Investigating the Novel Use of Seaweed Extracts as Biopesticides

Submitted to the Waterford Institute of Technology for the Degree of Doctor of Philosophy

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DECLARATION

I hereby certify that this material, which I now submit for assessment is entirely my own work and has not been taken from the work of others, save to the extent that such work has been cited and acknowledged within the text of my work.

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ACKNOWLEDGEMENTS

It's hard to believe four years has passed since I began my studies in WIT which has had both its highs and lows but have always being grateful of the opportunity that was given to me. I could not of completed this journey without the help of others who offered me guidance, support and friendship throughout the years of study and also the WIT scholarship programme for funding this research.

Foremost, I would like to express my sincere gratitude to Nick, Helen, Peter and Shiau Pin Tan (Graece) for their continuous support, patience and freedom to pursue my research, while silently and non-obtrusively ensuring that I stayed on course and do not deviate from the core of my research. Without this guidance, this thesis would not have been possible and I shall eternally be grateful to my supervisors for their assistance. I would like to give a special thanks to Graece who was an incredible mentor particularly in the area of microbiology which I had very little experience in. This was an essential part of my research and I thank you Graece for getting me through it and apologies for all the head-wrecking questions I asked along the way. My thesis wouldn't be near as good without your support and guidance.

A big thanks to the technicians, postgraduate and postdoctoral friends for all their help and encouragement. The lunch-time bants were a great way to distract from the pressures of research as we were all on this journey together.

To the "Glenmore girls" ye have being an incredible group of friends through our viber conversations and nights out which are an essential part of the research life in order to maintain that all in important work-life balance!!! Annabel where can I start who would of thought I would of made such an amazing friend throughout my studies with your incredible smarts and your never ending encouragement. You have made me a much more open-minded person. And not forgetting the countless tea breaks and chats about the weekend gossip which have made my time here at WIT so much more enjoyable. Thank you ;).

Michael you have being my rock throughout this journey. Your emotional support and understanding especially through the turmoil times have made you one of my greatest supporters in this thesis. I can't wait to see what the next chapter in life has for us.

Last but not the least, I would like to thank my family; my parents Declan and Eileen and my sisters Orla and Elaine for their continuous support over the years. I know it took awhile and I love that the bank of Declan never charged interest lol! So therefore it only seems right that I dedicate this dissertation to my parents for their endless support and encouragement, as without them this journey would not of been possible.

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2D COSY	2-dimensional correlation spectroscopy
ATR	Attenuated total reflectance
BHI	Brain heart infusion
CAGR	Compound annual growth rate
CFU	Colony forming unit
CLSI	Clinical and laboratory standards institute
DAFM	Department of agriculture, food and the marine
DEPT	Distortionless enhancement by polarization transfer
DPPH	2,2-diphenyl-1-picryl-hydrazyl
Dw	Dry weight
EPA	Environmental protection agency
EC	European commission
EUCAST	European committee on antimicrobial susceptibility testing
EIRC	Eco-innovation research centre
EU	European Union
FDA	Fluorescein diacetate
FSC	Forest stewardship council
FTIR	Fourier transform infrared spectroscopy
FTIR-ATR	Fourier transform infrared spectroscopy-attenuated total reflectance
g	Gravity
GC-MS	Gas chromatography mass spectroscopy
HPLC	High performance liquid chromatography
IR	Infrared spectroscopy
KOH	Potassium hydroxide
LC	Lethal concentration

LC-ESI-MS	Liquid chromatography electrospray ionisation mass spectroscopy
LC-MS	Liquid chromatography mass spectroscopy
MAE	Microwave-assisted extraction
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MCT	Mercury cadmium telluride
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MRD	Maximum recovery diluent
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Murashige and skoog basal salt mixture
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PCD	Programmed cell death
PDI	Percent disease index
PGE	Phloroglucinol equivalents
PPP	Plant protection product
PTFE	Polytetrafluoroethylene
ROS	Reactive oxygen species
RPM	Revolutions per minute
SE	Standard error
SD	Standard deviation
SFE	Supercritical fluid extraction
SFM	Sustainable forest management

SPE	Solid phase extraction
TCD	Total cell death
TDF	Total dietary fiber
TLC	Thin layer chromatography
TPC	Total phenol content
TTC	2, 3, 5-triphenyl-tetrazolium chloride
UAE	Ultrasound-assisted extraction
UCL	Upper confident level
UV-Visible	Ultraviolet-visible
WHO	World health organisation
WIT	Waterford institute of technology

ABSTRACT

Seaweeds grow in relative abundance along the Irish coastline with about 40,000 tonnes of natural seaweed harvested in Ireland annually, estimated to be worth €18 million. In the last three decades, the discovery of metabolites with biological activities from macroalgae has increased significantly. The vast array of phytochemicals produced by seaweeds include those that exhibit antifungal and antibacterial properties. Such properties have been exploited by researchers in the search for novel antimicrobial compounds. However, there is also a significant requirement for the discovery of compounds active against phytopathogens arising from a multitude of concerns including; climate change, emergence of resistance against current treatments, controlling of invasive species and the negative effects associated with the use of synthetic pesticides. It is estimated that plant diseases can affect 30% of the crop harvest if not managed correctly and efficiently. These needs have driven the main objectives of the present study: (1) to screen seaweeds collected from the South-East coast of Ireland against fungal and bacterial plant pathogens, (2) to compare activity to commercially available plant protection products and (3) to isolate and identify the antimicrobial compounds from the most promising crude extracts.

The initial antifungal screen using the poisoned food technique, found the methanol extract of the green seaweed, *Ulva lactuca* exhibited the strongest antifungal activity against the destructive root rot fungus *Heterobasidion annosum sensu stricto*. Additionally, the poisoned food technique was reported to be the optimum test method for this particular extract and fungal strain. The efficacy of this active seaweed extract was compared to the chemical Plant Protection Product urea; the main chemical method currently in use for controlling this worldwide pathogen. The protectant activity of both the extracts and urea were evaluated on disks of *Picea sitchensis* (Sitka spruce), a species sensitive to infection from *H. annosum s.s.* The data demonstrated that the extracts exhibited strong protectant activity at 20 mg/mL after 18 h and 24 h soaking periods. Highlighting a potential biopesticide product for use in forestry which is particularly relevant as result of the predicted pressures associated with climate change making trees more susceptible to disease and encouraging the introduction of invasive species.

Crude seaweed extracts were also tested against nine quarantine bacterial plant pathogens using the disk diffusion assay. The seaweeds were found to exhibit a broad

spectrum of activity, with methanol found to be the optimum solvent for extracting antimicrobial compounds from the different seaweed species. *Polysiphonia lanosa* showed activity against the majority of the tested organisms; particularly the methanol extracts which proved the most potent with an inhibition zone of 15.83 ± 0.41 mm exhibited against *Xanthomonas arboricola*. The minimum inhibitory concentration of the most effective extract, the methanol extract of *P. lanosa,* was determined to be 6.25 mg/mL with the same concentration also found to exhibit antibiofilm activity (>80% inhibition) against *Xanthomonas fragariae* in a dose response manner. Unfortunately, the crude methanol extract of *P. lanosa* was found to be phytotoxic to the model plant species *Arabidopsis thaliana* at and below its minimