015.07.076

124. Li QZ, Wu D, Zhou S, Liu YF, Li ZP, Feng J, et al. Structure elucidation of a bioactive polysaccharide from fruiting bodies of Hericium erinaceus in different maturation stages. Carbohydr Polym. 2016;144:196–204.

125. Boateng J., Matthews KH, Stevens HNE, Eccleston GM. Wound Healing Dressings and Drug Delivery Systems: A Review. J Pharm Sci. 2008;97(8):2892–923.

126. Singer AJ, Clark R a F. Cutaneous Wound Healing. N Engl J Med [Internet]. 1999;341(10):738–46. Available from: http://www.nejm.org/doi/full/10.1056/NEJM199909023411006

127. Bhatnagar M, Bhatnagar A. Wound Dressings from Algal Polymers. In: Se-Kwon Kim, Chojnacka K, editors. Marine Algae Extracts: Processes, Products, and Applications. First Edit. 2015. p. 524.

128. Velnar T, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. J Int Med Res [Internet]. 2009;37(5):1528–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19930861

129. Swanson T, Keast D, Cooper R, Black J, Fletcher J, Swanson T. Clinical practice Ten top tips: identification of wound infection in a chronic wound. 2015;2(1):20–5.

130. Martin P, Martin P. Wound Healing — Aiming for Perfect Skin Regeneration. 2014;75(1997).

131. Wolberg AS. Thrombin generation and fibrin clot structure. Blood Rev. 2007;21(3):131–42.

132. Clark R, editor. The Molecular and Cellular Biology of Wound Repair. 2 nd. New York: Plenum; 1996. 3–50 p.

133. Romanelli M, Vowden K, Weir D. Exudate management made easy. Wounds Int. 2010;1(2):1–6.

134. Olczyk P, Mencner L, Komosinska-Vassev K. The role of the extracellular matrix components in cutaneous wound healing. Biomed Res Int [Internet]. 2014;2014:747584. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24772435

135. Nahrendorf M, Pittet MJ, Swirski FK. Monocytes: Protagonists of infarct inflammation and repair after myocardial infarction. Circulation. 2010;121(22):2437–45.

136. Manuscript A. Inflammation and wound healing : The role of the macrophage. 2013;16(2008):19–25.

137. Greenhalgh DG. The role of apoptosis in wound healing. Int J Biochem Cell Biol [Internet]. 1998;30(9):1019–30. Available from: http://www.sciencedirect.com/science/article/pii/S1357272598000582

138. Shaw TJ, Martin P. Wound repair at a glance. J Cell Sci. 2009;122(Pt 18):3209–13.

139. Yang J, Zhang L, Yu C, Yang X-F, Wang H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark Res [Internet]. 2014;2(1):1. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3892095&tool=pmcentrez&rendertype=abstract

140. Parihar A, Eubank TD, Doseff AI. Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. J Innate Immun. 2010;2(3):204–15.

141. Swirski FK, Robbins CS. Neutrophils usher monocytes into sites of

inflammation. Circ Res. 2013;112(5):744–5.

142. Gurtner G, Werner S, Barrandon Y, Longaker M. Wound repair and regeneration. Nature [Internet]. 2008;453(7193):314–21. Available from: http://dx.doi.org/10.1038/nature07039

143. Enoch S, Leaper DJ. Basic science of wound healing. Surg [Internet]. 2008;26(2):31–7. Available from: http://www.sciencedirect.com/science/article/pii/S026393190700316X

144. Lipsky B a, Hoey C. Topical antimicrobial therapy for treating chronic wounds. Clin Infect Dis [Internet]. 2009;49(10):1541–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19842981

145. Cadogan J, Baldwin D, Carpenter S, Davey J, Harris H, Wicks G. Identification, diagnosis and treatment of wound infection. Nurs Stand [Internet]. 2011;26(11):44–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22206103

146. Dryden MS. Complicated skin and soft tissue infection. J Antimicrob Chemother. 2010;65(SUPPL. 3):35–44.

147. Livermore DM, Mushtaq S, Warner M, James D, Kearns A, Woodford N. Pathogens of skin and skin-structure infections in the UK and their susceptibility to antibiotics, including ceftaroline. J Antimicrob Chemother. 2015;70(10):2844–53.

148. Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev. 2001;14(2):244–69.

149. C.O.Nwachukwu BAW and. A survey of common pathogens in wound in patients at the University of Port Harcourt Teaching Hospital (U.P.T.H), Port Harcourt.pdf. West Afr Med J. 2003;22:50–4.

150. Azene MK, Beyene B a. Bacteriology and antibiogram of pathogens from wound infections at Dessie Laboratory, north east Ethiopia. Tanzan J Health Res. 2011;13(4):unpaginated.

151. Dowd SE, Sun Y, Secor PR, Rhoads DD, Wolcott BM, James GA, et al. Survey

of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiol. 2008;8:43.

152. European Centre for Disease Prevention and Control. Point prevalence survey of healthcare-associated infections and antimicrobial use in European long-term care facilities [Internet]. 2013. Available from: https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/healthcare-associated-infections-point-prevalence-survey-long-term-care-facilities-2013.pdf

153. HSE. Staphylococcus Aureus [Internet]. 2013. Available from: http://www.hse.ie/eng/health/az/S/Staphylococcal-infections/

154. Somerville G a, Proctor R a. The Biology of Staphylococci [Internet]. Staphylococci in Human Disease. 2009. 3–18 p. Available from: http://books.google.com/books/about/The_staphylococci_in_human_disease.html?id=RClrAAAAMAAJ&pgis=1

155. Plata K, Rosato AE, Wegrzyn G. Staphylococcus aureus as an infectious agent: Overview of biochemistry and molecular genetics of its pathogenicity. Acta Biochim Pol. 2009;56(4):597–612.

156. Gould D CA. Staphylococcus aureus: A Reviewof the Literature.pdf. J Clin Nurs. 1995;4:5–12.

157. Staphylococcus aureus on mannitol agar mannitol agar [Internet]. 2013 [cited 2015 Oct 9]. p. Medical Labs Portal. Available from: http://www.medical-labs.net/staphylococcus-aureus-on-mannitol-agar-mannitol-agar-578/

158. Mulligan ME, Murray-Leisure K a, Ribner BS, Standiford HC, John JF, Korvick J a, et al. Methicillin-resistant Staphylococcus aureus: a consensus review of the microbiology, pathogenesis, and epidemiology with implications for prevention and management. Am J Med. 1993;94(March):313–28.

159. Gardete S, Tomasz A. Mechanisms of vancomycin resistance in <i>Staphylococcus aureus<\i>. J Clin Invest. 2014;124(7):2836–40.

160. Public Health Agency of Canada. Psuedomonas spp: Pathogen Safety Data Sheet

Data Sheet - Infectious Substances [Internet]. 2012 [cited 2015 Oct 9]. p. Public Health Agency of Canada. Available from: http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/pseudomonas-spp-eng.php

161. E.O Labs. Pseudomonas Selective Agar C.F.C - PP1051 [Internet]. E.O.Labs: Microculture and Media. 2015 [cited 2015 Oct 10]. Available from: http://www.eolabs.com/pseudomonas-selective-agar-cfc.html

162. Willcox MDP. Pseudomonas aeruginosa infection and inflammation during contact lens wear: a review. Optom Vis Sci. 2007;84(4):273–8.

163. Cummings BS, Mchowat J, Schnellmann RG. Phospholipase A 2 s in Cell Injury and Death. 2000;294(3):793–9.

164. Ferguson D. A study of clinical strains of Pseudomonas aeruginosa and the investigation of antibiotic resistance mechanisms in the multidrug resistant strain PA13 by. 2007;(November):297.

165. Oliver A, Mulet X, López-causapé C, Juan C. The increasing threat of Pseudomonas aeruginosa high-risk clones. Drug Resist Updat [Internet]. 2015;21–22:41–59. Available from: http://dx.doi.org/10.1016/j.drup.2015.08.002

166. Iii JPL, Zhanel GG, Clark NM. Emergence of Antimicrobial Resistance among Pseudomonas aeruginosa : Implications for Therapy. 2017;1(212):326–45.

167. Microbiology AS for. E.Coli on Maconkney Agar [Internet]. 2014 [cited 2016 Jan 14]. p. Microbeworld. Available from: http://www.microbeworld.org/component/jlibrary/?view=article&id=13045

168. Kaper JB, Nataro JP, Mobley HLT. Pathogenic Escherichia coli. Nat Rev Microbiol [Internet]. 2004;2(2):123–40. Available from: http://www.nature.com/doifinder/10.1038/nrmicro818

169. Reinthaler F., Posch J, Feierl G, Wüst G, Haas D, Ruckenbauer G, et al. Antibiotic resistance of E. coli in sewage and sludge. Water Res [Internet]. 2003;37(8):1685–90. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0043135402005699

170. Bjarnsholt T. The role of bacterial biofilms in chronic infections. APMIS Suppl

[Internet]. 2013;121(136):1–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23635385

171. Bjarnsholt T, Givskov M. The role of quorum sensing in the pathogenicity of the cunning aggressor Pseudomonas aeruginosa. Anal Bioanal Chem. 2007;387(2):409–14.

172. Costerton JW. Bacterial bofilms: a common cause of persistent infections. Science (80-). 1999;284(1999):1318–22.

173. Donlan RM. Biofilms: Microbial life on surfaces. Emerg Infect Dis. 2002;8(9):881–90.

174. Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JW. A review of the scientific evidence for biofilms in wounds. Wound Repair Regen. 2012;20(5):647–57.

175. Hiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents. 2010;35(4):322–32.

176. Stewart PS, William Costerton J. Antibiotic resistance of bacteria in biofilms. Lancet. 2001;358(9276):135–8.

177. Monroe D. Looking for chinks in the armor of bacterial biofilms. PLoS Biol. 2007;5(11):2458–61.

178. Wolcott RD, Rhoads DD. A study of biofilm-based wound management in subjects with critical limb ischaemia. J Wound Care. 2008;17(4):145–8, 150–2, 154–5.

179. Danese PN. Antibiofilm Approaches: Prevention of Catheter Colonization. Chem Biol. 2002;9(8):873–80.

180. Ammons MCB, Ward LS, James GA. Anti-biofilm efficacy of a lactoferrin/xylitol wound hydrogel used in combination with silver wound dressings. Int Wound J. 2011;8(3):268–73.

181. Khan F, Hashmi MU, Khalid N, Hayat MQ, Ikram A, Janjua HA. Controlled assembly of silver nano-fluid in Heliotropium crispum extract: a potent anti-

biofilm and bactericidal formulation. Appl Surf Sci [Internet]. 2016;387:317–31. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0169433216311631

182. Kalishwaralal K, Barathmanikanth S, Ram S, Pandian K, Deepak V, Gurunathan S. Silver nanoparticles impede the biofilm formation by Pseudomonas aeruginosa and Staphylococcus epidermidis. Colloids Surfaces B Biointerfaces [Internet]. 2010;79(2):340–4. Available from: http://dx.doi.org/10.1016/j.colsurfb.2010.04.014

183. Banat IM, De Rienzo MAD, Quinn GA. Microbial biofilms: biosurfactants as antibiofilm agents. Appl Microbiol Biotechnol. 2014;98(24):9915–29.

184. Ishwarya R, Vaseeharan B, Kalyani S, Banumathi B, Govindarajan M, Alharbi NS, et al. Facile green synthesis of zinc oxide nanoparticles using Ulva lactuca seaweed extract and evaluation of their photocatalytic, antibiofilm and insecticidal activity. J Photochem Photobiol B Biol [Internet]. 2018;178(October 2017):249–58. Available from: https://doi.org/10.1016/j.jphotobiol.2017.11.006

185. Yuvaraj N, Arul V. Preliminary screening of anti-biofilm , anti-Larval settlement and cytotoxic potential of seaweeds and seagrasses collected from Pondicherry and Rameshwaram coastal line , India. World J Fish Mar Sci. 2014;6(2):169–75.

186. Dabiri G, Damstetter E, Phillips T. Choosing a Wound Dressing Based on Common Wound Characteristics. Adv Wound Care [Internet]. 2016;5(1):32–41. Available from: http://online.liebertpub.com/doi/10.1089/wound.2014.0586

187. adpi: Resources for Schools [Internet]. 2015 [cited 2015 Oct 9]. p. Alexander Fleming and the discovery of penicillin. Available from: http://www.abpischools.org.uk/page/modules/infectiousdiseases_timeline/timeline6.cfm?coSiteNavigation_allTopic=1

188. Durckheimer BW, Blumbach J, Lattrell R. Recent Developments in the Field of B-Lactam Antibiotics. Angew Chemie Int Ed. 1985;24:180–202.

189. Tomasz a. The mechanism of the irreversible antimicrobial effects of penicillins: how the beta-lactam antibiotics kill and lyse bacteria. Annu Rev Microbiol. 1979;33:113–37.

190. Lin* XZ and J. Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria [Internet]. Frointers in Microbiology. 2013 [cited 2015 Oct 9]. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2013.00128/full

191. McDougal LK, Thornsberry C. The role of B-lactamase in staphylococcal resistance to penicillinase-resistant penicillins and cephalosporins. J Clin Microbiol. 1986;23(5):832–9.

192. Vu H, Nikaido H. Role of β-lactam hydrolysis in the mechanism of resistance of a β-lactamase-constitutive Enterobacter cloacae strain to expanded-spectrum β-lactams. Antimicrob Agents Chemother [Internet]. 1985;27(3):393–8. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-0021954359&partnerID=40&md5=9727bd03a3e61ba1735d6973026e5c5a

193. Anna Tribble and Uchechi Oranusi. B-Lactamase [Internet]. Biochemistry Protien Projects: Berea College. 2013 [cited 2015 Oct 9]. Available from: http://chemistry.berea.edu/~biochemistry/2013/uo_at/

194. Watanakunakorn C. Mode of action and in-vitro activity of vancomycin. J Antimicrob Chemother [Internet]. 1984;14 Suppl D:7–18. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6440886

195. Hammes WP, Neuhaus FC. On the mechanism of action of vancomycin: inhibition of peptidoglycan synthesis in Gaffkya homari. Antimicrob Agents Chemother. 1974;6(6):722–8.

196. Etscovitz E. The mode of action of sulfonamides. Tufts Med J. 1945;13(2):13–6.

197. Jersey B 224 with W at U of M and D of N. Antimicrobial Drugs Pt. 1 [Internet]. 2015 [cited 2015 Oct 9]. p. Study Blue. Available from: https://www.studyblue.com/notes/note/n/antimicrobial-drugs-pt-1/deck/907368

198. Combs MT, Castagnoli N, Glanville JO, Anderson MR. Optimal Analysis of Sulfonamides From Biological Matrices. 1997;

199. Miller-hjelle M a, Somaraju V. Synthetic Organic Antimicrobials: Sulfonamides, Trimethoprim, Nitrofurans, Quinolones, Methenamine. In: Modern

Pharmacology with Clinical Applications. 6th ed. Philadelphia : Lippincott Williams & Wilkins,; 2004. p. 515–25.

200. by Eric J. Rentz, DO Ms. An Indepth History of Silver [Internet]. 2015 [cited 2015 Oct 8]. Available from: http://www.hydrosolinfo.com/articles/history-of-silver.php

201. Chopra I. The increasing use of silver-based products as antimicrobial agents: a useful development or a cause for concern? J Antimicrob Chemother [Internet]. 2007;59(4):587–90. Available from: http://www.jac.oxfordjournals.org/cgi/doi/10.1093/jac/dkm006

202. Tan SP, McLoughlin P, O'Sullivan L, Prieto ML, Gardiner GE, Lawlor PG, et al. Development of a novel antimicrobial seaweed extract-based hydrogel wound dressing. Int J Pharm [Internet]. 2013;456(1):10–20. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0378517313007400

203. Hadrup N, Lam HR. Oral toxicity of silver ions, silver nanoparticles and colloidal silver - A review. Regul Toxicol Pharmacol [Internet]. 2014;68(1):1–7. Available from: http://dx.doi.org/10.1016/j.yrtph.2013.11.002

204. AshaRani P V, Low Kah Mun G, Hande MP, Valiyaveettil S. Cytotoxicity and genotoxicity of silver nanoparticles in human cells - SOM. ACS Nano [Internet]. 2009;3(2):279–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19236062

205. Deitch E a, Marino a a, Malakanok V, Albright J a. Silver nylon cloth: in vitro and in vivo evaluation of antimicrobial activity. J Trauma [Internet]. 1987;27(3):301–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3560272

206. Cavanagh MH, Burrell RE, Nadworny PL. Evaluating antimicrobial efficacy of new commercially available silver dressings. Int Wound J [Internet]. 2010;7(5):394–405. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20626470

207. Jung WK, Koo HC, Kim KW, Shin S, Kim SH, Park YH. Antibacterial activity and mechanism of action of the silver ion in Staphylococcus aureus and

Escherichia coli. Appl Environ Microbiol [Internet]. 2008;74(7):2171–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18245232

208. Modak SM, Fox CL. Binding of silver sulfadiazine to the cellular components of Pseudomonas aeruginosa. Biochem Pharmacol. 1973;22(19):2391–404.

209. Radulescu M, Andronescu E, Dolete G, Popescu R, Fufă O, Chifiriuc M, et al. Silver Nanocoatings for Reducing the Exogenous Microbial Colonization of Wound Dressings. Materials (Basel) [Internet]. 2016;9(5):345. Available from: http://www.mdpi.com/1996-1944/9/5/345

210. Massand S, Cheema F, Brown S, Davis WJ, Surgeon AP, Burkey B, et al. The use of a chitosan dressing with silver in the management of paediatric burn wounds: a pilot study. :26–30.

211. Sütterlin S. Aspects of Bacterial Resistance to Silver. 2015.

212. Leaper PD. Appropriate Use of Silver Dressings in Wounds. In: Wounds International. London; 2012.

213. Munteanu A, Ip F, Nitescu C. A modern method of treatment : The role of silver dressings in promoting healing and preventing pathological scarring in patients with burn wounds. J Med Life. 2016;9(3):306–15.

214. Trop M, Novak M, Rodl S, Hellbom B, Kroell W, Goessler W. Silver-Coated Dressing Acticoat Caused Raised Liver Enzymes and Argyria-like Symptoms in Burn Patient. J Trauma Inj Infect Crit Care [Internet]. 2006;60(3):648–52. Available from: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00005373-200603000-00028

215. Tonks a. Honey stimulates inflammatory cytokine production from monocytes. Cytokine [Internet]. 2003;21(5):242–7. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1043466603000929

216. Walker L. FDA Quietly Acknowledges Medical Benefits of Honey [Internet]. Natural News. [cited 2015 Oct 8]. Available from: http://www.naturalnews.com/022872_honey_FDA_benefits.html

217. Cutting KF. Antibacterial Honey (Medihoney^{TM}): in-vitro Activity Against Clinical Isolates of MRSA, VRE, and Other Multiresistant Gram-negative Organisms Including Pseudomonas aeruginosa. 2007;19(9).

218. Visavadia BG, Honeysett J, Danford MH. Manuka honey dressing: An effective treatment for chronic wound infections. Br J Oral Maxillofac Surg [Internet]. 2008;46(1):55–6. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0266435606001860

219. Guthrie HC, Martin KR, Taylor C, Spear AM, Whiting R, Macildowie S, et al. A pre-clinical evaluation of silver, iodine and Manuka honey based dressings in a model of traumatic extremity wounds contaminated with Staphylococcus aureus. Injury [Internet]. 2014;45(8):1171–8. Available from: http://dx.doi.org/10.1016/j.injury.2014.05.007

220. Yusof N, Ainul Hafiza a. H, Zohdi RM, Bakar MZ a. Development of honey hydrogel dressing for enhanced wound healing. Radiat Phys Chem [Internet]. 2007;76(11–12):1767–70. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0969806X0700237X

221. Molan PC. The Evidence Supporting the Use of Honey as a Wound Dressing. Int J Low Extrem Wounds [Internet]. 2006;5(1):40–54. Available from: http://ijl.sagepub.com/cgi/doi/10.1177/1534734605286014

222. Derma Sciences Europe Ltd. About Medihoney [Internet]. 2017 [cited 2017 Jul 7]. Available from: http://outside-us.dermasciences.com/medihoney

223. Pangestuti R, Kim SK. Biological activities and health benefit effects of natural pigments derived from marine algae. J Funct Foods [Internet]. 2011;3(4):255–66. Available from: http://dx.doi.org/10.1016/j.jff.2011.07.001

224. Sciences A. Honey As Complementary Medicine : - a Review. Int J Pharma Bio Sci. 2012;3(2):12–31.

225. Molan PC. THE ANTIBACTERIAL ACTIVITY OF HONEY. Variation in the potency of the antibacterial activity Variation in antibacterial activity. Bee World. 1992;73(2):59–76.

226. IndustryARC. Bioactive Wound Care Market to Reach 4.9 Billion USD by 2020 - IndustryARC Research [Internet]. 2016 [cited 2017 Aug 1]. p. Cision PR Newswire. Available from: http://www.prnewswire.com/news-releases/bioactive-wound-care-market-to-reach-49-billion-usd-by-2020---industryarc-research-570105551.html

227. McDonnell G, Russell AD. Antiseptics and disinfectants: Activity, action, and resistance. Clin Microbiol Rev. 2001;12(1):227–8.

228. Gottardi W. Iodine and Iodine Compounds. In: Febiger L, editor. Disinfection, sterilization, and preservation. 4th ed. Philadelphia; 1991.

229. Bradshaw CE. An in vitro comparison of the antimicrobial activity of honey, iodine and silver wound dressings. Biosci Horizons. 2011;4(1):61–70.

230. Leaper DJ, Duani P. Topical antimicrobial therapy of chronic wounds healing by secondary intention using iodine products. Int Wound J. 2008;5(2):361–8.

231. Russell AD. Similarities and differences in the responses of microorganisms to biocides. J Antimicrob Chemother. 2003;52(5):750–63.

232. Uchiyama S, Dahesh S, Nizet V, Kessler J. Enhanced topical delivery of non-complexed molecular iodine for Methicillin-resistant Staphylococcus aureus decolonization. Int J Pharm [Internet]. 2019;554(May 2018):81–6. Available from: https://doi.org/10.1016/j.ijpharm.2018.11.004

233. Vermeulen H, Westerbos SJ, Ubbink DT. Benefit and harm of iodine in wound care : a systematic review. J Hosp Infect [Internet]. 2010;76(3):191–9. Available from: http://dx.doi.org/10.1016/j.jhin.2010.04.026

234. Baliano AP, Pimentel EF, Buzin AR, Vieira TZ, Romão W, Tose L V., et al. Brown seaweed Padina gymnospora is a prominent natural wound-care product. Rev Bras Farmacogn [Internet]. 2016;26(6):714–9. Available from: http://dx.doi.org/10.1016/j.bjp.2016.07.003

235. Pérez MJ, Falqué E, Domínguez H. Antimicrobial action of compounds from marine seaweed. Mar Drugs. 2016;14(3):1–38.

236. Paul W, Sharma C. Chitosan and alginate wound dressings: a short review.

Trends Biomater Artif Organs [Internet]. 2004;18(1):18–23. Available from: http://medind.nic.in/taa/t04/i1/taat04i1p18.pdf

237. Boateng J, Catanzano O. Advanced Therapeutic Dressings for Effective Wound Healing-A Review. J Pharm Sci [Internet]. 2015;104:n/a-n/3653-3680. Available from: http://doi.wiley.com/10.1002/jps.24610

238. GlobeNewswire Inc. Bioactive Wound Care Market To Reach USD 13.50 Billion By 2026 [Internet]. West. 2019 [cited 2019 Jun 26]. p. Reports and Data. Available from: https://www.globenewswire.com/news-release/2019/05/16/1826492/0/en/Bioactive-Wound-Care-Market-To-Reach-USD-13-50-Billion-By-2026-Reports-And-Data.html

239. Abdelrahman T, Newton H. Wound dressings: Principles and practice. Surgery [Internet]. 2011;29(10):491–5. Available from: http://dx.doi.org/10.1016/j.mpsur.2011.06.007

240. Benbow M. Exploring the concept of moist wound healing and its application in practice. Br J Nurs. 2008;17(15):s4–16.

241. Sood A, Granick MS, Tomaselli NL. Wound Dressings and Comparative Effectiveness Data. Adv Wound Care [Internet]. 2014;3(8):511–29. Available from: http://online.liebertpub.com/doi/abs/10.1089/wound.2012.0401

242. Jørgensen B, Friis GJ, Gottrup F. Pain and quality of life for patients with venous leg ulcers: Proof of concept of the efficacy of Biatain®-Ibu, a new pain reducing wound dressing. Wound Repair Regen. 2006;14(3):233–9.

243. Sezer AD, Cevher E, Hatipoğlu F, Oğurtan Z, Baş AL, Akbuğa J. Preparation of fucoidan-chitosan hydrogel and its application as burn healing accelerator on rabbits. Biol Pharm Bull. 2008;31(12):2326–33.

244. Liakos I, Rizzello L, Scurr DJ, Pompa PP, Bayer IS, Athanassiou A. All-natural composite wound dressing films of essential oils encapsulated in sodium alginate with antimicrobial properties. Int J Pharm [Internet]. 2014;463(2):137–45. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0378517313009605

245. Rossi S, Marciello M, Sandri G, Ferrari F, Bonferoni MC, Papetti A, et al.

Wound dressings based on chitosans and hyaluronic acid for the release of chlorhexidine diacetate in skin ulcer therapy. Pharm Dev Technol [Internet]. 2007;12(4):415–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17763146

246. Broussard KC, Powers JG. Wound dressings: Selecting the most appropriate type. Am J Clin Dermatol. 2013;14(6):449–59.

247. Adis Medical Writers. Select appropriate wound dressings by matching the properties of the dressing to the type of wound. Drugs Ther Perspect. 2014;30(6):213–7.

248. Murphy PS, Evans GRD. Advances in Wound Healing: A Review of Current Wound Healing Products. Plast Surg Int [Internet]. 2012;2012:1–8. Available from: http://www.hindawi.com/journals/psi/2012/190436/

249. Zilberman M, Egozi D, Shemesh M, Keren A, Mazor E, Baranes-Zeevi M, et al. Hybrid wound dressings with controlled release of antibiotics: Structure-release profile effects and in vivo study in a guinea pig burn model. Acta Biomater [Internet]. 2015;22:155–63. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1742706115002007

250. Thompson D, Oster G. Use of Terfenadine and Contraindicated Drugs. JAMA J Am Med Assoc. 2011;276(12):953.

251. Tan HL, Lin WT, Tang TT. The use of antimicrobial-impregnated PMMA to manage periprosthetic infections: Controversial issues and the latest developments. Int J Artif Organs. 2012;35(10):832–9.

252. Neill JIMO. Tackling Drug-Resistant Infections Globally : Final Report and Recommendations. 2016.

253. Todd J. Polymethylmethacrylate : Properties and Contemporary Uses in Orthopaedics Abstract. J Am Acad Orthop Surg. 2010;18(5):297–305.

254. Elsner JJ, Berdicevsky I, Zilberman M. In vitro microbial inhibition and cellular response to novel biodegradable composite wound dressings with controlled release of antibiotics. Acta Biomater [Internet]. 2011;7(1):325–36. Available

from: http://dx.doi.org/10.1016/j.actbio.2010.07.013

255. Rowan MP, Cancio LC, Elster E a, Burmeister DM, Rose LF, Natesan S, et al. Burn wound healing and treatment: review and advancements. Crit Care [Internet]. 2015;19(1):243. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4464872&tool=pmcentrez&rendertype=abstract

256. Dhivya S, Viswanadha VP, Elango S. Wound dressings – a review. BioMedicine. 2015;5(4):24–8.

257. Gustaite S, Kazlauske J, Bobokalonov J, Perni S, Dutschk V, Liesiene J, et al. Characterization of cellulose based sponges for wound dressings. Colloids Surfaces A Physicochem Eng Asp [Internet]. 2015;480:336–42. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0927775714006967

258. Tavakoli J, Tang Y. Honey/PVA hybrid wound dressings with controlled release of antibiotics: Structural, physico-mechanical and in-vitro biomedical studies. Mater Sci Eng C [Internet]. 2017;77:318–25. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0928493116320938

259. Yamanaka S, Watanabe K, Kitamura N, Iguchi M, Mitsuhashi S, Nishi Y, et al. The structure and mechanical properties of sheets prepared from bacterial cellulose. J Mater Sci. 1989;24:3141–5.

260. Mogoşanu GD, Grumezescu AM. Natural and synthetic polymers for wounds and burns dressing. Int J Pharm [Internet]. 2014;463(2):127–36. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0378517313010831

261. Turner NJ, Badylak SF. The Use of Biologic Scaffolds in the Treatment of Chronic Nonhealing Wounds. Adv Wound Care. 2014;4(8):490–500.

262. Ul-Islam M, Khan T, Khattak WA, Park JK. Bacterial cellulose-MMTs nanoreinforced composite films: novel wound dressing material with antibacterial properties. Cellulose [Internet]. 2013;20(2):589–96. Available from: http://link.springer.com/10.1007/s10570-012-9849-3

263. Ip M, Lui SL, Poon VKM, Lung I, Burd A. Antimicrobial activities of silver

dressings: An in vitro comparison. J Med Microbiol. 2006;55(1):59–63.

264. Austrainian Home Nursing Supplies. Acticoat 3 Day Silver Dressing 5cm x 5cm – Box of 5 [Internet]. 2018 [cited 2019 Jun 26]. Available from: https://www.ahns.com.au/product/wound-care/acticoat-3day-silver-dressing-05cm-x-05cm-box-of-5/

265. Dessy LA, Serratore F, Corrias F, Parisi P, Mazzocchi M, Carlesimo B. Retention of polyurethane foam fragments during VAC therapy: A complication to be considered. Int Wound J. 2015;12(2):132–6.

266. Sibbald RG, Coutts P, Woo KY. Reduction of Bacterial Burden and Pain in Chronic Wounds Using a New Polyhexamethylene Biguanide Antimicrobial Foam Dressing-Clinical Trial Results. Adv Skin Wound Care. 2011;24(2):78–84.

267. Han SS. Topical formulations of water-soluble chitin as a wound healing assistant. Fibers Polym [Internet]. 2005;6(3):219–23. Available from: http://link.springer.com/10.1007/BF02875645

268. Anaya P, Cárdenas G, Lavayen V, García A, O'Dwyer C. Chitosan gel film bandages: Correlating structure, composition, and antimicrobial properties. J Appl Polym Sci. 2013;128(6):3939–48.

269. Tanihara M, Suzuki Y, Nishimura Y, Suzuki K, Kakimaru Y, Fukunishi Y. A novel microbial infection-responsive drug release system. J Pharm Sci. 1999;88(5):510–4.

270. Sezer AD, Hatipoğlu F, Cevher E, Oğurtan Z, Baş AL, Akbuğa J. Chitosan film containing fucoidan as a wound dressing for dermal burn healing: preparation and in vitro/in vivo evaluation. AAPS PharmSciTech. 2007;8(2):Article 39.

271. Andersen T, Auk-Emblem P, Dornish M. 3D Cell Culture in Alginate Hydrogels. Microarrays [Internet]. 2015;4(2):133–61. Available from: http://www.mdpi.com/2076-3905/4/2/133/htm

272. Kadam SU, Tiwari BK, Smyth TJ, O'Donnell CP. Optimization of ultrasound assisted extraction of bioactive components from brown seaweed Ascophyllum nodosum using response surface methodology. Ultrason Sonochem [Internet].

2015;23:308–16. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1350417714003137

273. Tan SP. Extraction, Isolation and Identification of Antimicrobial Compounds from Irish Seaweeds and their Potential Use in Wound Dressings. Waterford Institute of Technology; 2013.

274. Ponce NM a, Pujol C a., Damonte EB, Flores ML, Stortz C a. Fucoidans from the brown seaweed Adenocystis utricularis: Extraction methods, antiviral activity and structural studies. Carbohydr Res. 2003;338:153–65.

275. Ovington LG. Advances in wound dressings. Clin Dermatol. 2007;25(1):33–8.

276. Kamoun E a., Kenawy E-RS, Tamer TM, El-Meligy M a., Mohy Eldin MS. Poly (vinyl alcohol)-alginate physically crosslinked hydrogel membranes for wound dressing applications: Characterization and bio-evaluation. Arab J Chem [Internet]. 2015;8(1):38–47. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1878535213004310

277. Balakrishnan B, Mohanty M, Umashankar P, Jayakrishnan a. Evaluation of an in situ forming hydrogel wound dressing based on oxidized alginate and gelatin. Biomaterials [Internet]. 2005;26(32):6335–42. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0142961205003194

278. Vieira MGA, Da Silva MA, Dos Santos LO, Beppu MM. Natural-based plasticizers and biopolymer films: A review. Eur Polym J [Internet]. 2011;47(3):254–63. Available from: http://dx.doi.org/10.1016/j.eurpolymj.2010.12.011

279. Hashemi SA, Madani SA, Abediankenari S. The Review on Properties of Aloe Vera in Healing of Cutaneous Wounds. Biomed Res Int [Internet]. 2015;2015:714216. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4452276&tool=pmcentrez&rendertype=abstract

280. Chandika P, Ko SC, Jung WK. Marine-derived biological macromolecule-based biomaterials for wound healing and skin tissue regeneration. Int J Biol Macromol [Internet]. 2015;77:24–35. Available from:

http://dx.doi.org/10.1016/j.ijbiomac.2015.02.050

281. Dhargalkar VK, Kavlekar D. Seaweeds – A field Manual - National Institute of Oceanography. Natl Inst Oceanogr. 2004;

282. Burgess JG, Jordan EM, Bregu M, Mearns-Spragg A, Boyd KG. Microbial antagonism: a neglected avenue of natural products research. Prog Ind Microbiol. 1999;35:27–32.

283. Singh SB, Young K, Silver LL. What is an "Ideal" Antibiotic? Discovery Challenges and Path forward. Biochem Pharmacol [Internet]. 2017;(January). Available from: http://dx.doi.org/10.1016/j.bcp.2017.01.003

284. European Centre for Disease Prevention and Control. Antimicrobial Resistance and Healthcare-associated Infections Programme [Internet]. 2019 [cited 2019 May 4]. Available from: https://ecdc.europa.eu/en/about-uswho-we-aredisease-programmes/antimicrobial-resistance-and-healthcare-associated-infections

285. The Department of Health. Ireland's National Action Plan on Antimicrobial Resistance 2017 – 2020. 2017.

286. Hornsey IS, Hide D. The production of antimicrobial compounds by British marine algae I. Antibiotic-producing marine algae. Br Phycol J. 1974;9(4):353–61.

287. Cox S, Hamilton Turley G, Rajauria G, Abu-Ghannam N, Jaiswal AK. Antioxidant potential and antimicrobial efficacy of seaweed (Himanthalia elongata) extract in model food systems. J Appl Phycol. 2014;26(4):1823–31.

288. Baleta FN, Bolaños JM, Ruma OC, Baleta AN, Cairel JD. Phytochemicals screening and antimicrobial properties of Sargassum oligocystum and Sargassum crassifolium Extracts. 2017;5(1):382–7.

289. Lustigman B, Brown C. Antibiotic production by marine algae isolated from the New York/New Jersey coast. Bull Environ Contam Toxicol. 1991;46(3):329–35.

290. Wahl M, ShahnazL., Dobretsov S, Saha M, Symanowski F, DavidK., et al. Ecology of antifouling resistance in the bladder wrack Fucus vesiculosus: Patterns of microfouling and antimicrobial protection. Mar Ecol Prog Ser.

2010;411:33–48.

291. Saha M, Rempt M, Grosser K, Pohnert G, Weinberger F. Surface-associated fucoxanthin mediates settlement of bacterial epiphytes on the rockweed Fucus vesiculosus. Biofouling. 2011;27(4):423–33.

292. El Wahidi M, El Amraoui B, El Amraoui M, Bamhaoud T. Screening of antimicrobial activity of macroalgae extracts from the moroccan Atlantic coast. Ann Pharm Fr [Internet]. 2015;73(3):190–6. Available from: http://dx.doi.org/10.1016/j.pharma.2014.12.005

293. Paterson GK, Harrison EM, Holmes MA. The emergence of mecC methicillin-resistant Staphylococcus aureus. Trends Microbiol [Internet]. 2014;22(1):42–7. Available from: http://dx.doi.org/10.1016/j.tim.2013.11.003

294. Hasan R, Acharjee M, Noor R. Prevalence of vancomycin resistant Staphylococcus aureus (VRSA) in methicillin resistant S. aureus (MRSA) strains isolated from burn wound infections. Tzu Chi Med J [Internet]. 2016;28(2):49–53. Available from: http://dx.doi.org/10.1016/j.tcmj.2016.03.002

295. Khan MF. Brief History of Staphylococcus aureus: A Focus to Antibiotic Resistance. EC Microbiol. 2017;2:36–9.

296. Zeana C, Kubin CJ, Della-Latta P, Hammer SM. Vancomycin-resistant Enterococcus faecium meningitis successfully managed with linezolid: case report and review of the literature. ClinInfectDis [Internet]. 2001;33(1058–4838):477–82. Available from: c:%5CKarsten%5CPDFs%5CInfektiologie-PDFs%5CInfekt-2001%5CZeana et al.-Vancomycin-resistant E.faecium meningitis successfully managed with linezolid.pdf

297. Doern CD, Park JY, Gallegos M, Alspaugh D, Burnham CAD. Investigation of linezolid resistance in staphylococci and enterococci. J Clin Microbiol. 2016;54(5):1289–94.

298. Diekema DJ, Jones RN. New drug classes Oxazolidinone antibiotics. Lancet. 2001;358:1975–82.

299. EUCAST. Antimicrobial susceptibility testing EUCAST disk diffusion method -

Version 6.0. 2017;0(January):1–22.

300. Clinical Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically ; Approved Standard — Ninth Edition. Vol. 32. 2012.

301. Vadivambal R, Jayas DS. Changes in quality of microwave-treated agricultural products-a review. Biosyst Eng. 2007;98(1):1–16.

302. Catarino MD, Silva AMS, Cardoso SM. Phycochemical constituents and biological activities of Fucus spp. Mar Drugs. 2018;16(8).

303. Wang T, Jónsdóttir R, Liu H, Gu L, Kristinsson HG, Raghavan S, et al. Antioxidant capacities of phlorotannins extracted from the brown algae Fucus vesiculosus. J Agric Food Chem. 2012;60(23):5874–83.

304. Zvyagintseva TN, Shevchenko NM, Popivnich IB, Isakov V V., Scobun AS, Sundukova E V., et al. A new procedure for the separation of water-soluble polysaccharides from brown seaweeds. Carbohydr Res. 1999;322(1–2):32–9.

305. Fisher Scientific. Summary of Key Physical Data for Solvents [Internet]. 2019 [cited 2019 Sep 24]. p. Technical Tools. Available from: https://www.fishersci.no/no/en/scientific-products/technical-tools/summary-key-physical-data-solvents.html

306. Águila-Ramírez RN, Arenas-gonzález A, Hernández-guerrero CJ, González-acosta B, Borges-souza JM, Véron B, et al. Antimicrobial and antifouling activities achieved by extracts of seaweeds from Gulf of California , Mexico Actividades antimicrobiana y anti-incrustante obtenidas de los extractos de algas marinas del Golfo de California , México. Hidrobiologica. 2012;22(1):8–15.

307. Vijayabaskar P, Vaseela N, Thirumaran G. Potential antibacterial and antioxidant properties of a sulfated polysaccharide from the brown marine algae Sargassum swartzii. Chin J Nat Med. 2012;10(6):421–8.

308. Black WAP. Seasonal variation in the chemical composition of some of the littoral seaweeds common to Scotland. JSCI. 1949;183–9.

309. Munda IM, Kremer BP. Chemical composition and physiological properties of

fucoids under conditions of reduced salinity. Mar Biol. 1977;42(1):9–15.

310. Wijesinghe W a JP, Jeon YJ. Biological activities and potential industrial applications of fucose rich sulfated polysaccharides and fucoidans isolated from brown seaweeds: A review. Carbohydr Polym [Internet]. 2012;88(1):13–20. Available from: http://dx.doi.org/10.1016/j.carbpol.2011.12.029

311. Rodríguez-Jasso RM, Mussatto SI, Pastrana L, Aguilar CN, Teixeira JA. Extraction of sulfated polysaccharides by autohydrolysis of brown seaweed Fucus vesiculosus. J Appl Phycol. 2013;25(1):31–9.

312. Jun J-Y, Jung M-J, Jeong I-H, Yamazaki K, Kawai Y, Kim B-M. Antimicrobial and Antibiofilm Activities of Sulfated Polysaccharides from Marine Algae against Dental Plaque Bacteria. Mar Drugs [Internet]. 2018;16(9):301. Available from: http://www.mdpi.com/1660-3397/16/9/301

313. Marudhupandi T, Kumar TTA. Antibacterial effect of fucoidan from Sargassum wightii against the chosen human bacterial pathogens. Int Curr Pharm J. 2013;2(10):156–8.

314. Dierick N, Ovyn A, De Smet S. In vitro assessment of the effect of intact marine brown macro-algae Ascophyllum nodosum on the gut flora of piglets. Livest Sci [Internet]. 2010;133(1–3):154–6. Available from: http://dx.doi.org/10.1016/j.livsci.2010.06.051

315. Le Tutour B, Benslimane F, Gouleau MP, Gouygou JP, Saadan B, Quemeneur F. Antioxidant and pro-oxidant activities of the brown algae, Laminaria digitata, Himanthalia elongata, Fucus vesiculosus, Fucus serratus and Ascophyllum nodosum. J Appl Phycol. 1998;10(2):121–9.

316. Costa E, Domingues P, Melo T, Coelho E, Pereira R, Calado R, et al. Lipidomic Signatures Reveal Seasonal Shifts on the Relative Abundance of High-Valued Lipids from the Brown Algae Fucus vesiculosus. Mar Drugs. 2019;17(335):1–23.

317. Farvin KHS, Jacobsen C. Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. Food Chem [Internet]. 2013;138(2–3):1670–81. Available from: http://dx.doi.org/10.1016/j.foodchem.2012.10.078

318. Kim S, Woo S, Yun H, Yum S, Choi E, Do J, et al. Total Phenolic Contents and Biological Activities of Korean Seaweed Extracts. Food Sci Biotechnol. 2005;14(6):1–5.

319. Clinical and Laboratory Standards Institute. IQCP for Disk Diffusion Antimicrobial Susceptibility Testing (AST). 2011.

320. Haste NM, Hughes CC, Tran DN, Fenical W, Jensen PR, Nizet V, et al. Pharmacological properties of the marine natural product marinopyrrole a against methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2011;55(7):3305–12.

321. Liñares J. The VISA/GISA problem: therapeutic implications. Clin Microbiol Infect [Internet]. 2001 [cited 2016 Nov 22];7:8–15. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1198743X15301117

322. Mayhall CG. The epidemiology of burn wound infections: then and now. ClinInfectDis [Internet]. 2003;37(1537-6591 (Electronic)):543–50. Available from: c:%5CKarsten%5CPDFs%5CInfektiologie-PDFs%5CInfekt-2003%5CMayhall-The epidemiology of burn wound infections- then and now.pdf

323. Val AG Del, Platas G, Basilio A, Cabello A, Gorrochategui J, Suay I, et al. Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria (Canary Islands, Spain). Int Microbiol. 2001;4(1):35–40.

324. Capello C, Fischer U, Hungerbühler K. What is a green solvent? A comprehensive framework for the environmental assessment of solvents. Green Chem. 2007;9(9):927–34.

325. Soni & Associates Inc. GRAS Notification for Fucoidan Concentrate from Fucus vesiculosus [Internet]. 2016. p. 1–68. Available from: http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm

326. Isnansetyo A, Laili Lutfia FN, Nursid M, T T, Susidarti RA. Cytotoxicity of Fucoidan from Three Tropical Brown Algae Against Breast and Colon Cancer Cell Lines. Pharmacogn J. 2016;9(1):14–20.

327. Choi Y, Lee SM, Chun J, Lee HB, Lee J. Influence of heat treatment on the antioxidant activities and polyphenolic compounds of Shiitake (Lentinus edodes) mushroom. Food Chem. 2006;99(2):381–7.

328. Donghai LI, Xiang R. Heat Denaturation of Protein Structures and Chlorophyll States in PSII Membranes *. TSINGHUA Sci Technol. 2002;7(4):407–10.

329. Shin HY, Lim SM, Bae SY, Oh SC. Thermal decomposition and stability of fatty acid methyl esters in supercritical methanol. J Anal Appl Pyrolysis [Internet]. 2011;92(2):332–8. Available from: http://dx.doi.org/10.1016/j.jaap.2011.07.003

330. Nishiguchi T, Jiang Z, Ueno M, Takeshita S, Cho K, Roh SW, et al. Reevaluation of bactericidal, cytotoxic, and macrophage-stimulating activities of commercially available Fucus vesiculosus fucoidan. Algae. 2014;29(3):237–47.

331. Senthilkumar K, Manivasagan P, Venkatesan J, Kim SK. Brown seaweed fucoidan: Biological activity and apoptosis, growth signaling mechanism in cancer. Int J Biol Macromol [Internet]. 2013;60:366–74. Available from: http://dx.doi.org/10.1016/j.ijbiomac.2013.06.030

332. Tajbakhsn S, Pouyan M, Zandi K, Bahramiian P, Sartavi K, Fouladwand M, et al. In vitro study of anibacterial activity of the alga Sargassum oligocystum from the Persian Gulf. Eur Rev Med Pharmacol Sci. 2011;15:293–8.

333. Sandsdalen E, Haug T, Stensvag K. The antibacterial effect of polyhydroxylated fucophlorethol from the marine broown alga, Fucus vesiculosus. World J Microbiol Biotechnol. 2003;1621:777–82.

334. Choi S, Jang E-J, Cha J-D. Synergistic Effect between Fucoidan and Antibiotics against Clinic Methicillin-Resistant Staphylococcus aureus. Adv Biosci Biotechnol [Internet]. 2015;6(April):275–85. Available from: http://www.scirp.org/journal/abb%5Cnhttp://dx.doi.org/10.4236/abb.2015.64027%5Cnhttp://creativecommons.org/licenses/by/4.0/

335. Singh RP, Kumari P, Reddy CRK. Antimicrobial compounds from seaweeds-associated bacteria and fungi. Appl Microbiol Biotechnol. 2014;99(4):1571–86.

336. Wolcott RD, Kennedy JP, Dowd SE. Regular debridement is the main tool for

maintaining a healthy wound bed in most chronic wounds. J Wound Care. 2014;18(2):54–6.

337. Attinger C, Wolcott R. Clinically Addressing Biofilm in Chronic Wounds. Adv Wound Care. 2012;1(3):127–32.

338. Burger A. Biofilm formation mechanisms and targets for developing antibiofilm agents. J Med Chem. 2005;6(6):829–829.

339. Kavita K, Singh VK, Mishra A, Jha B. Characterisation and anti-biofilm activity of extracellular polymeric substances from Oceanobacillus iheyensis. Carbohydr Polym [Internet]. 2014;101(1):29–35. Available from: http://dx.doi.org/10.1016/j.carbpol.2013.08.099

340. Prabhakaran S, Rajaram R, Balasubramanian V, Mathivanan K. Antifouling potentials of extracts from seaweeds, seagrasses and mangroves against primary biofilm forming bacteria. Asian Pac J Trop Biomed [Internet]. 2012;2(1 SUPPL.):S316–22. Available from: http://dx.doi.org/10.1016/S2221-1691(12)60181-6

341. Busetti A, Thompson TP, Tegazzini D, Megaw J, Maggs CA, Gilmore BF. Antibiofilm activity of the brown alga Halidrys siliquosa against clinically relevant human pathogens. Mar Drugs. 2015;13(6):3581–605.

342. Deepa S, Venkateshwaran P, Vinithkumar NV, Kirubagaran R. Bioactive Propensity of Macroalgae from the Andaman & Nicobar Islands. Pharmacogn J. 2017;9(6):815–20.

343. Villa F, Cappitelli F. Plant-derived bioactive compounds at sub-lethal concentrations : towards smart biocide-free antibiofilm strategies. Phytochem Rev. 2013;12(1):245–54.

344. Hacker RG, Rachid S, Ohlsen K, Witte W. Effect of Subinhibitory Antibiotic Concentrations on Polysaccharide Intercellular Adhesin Expression in Biofilm-Forming Staphylococcus epidermidis. 2000;44(12):3357–63.

345. Mcaleese F, Wu SW, Sieradzki K, Dunman P, Murphy E, Projan S, et al. Overexpression of Genes of the Cell Wall Stimulon in Clinical Isolates of

Staphylococcus aureus Exhibiting Vancomycin-Intermediate- S . aureus -Type Resistance to Vancomycin. 2006;188(3):1120–33.

346. Mirani ZA, Jamil N. Effect of sub-lethal doses of vancomycin and oxacillin on biofilm formation by vancomycin intermediate resistant Staphylococcus aureus. 2011;(iii):191–5.

347. Riss TL, Moravec RA, Niles AL. Cytotoxicity Testing: Measuring Viable Cells, Dead Cells, and Detecting Mechanism of Cell Death. In: Stoddart MJ, editor. Mammalian Cell Viablilty: Methods in Molecula Biology [Internet]. Springer; 2011. p. 103–14. Available from: http://link.springer.com/10.1007/978-1-61779-108-6

348. International Organization for Standardization. ISO 10993-1 : Biological evaluation of medical devices — Part 1: Evaluation and testing within a risk management process. Int Stand. 2009;(Part 1):1–28.

349. Merck. Cell Viability and Proliferation Assays [Internet]. 2019 [cited 2019 May 3]. Available from: https://www.sigmaaldrich.com/technical-documents/articles/biofiles/cell-viability-and-proliferation.html

350. Choi S, Chung MH. A review on the relationship between aloe vera components and their biologic effects. Semin Integr Med. 2003;1(1):53–62.

351. Domingue G, Ellis B, Dasgupta M, Costerton JW. Testing antimicrobial susceptibilities of adherent bacteria by a method that incorporates guidelines of the National Committee for Clinical Laboratory Standards. J Clin Microbiol. 1994;32(10):2564–8.

352. Invitrogen & Gibco. Cell Culture Basics Handbook [Internet]. 2015. Available from: https://www.vanderbilt.edu/viibre/CellCultureBasicsEU.pdf

353. Kırmusaoğlu S. The Methods for Detection of Biofilm and Screening Antibiofilm Activity of Agents. In: Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods. 2016. p. 13.

354. Dobretsov S, Teplitski M, Paul V. Mini-review: Quorum sensing in the marine environment and its relationship to biofouling. Biofouling. 2009;25(5):413–27.

355. Salta M, Wharton JA, Dennington SP, Stoodley P. Anti-Biofilm Performance of Three Natural Products against Initial Bacterial Attachment. 2013;21757–80.

356. Saha M, Wahl M. Seasonal variation in the antifouling defence of the temperate brown alga Fucus vesiculosus. Biofouling. 2013;29(6):661–8.

357. Roy R, Tiwari M, Donelli G, Tiwari V. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence. 2018;9(1):522–54.

358. De Oliveira JFA, Saito Â, Bido AT, Kobarg J, Stassen HK, Cardoso MB. Defeating Bacterial Resistance and Preventing Mammalian Cells Toxicity Through Rational Design of Antibiotic-Functionalized Nanoparticles. Sci Rep. 2017;7(1):1–10.

359. Dillehay TD, Ramírez C, Pino M, Collins MB, Rossen J, Pino-Navarro JD. Monte Verde: seaweed, food, medicine, and the peopling of South America. Science [Internet]. 2008;320(5877):784–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18467586

360. Kendig EL, Le HH, Belcher SM. Defining hormesis: Evaluation of a complex concentration response phenomenon. Int J Toxicol. 2010;29(3):235–46.

361. Jones RA, Hoffman LR, Zhang Z, Miller SI, D'Argenio DA, MacCoss MJ. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature. 2005;436(7054):1171–5.

362. Linares JF, Gustafsson I, Baquero F, Martinez JL. Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci. 2006;103(51):19484–9.

363. Meerloo J Van, Kaspers GJL, Cloos J. Cancer Cell Culture: Cell senstitivity assays: The MTT assay. 2011;731(July 2015):237–45. Available from: http://link.springer.com/10.1007/978-1-61779-080-5

364. American Tissue Culture Collection. MTT Cell Proliferation Assay Instruction Guide. 6597:1–6. Available from: https://www.atcc.org/~/media/DA5285A1F52C414E864C966FD78C9A79.ashx

365. American Tissue Culture Collection. THP-1 (ATCC® TIB-202™) [Internet].

[cited 2017 Aug 15]. Available from: https://www.lgcstandards-atcc.org/products/all/TIB-202.aspx?geo_country=ie#culturemethod

366. Bosshart H, Heinzelmann M. THP-1 cells as a model for human monocytes. Ann Transl Med. 2016;4(21):438–438.

367. Jiao ZH, Li M, Feng YX, Shi JC, Zhang J, Shao B. Hormesis effects of silver nanoparticles at non-cytotoxic doses to human hepatoma cells. PLoS One. 2014;9(7).

368. Liu J, Simmons SO, Pei R. Regulation of IL-8 promoter activity by verrucarin A in human monocytic THP-1 cells. J Toxicol Environ Heal - Part A Curr Issues. 2014;77(19):1125–40.

369. Kingkaew J, Kirdponpattara S, Sanchavanakit N, Pavasant P, Phisalaphong M. Effect of molecular weight of chitosan on antimicrobial properties and tissue compatibility of chitosan-impregnated bacterial cellulose films. Biotechnol Bioprocess Eng. 2014;19(3):534–44.

370. American Tissue Culture Collection. Hep G2 [HEPG2] (ATCC® HB-8065™) [Internet]. 2016 [cited 2019 Apr 25]. Available from: https://www.lgcstandards-atcc.org/products/all/HB-8065.aspx?geo_country=ie

371. Kim EJ, Park SY, Lee JY, Park JHY. Fucoidan present in brown algae induces apoptosis of human colon cancer cells. BMC Gastroenterol. 2010;10.

372. Ale MT, Maruyama H, Tamauchi H, Mikkelsen JD, Meyer AS. Fucoidan from Sargassum sp. and Fucus vesiculosus reduces cell viability of lung carcinoma and melanoma cells in vitro and activates natural killer cells in mice in vivo. Int J Biol Macromol [Internet]. 2011;49(3):331–6. Available from: http://dx.doi.org/10.1016/j.ijbiomac.2011.05.009

373. Teruya T, Konishi T, Uechi S, Tamaki H, Tako M. Anti-proliferative activity of oversulfated fucoidan from commercially cultured Cladosiphon okamuranus TOKIDA in U937 cells. Int J Biol Macromol. 2007;41(3):221–6.

374. Wu L, Sun J, Su X, Yu Q, Yu Q, Zhang P. A review about the development of fucoidan in antitumor activity: Progress and challenges. Carbohydr Polym

[Internet]. 2016;154:96–111. Available from: http://dx.doi.org/10.1016/j.carbpol.2016.08.005

375. Noda M. Overview: molecular basis of neural circuit formation. Tanpakushitsu Kakusan Koso. 2004;49(3 Suppl):301–2.

376. Castejón N, Luna P, Señoráns FJ. Alternative oil extraction methods from Echium plantagineum L. seeds using advanced techniques and green solvents. Food Chem [Internet]. 2018;244(July 2017):75–82. Available from: http://dx.doi.org/10.1016/j.foodchem.2017.10.014

377. Tao Y, Xu W. Microwave-assisted solubilization and solution properties of hyperbranched polysaccharide. Carbohydr Res [Internet]. 2008;343(18):3071–8. Available from: http://dx.doi.org/10.1016/j.carres.2008.09.009

378. Bagherian H, Zokaee Ashtiani F, Fouladitajar A, Mohtashamy M. Comparisons between conventional, microwave- and ultrasound-assisted methods for extraction of pectin from grapefruit. Chem Eng Process Process Intensif [Internet]. 2011;50(11–12):1237–43. Available from: http://dx.doi.org/10.1016/j.cep.2011.08.002

379. Michalak I, Tuhy Ł, Chojnacka K. Seaweed extract by microwave assisted extraction as plant growth biostimulant. Open Chem. 2015;13(1):1183–95.

380. Zhang R, Yuen AKL, Magnusson M, Wright T, Nys R De, Masters AF, et al. A comparative assessment of the activity and structure of phlorotannins from the brown seaweed Carpophyllum flexuosum. Algal Res. 2018;29(August 2017):130–41.

381. Bekdeşer B, Durusoy N, Özyürek M, Güçlü K, Apak R. Optimization of microwave-assisted extraction of polyphenols from herbal teas and evaluation of their in vitro hypochlorous acid scavenging activity. J Agric Food Chem. 2014;62(46):11109–15.

382. Xiao X, Si X, Yuan Z, Xu X, Li G. Isolation of fucoxanthin from edible brown algae by microwave-assisted extraction coupled with high-speed countercurrent chromatography. J Sep Sci. 2012;35(17):2313–7.

383. Balasubramanian P, Praveen AM, Parvathy K, Jayabalan R. Evaluation of antioxidant properties of Brown Seaweed Polysaccharides (BSP) for prebiotic studies. In: International Conference on Recent Advances in Food Processing Technology (ICRAFPT 2018). Thanjavur ,Tamil Nadu, India.; 2018.

384. CEM. Operation Manual Microwave Accelerated Reaction System. North Carolina; 2001.

385. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity : A review. J Pharm Anal [Internet]. 2016;6(2):71–9. Available from: http://dx.doi.org/10.1016/j.jpha.2015.11.005

386. Toit EA, Rautenbach M. A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. J Microbiol Methods. 2000;42:159–65.

387. Parys S, Rosenbaum A, Kehraus S, Reher G, Glombitza KW, König GM. Evaluation of quantitative methods for the determination of polyphenols in algal extracts. J Nat Prod. 2007;70(12):1865–70.

388. Sugiono, Bambang Widjanarko S, Adi Soehono L. Extraction optimization by response surface methodology and characterization of Fucoidanfrom brown seaweed Sargassum polycystum. Int J ChemTech Res. 2014;6(1):195–205.

389. Spigno G, De Faveri DM. Microwave-assisted extraction of tea phenols: A phenomenological study. J Food Eng [Internet]. 2009;93(2):210–7. Available from: http://dx.doi.org/10.1016/j.jfoodeng.2009.01.006

390. Zheng X, Yin F, Liu C, Xu X. Effect of Process Parameters of Microwave Assisted Extraction (MAE) on Polysaccharides Yield from Pumpkin. J Northeast Agric Univ (English Ed [Internet]. 2012;18(2):79–86. Available from: http://dx.doi.org/10.1016/S1006-8104(12)60014-2

391. Putnik P, Kovačević DB, Penić M, Fegeš M, Dragović-Uzelac V. Microwave-Assisted Extraction (MAE) of Dalmatian Sage Leaves for the Optimal Yield of Polyphenols: HPLC-DAD Identification and Quantification. Food Anal Methods. 2016;9(8):2385–94.

392. Chan CH, Yusoff R, Ngoh GC, Kung FWL. Microwave-assisted extractions of active ingredients from plants. J Chromatogr A [Internet]. 2011;1218(37):6213–25. Available from: http://dx.doi.org/10.1016/j.chroma.2011.07.040

393. Al-Harahsheh M, Kingman SW. Microwave-assisted leaching - A review. Hydrometallurgy. 2004;73(3–4):189–203.

394. Bhuyan DJ, Van Vuong Q, Chalmers AC, van Altena IA, Bowyer MC, Scarlett CJ. Microwave-assisted extraction of Eucalyptus robusta leaf for the optimal yield of total phenolic compounds. Ind Crops Prod [Internet]. 2015;69:290–9. Available from: http://dx.doi.org/10.1016/j.indcrop.2015.02.044

395. Wang Y, You J, Yu Y, Qu C, Zhang H, Ding L, et al. Analysis of ginsenosides in Panax ginseng in high pressure microwave-assisted extraction. Food Chem. 2008;110(1):161–7.

396. Rodriguez-Jasso R, Mussatto S, Pastrana L, Aguilar C, Teixeira J. Chemical composition and antioxidant activity of sulphated polysaccharides extracted from Fucus vesiculosus using different hydrothermal processes. Chem Pap [Internet]. 2014;68(2):203–9. Available from: http://www.degruyter.com/view/j/chempap.2014.68.issue-2/s11696-013-0430-9/s11696-013-0430-9.xml

397. Wang T, Jónsdóttir R, Ólafsdóttir G. Total phenolic compounds , radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chem [Internet]. 2009;116(1):240–8. Available from: http://dx.doi.org/10.1016/j.foodchem.2009.02.041

398. Cho M, Lee H, Kang I, Won M, You S. Antioxidant properties of extract and fractions from Enteromorpha prolifera, a type of green seaweed. Food Chem [Internet]. 2011;127(3):999–1006. Available from: http://dx.doi.org/10.1016/j.foodchem.2011.01.072

399. Money Guide Ireland. Who Has The Cheapest Electricity Prices in Ireland? [Internet]. 2019 [cited 2019 Jun 19]. Available from: http://www.moneyguideireland.com/electricity-prices

400. Yao H, Du X, Yang L, Wang W, Yang F, Zhao C, et al. Microwave-assisted

method for simultaneous extraction and hydrolysis for determination of flavonol glycosides in Ginkgo foliage using bronsted acidic ionic-liquid [HO3S(CH2)4]HSO4 aqueous solutions. Int J Mol Sci. 2012;13(7):8775–88.

401. Zhang HF, Yang XH, Wang Y. Microwave assisted extraction of secondary metabolites from plants: Current status and future directions. Trends Food Sci Technol [Internet]. 2011;22(12):672–88. Available from: http://dx.doi.org/10.1016/j.tifs.2011.07.003

402. Moroney NC, O'Grady MN, Lordan S, Stanton C, Kerry JP. Seaweed polysaccharides (laminarin and fucoidan) as functional ingredients in pork meat: An evaluation of anti-oxidative potential, thermal stability and bioaccessibility. Mar Drugs. 2015;13(4):2447–64.

403. Pi??eiro Z, Marrufo-Curtido A, Vela C, Palma M. Microwave-assisted extraction of stilbenes from woody vine material. Food Bioprod Process [Internet]. 2017;103:18–26. Available from: http://dx.doi.org/10.1016/j.fbp.2017.02.006

404. Xiao W, Han L, Shi B. Microwave-assisted extraction of flavonoids from Radix Astragali. Sep Purif Technol. 2008;62(3):614–8.

405. Khajeh M, Akbari Moghaddam AR, Sanchooli E. Application of Doehlert design in the Optimization of microwave-assisted extraction for determination of zinc and copper in cereal samples using FAAS. Food Anal Methods. 2010;3(3):133–7.

406. Routray W, Orsat V. Microwave-Assisted Extraction of Flavonoids: A Review. Food Bioprocess Technol. 2012;5(2):409–24.

407. Guo Z, Jin Q, Fan G, Duan Y, Qin C, Wen M. Microwave-assisted extraction of effective constituents from a Chinese herbal medicine Radix puerariae. Anal Chim Acta. 2001;436(1):41–7.

408. Sparr E, Erland B. Analytical-scale microwave-assisted extraction. J Chromatogr A. 2000;902:227–50.

409. Fernández P, Vázquez C, Lorenzo RA, Carro AM, Álvarez I, Cabarcos P. Experimental design for optimization of microwave-assisted extraction of benzodiazepines in human plasma. Anal Bioanal Chem. 2010;397(2):677–85.

410. Vongsangnak W, Gua J, Chauvatcharin S, Zhong JJ. Towards efficient extraction of notoginseng saponins from cultured cells of Panax notoginseng. Biochem Eng J. 2004;18(2):115–20.

411. Vázquez Blanco E, López Mahía P, Muniategui Lorenzo S, Prada Rodríguez D, Fernández Fernández E. Optimization of microwave-assisted extraction of hydrocarbons in marine sediments: comparison with the Soxhlet extraction method. Fresenius J Anal Chem [Internet]. 2000;366(3):283–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11225673

412. Gao M, Song BZ, Liu CZ. Dynamic microwave-assisted extraction of flavonoids from Saussurea medusa Maxim cultured cells. Biochem Eng J. 2006;32(2):79–83.

413. Mandal V, Mohan Y, Hemalatha S. Microwave Assisted Extraction – An Innovative and Promising Extraction Tool for Medicinal Plant Research. Pharmacogn Rev [Internet]. 2007;1(1):7–18. Available from: http://www.sineo.cn/pic/article/42.pdf

414. Burke AC. Antibiotic Side Effects. Semin Respir Crit Care Med. 2000;21(1):1–10.

415. D'Amico R, Pifferi S, Leonetti C, Torri V, Tinazzi A, Liberati A. Effectiveness of antibiotic prophylaxis in critically ill adult patients: Systematic review of randomised controlled trials. Br Med J. 1998;316(7140):1275–85.

416. Krueger WA, Lenhart FP, Neeser G, Ruckdeschel G, Schreckhase H, Eissner HJ, et al. Influence of combined intravenous and topical antibiotic prophylaxis on the incidence of infections, organ dysfunctions, and mortality in critically III surgical patients: A prospective, stratified, randomized, double-blind, placebo-controlled clinical tr. Am J Respir Crit Care Med. 2002;166(8):1029–37.

417. Rainnie DJ, Bragg P, Bragg, P D and Rainnie DJ. The effect of silver ions on the respiratory chain of Escherichia coli. Can J Microbiol [Internet]. 1974;20(6):883–9. Available from: http://www.thesilveredge.com/pdf/The-Effect-of-Silver-Ions.pdf

418. Atiyeh BS, Costagliola M, Hayek SN, Dibo SA. Effect of silver on burn wound

infection control and healing: Review of the literature. Burns. 2007;33(2):139–48.

419. Poon VKM, Burd A. In vitro cytotoxity of silver: Implication for clinical wound care. Burns. 2004;30(2):140–7.

420. Gowda S, Desai PB, Hull V V, Math A a K, Vernekar SN, Kulkarni SS. A review on laboratory liver function tests. Pan Afr Med J [Internet]. 2009;3(November):17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21532726%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2984286

421. FDA. Derma Sciences Medihoney Primary Dressings with Active Manuka Honey. 501K. 2007;1–6.

422. FDA. 510K summary Amerigel [Internet]. Vol. 10. 2009. Available from: https://www.accessdata.fda.gov/cdrh_docs/pdf9/k092086.pdf

423. Moore J, Perkins A. Evaluating Antimicrobial Efficacy and Cost of 3 Dressings Containing Silver Versus a Novel Antimicrobial Hydrogel Impregnated Gauze Dressing Containing Oakin, an Oak Extract. Adv Skin Wound Care [Internet]. 2010;23(12):544–51. Available from: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00129334-201012000-00008

424. Kavoosi G, Dadfar SMM, Purfard AM. Mechanical, Physical, Antioxidant, and Antimicrobial Properties of Gelatin Films Incorporated with Thymol for Potential Use as Nano Wound Dressing. J Food Sci. 2013;78(2).

425. Orafidiya LO, Oyedele AO, Shittu AO, Elujoba AA. The formulation of an effective topical antibacterial product containing Ocimum gratissimum leaf essential oil. Int J Pharm. 2001;224(1–2):177–83.

426. Edwards-Jones V, Buck R, Shawcross SG, Dawson MM, Dunn K. The effect of essential oils on methicillin-resistant Staphylococcus aureus using a dressing model. Burns. 2004;30(8):772–7.

427. Seaman S. Dressing selection in chronic wound management. J Am Podiatr Med

Assoc. 2002;92(1):24–33.

428. Bennett-Marsden M. How to select a wound dressing. Clin Pharm. 2010;2(11):363–5.

429. Wang T, Zhu XK, Xue XT, Wu DY. Hydrogel sheets of chitosan, honey and gelatin as burn wound dressings. Carbohydr Polym [Internet]. 2012;88(1):75–83. Available from: http://dx.doi.org/10.1016/j.carbpol.2011.11.069

430. Lai HL, Abu'Khalil A, Craig DQM. The preparation and characterisation of drug-loaded alginate and chitosan sponges. Int J Pharm. 2003;251(1–2):175–81.

431. Ma L, Yu W, Ma X. Preparation and characterization of novel sodium alginate/chitosan two ply composite membranes. J Appl Polym Sci. 2007;106(1):394–9.

432. Sj H, Dw W, Dw T, Ke H, Sl P. An In vitro Comparison of Two Silver-containing Antimicrobial Wound Dressings . In Vitro. 2012;(January):22247408–22247408.

433. Blair SE, Carter DA. The potential for honey in the management of wounds and infection. Aust Infect Control [Internet]. 2007;10(1):24–31. Available from: http://dx.doi.org/10.1071/HI05024

434. Lusby PE, Coombes A, Wilkinson JM. Honey: a potent agent for wound healing? J Wound Ostomy Continence Nurs. 2002;29(6):295–300.

435. Bizerra FC, Da Silva PI, Hayashi MAF. Exploring the antibacterial properties of honey and its potential. Front Microbiol. 2012;3(November):2–3.

436. Cooper R. Iodine Revisited. J Chem Phys [Internet]. 2007;0(0):1–14. Available from: http://scitation.aip.org/content/aip/journal/jcp/55/1/10.1063/1.1675521

437. Moore SL, Payne, David N. Types of Antimicrobial Agents. In: Fraise AP, Lambert PA, Maillard J-Y, editors. Principles and Practice of Disinfection, Preservation and Sterilization. 2008.

438. Carroll Ai, Wynne M. HSE National Wound Management Guidelines. 2018;127–200.

439. Aishara Atiyeh, Juan P Barret HD. Best Practice Guidelines: Effective Skin and Wound Management of Non-Complex Burns Best Practice Guidelines: Effective Skin and Wound Management of Non-Complex Burns Best Practice Guidelines: Effective Skin and Wound Management of Non-Complex Burns C Best. Wound Int [Internet]. 2014; Available from: http://www.woundcare-bbraun.com

440. Castro R, Zarra I, Lamas J. Water-soluble seaweed extracts modulate the respiratory burst activity of turbot phagocytes. Aquaculture. 2004;229(1–4):67–78.

441. Dai T, Tanaka M, Huang Y-Y, Hamblin MR. Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects. Vol. 9, Expert Rev Anti Infect Ther. 2011. 857–879 p.

442. Muzzarelli R, Tarsi R, Filippini O, Giovanetti E, Biagini G, Varaldo PE. Antimicrobial Properties of N-Carboxybutyl Chitosan. Antimicrob Agents Chemother. 1990;34(10):2019–23.

443. Laurienzo P. Marine polysaccharides in pharmaceutical applications: An overview. Mar Drugs. 2010;8(9):2435–65.

444. Qin C, Li H, Xiao Q, Liu Y, Zhu J, Du Y. Water-solubility of chitosan and its antimicrobial activity. Carbohydr Polym. 2006;63(3):367–74.

445. Jeffery SLA. Non-adherent and flexible - using Cutimed Sorbact as a filler and liner with NPWT. J Wound Care [Internet]. 2014;23(5 Suppl):S3-15. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25188938

446. Kravanja G, Primoži M, Knez Ž, Leitgeb M. Chitosan-Based (Nano)Materials for Novel Biomedical Applications. Molecules. 2019;24:151–83.

447. Nalawade T, Sogi SP, Bhat K. Bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms. J Int Soc Prev Community Dent. 2015;5(2):114.

448. Snejdrova E, Dittrich M. Pharmaceutically Used Plasticizers. Recent Adv Plast. 2012;45–68.

449. Momoh FU, Boateng JS, Richardson SCW, Chowdhry BZ, Mitchell JC.

Development and functional characterization of alginate dressing as potential protein delivery system for wound healing. Int J Biol Macromol [Internet]. 2015;81:137–50. Available from: http://dx.doi.org/10.1016/j.ijbiomac.2015.07.037

450. Prajapati BG, Sawant KK. Poly electrolyte complex of chitosan alginate for local drug delivery. Int J ChemTech Res. 2009;1(3):643–8.

451. Olijtzky I. Antimicrobial Properties of a Propylene Glycol Based Topical Therapeutic Agent. J Pharm Sci. 1965;54(5):5–6.

452. Joseph M. Catanzaro M, J. Graham Smith, Jr., MD Augusta G. Propylene glycol dermatitis. J Am Acad Dermatol. 1991;24(2):90–5.

453. Zheng LY, Zhu JF. Study on antimicrobial activity of chitosan with different molecular weights. Carbohydr Polym. 2003;54(4):527–30.

454. Campaniello D, Bevilacqua A, Sinigaglia M, Corbo MR. Chitosan: Antimicrobial activity and potential applications for preserving minimally processed strawberries. Food Microbiol. 2008;25(8):992–1000.

455. Bowler PG, Jones SA, Walker M, Parsons D. Microbicidal Properties of a Silver-Containing Hydrofiber® Dressing Against a Variety of Burn Wound Pathogens. J Burn Care Rehabil. 2004;25(2):192–6.

456. Toppo FA, Pawar RS. Novel Drug Delivery Strategies and Approaches for Wound Healing Managements. J Crit Rev [Internet]. 2015;2(2):12–20. Available from: https://innovareacademics.in/journals/index.php/jcr/article/view/6038/5251

457. Minden-Birkenmaier B, Bowlin G. Honey-Based Templates in Wound Healing and Tissue Engineering. Bioengineering. 2018;5(2):46.

458. Sinno S. Honey and Wound Healing An Overview. J Clin Derm. 2015;12(3):1–10.

459. Sivamani RK, Ma BR, Wehrli LN, Maverakis E. Phytochemicals and Naturally Derived Substances for Wound Healing. Adv wound care [Internet]. 2012;1(5):213–7. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3839002&tool=pmce

ntrez&rendertype=abstract

460. Ghisalberti EL. Detection and Isolation of Bioactive natural products. In: Colegate SM, Molyneux RJ, editors. Bioactive naural products; detection, isolation and structural determination. 2nd ed. Florida, USA: CRC Press, Taylor & Francis Group; 2008. p. 11–76.

461. Ebada SS, Edrada RA, Lin W, Proksch P. Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates. Nat Protoc [Internet]. 2008;3(12):1820–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18989260

462. Papista E, Acs E, Boddi B. Chlorophyll- a determination with ethanol – a critical test. Hydrobiologia. 2002;485:191–8.

463. Christen P. Recent Extraction Techniques for Natural Products : Microwave-assisted Extraction and Pressurised Solvent Extraction. 2002;113:105–13.

464. Lake RJ, Mccombs JD, Munro MHG, Perry B. Reverse Phase Flash Chromatography: A Method for the Rapid Partitioning of Natural Product Extracts. J Nat Prod. 1987;50:290–2.

465. Sathya R, Kanaga N, Sankar P, Jeeva S. Antioxidant properties of phlorotannins from brown seaweed Cystoseira trinodis (Forsskal) C . Agardh. Arab J Chem [Internet]. 2017;10:S2608–14. Available from: http://dx.doi.org/10.1016/j.arabjc.2013.09.039

466. Sheeja L, Lakshmi D, Bharadwaj S, Parveen KS. Anticancer activity of phytol purified from Gracilaria edulis against human breast cancer cell line (MCF-7). 2016;19(4).

467. Vieira FA, Guilherme RJR, Neves MC, Abreu H, Rodrigues ERO, Maraschin M, et al. Single-step extraction of carotenoids from brown macroalgae using non-ionic surfactants. 2017;172:268–76.

468. Rajauria G, Abu-ghannam N. Isolation and Partial Characterization of Bioactive Fucoxanthin from Himanthalia elongata Brown Seaweed : A TLC-Based Approach. Int J Anal Chem. 2013;6.

469. Sigma-Aldrich. Preparative TLC Plates [Internet]. 2019 [cited 2019 Jun 10]. Available from: https://www.sigmaaldrich.com/analytical-chromatography/analytical-products.html?TablePage=18169737

470. Andersen NH. Preparative thin-layer and column chromatography. 1969;10:316–9.

471. Rajauria G, Foley B, Abu-ghannam N. Identification and characterization of phenolic antioxidant compounds from brown Irish seaweed Himanthalia elongata using LC-DAD – ESI-MS / MS. Innov Food Sci Emerg Technol [Internet]. 2016;37:261–8. Available from: http://dx.doi.org/10.1016/j.ifset.2016.02.005

472. Leyton A, Pezoa-conte R, Barriga A, Buschmann AH, Mäki-arvela P, Mikkola J, et al. Identification and efficient extraction method of phlorotannins from the brown seaweed Macrocystis pyrifera using an orthogonal experimental design. Algal Res [Internet]. 2016;16:201–8. Available from: http://dx.doi.org/10.1016/j.algal.2016.03.019

473. Dewald HD. Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation & Applications. J Chem Educ. 1999;76(1):45701.

474. Elipe VM. Advantages and disadvantages of nuclear magnetic resonance spectroscopy as a hyphenated technique. Anal Chim Acta. 2003;497:1–25.

475. Mcmaster C. Stains for Developing TLC Plates [Internet]. [cited 2019 Jun 10]. Available from: https://www.chemistry.mcmaster.ca/adronov/resources/Stains_for_Developing_TLC_Plates.pdf

476. Chan SH. A review on solubility and polymerization of silica. Geothermics. 1989;18(1–2):49–56.

477. Silicycle. Thin layer chromatography hand book. North America; 1999.

478. Simmons TA, Limbach PA. Influence of Co-matrix proton affinity on oligonucleotide ion stability in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Am Soc Mass Spectrom. 1998;9(7):668–75.

479. Soczewifiski E, Wawrzynowicz T. Thin-layer chromatography as a pilot

technique for the optimization of column chromatography. J Chronwrograpl~y,. 1981;18:729–32.

480. Li R, Wang Y, Tan J, Tang S, Jiang Z, Di S, et al. Simultaneous determination of synthetic edible pigments in beverages by titania-based RP-HPLC. Arab J Chem [Internet]. 2019;0–6. Available from: https://doi.org/10.1016/j.arabjc.2019.05.001

481. Jiang Z, Kempinski C, Chappell J. Extraction and Analysis of Terpenes/Terpenoids. Curr Protoc Plant Biol. 2016;1(2):345–58.

482. Jiang Z, Chen Y, Yao F, Chen W, Zhong S, Zheng F, et al. Antioxidant, antibacterial and antischistosomal activities of extracts from Grateloupia livida (Harv). Yamada. PLoS One. 2013;8(11).

483. González-Burgos E, Gómez-Serranillos MP. Terpene Compounds in Nature: A Review of Their Potential Antioxidant Activity. Curr Med Chem [Internet]. 2012;19(31):5319–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22963623

484. Sadek P. Solvent Miscibility and Viscosity Chart. In: The HPLC Solvent Guide Wiley-Interscience. 2002. p. 2.

485. Bansemir A, Just N, Michalik M, Lindequist U, Lalk M. Extracts and sesquiterpene derivatives from the red alga laurencia chondrioides with antibacterial activity against fish and human pathogenic bacteria. Chem Biodivers. 2004;1(3):463–7.

486. Xu N, Fan X, Yan X, Li X, Niu R, Tseng CK. Antibacterial bromophenols from the marine red alga Rhodomela confervoides. Phytochemistry. 2003;62(8):1221–4.

487. Jassbi AR, Zamanizadehnajari S, Azar PA, Tahara S. Antibacterial diterpenoids from Astragalus brachystachys. Zeitschrift fur Naturforsch - Sect C J Biosci. 2002;57(11–12):1016–21.

488. Chakraborty K, Lipton AP, Paulraj R, Chakraborty RD. Guaiane sesquiterpenes from seaweed Ulva fasciata Delile and their antibacterial properties. Eur J Med

Chem [Internet]. 2010;45(6):2237–44. Available from: http://dx.doi.org/10.1016/j.ejmech.2010.01.065

489. Chakraborty K, Lipton AP, Paul Raj R, Vijayan KK. Antibacterial labdane diterpenoids of Ulva fasciata Delile from southwestern coast of the Indian Peninsula. Food Chem [Internet]. 2010;119(4):1399–408. Available from: http://dx.doi.org/10.1016/j.foodchem.2009.09.019

490. Jill Venton B. Ultraviolet-Visible (UV-Vis) Spectroscopy. J Vis Exp. 2017;(Science Education Collection).

491. Badertscher M, Buhlmann P, Pretsch E. Structure Determination of Organic Compounds [Internet]. 4th ed. Organic Chemistry: An Intermediate Text. Zurich: Springer; 2009. Available from: https://onlinelibrary.wiley.com/doi/pdf/10.1002/0471648736.ch11%0Ahttp://link.springer.com/10.1007/978-3-540-93810-1

492. Kalsi PS. Ultraviolet and Visable Spectroscopy. In: Kalsi PS, editor. Spectroscopy of Organic Compounds. 6th ed. New Delhi, India: New Age International Ltd.; 2007. p. 9–65.

493. Mmola M, Le Roes-Hill M, Durrell K, Bolton JJ, Sibuyi N, Meyer ME, et al. Enhanced antimicrobial and anticancer activity of silver and gold nanoparticles synthesised using Sargassum incisifolium aqueous extracts. Molecules. 2016;21(12):1–14.

494. Venkatesan M, Arumugam V, Pugalendi R, Ramachandran K, Sengodan K, Vijayan SR, et al. Antioxidant, anticoagulant and mosquitocidal properties of water soluble polysaccharides (WSPs) from Indian seaweeds. Process Biochem [Internet]. 2019;(January):0–1. Available from: https://doi.org/10.1016/j.procbio.2019.05.029

495. Jose GM, Raghavankutty M, Kurup GM. Sulfated polysaccharides from Padina tetrastromatica induce apoptosis in HeLa cells through ROS triggered mitochondrial pathway. Process Biochem [Internet]. 2018;68(February):197–204. Available from: https://doi.org/10.1016/j.procbio.2018.02.014

496. Fitton J, Dell'Acqua G, Gardiner V-A, Karpiniec S, Stringer D, Davis E. Topical

Benefits of Two Fucoidan-Rich Extracts from Marine Macroalgae. Cosmetics. 2015;2(2):66–81.

497. Hoysecroft CE, Constable EC. The use of IR spectroscopy as an analytical tool. In: Pearson, editor. Chemistry. 2 nd. Essex: Prentice Hall; 2002. p. 330–4.

498. Dantas-Santos N, Almeida-Lima J, Vidal AAJ, Gomes DL, Oliveira RM, Pedrosa SS, et al. Antiproliferative activity of fucan nanogel. Mar Drugs. 2012;10(9):2002–22.

499. Sinurat E, Saepudin E, Peranginangin R, Hudiyono S. Immunostimulatory activity of brown seaweed-derived fucoidans at different molecular weights and purity levels towards white spot syndrome virus (WSSV) in shrimp litopenaeus vannamei. J Appl Pharm Sci. 2016;6(10):082–91.

500. Delma CR, Somasundaram ST, Srinivasan GP, Khursheed M, Bashyam MD, Aravindan N. Fucoidan from turbinaria conoides: A multifaceted “deliverable” to combat pancreatic cancer progression. Int J Biol Macromol [Internet]. 2015;74:447–57. Available from: http://dx.doi.org/10.1016/j.ijbiomac.2014.12.031

501. Sinurat E, Peranginangin R. Purification and Characterisation of Fucoidan from the Brown Seaweed Sargassum binderi sonder. Squalen Bull Mar Fish Postharvest Biotechnol. 2015;10(2):79–87.

502. Zein S, Haddad M, Krivoruchko E, Sobolev AP, Azar S, Kanaan H. A New Molecule of Water Soluble Polysaccharide Isolated from Eucalyptus Growing in Lebanon. Eur J Med Res. 2018;5(1):48–54.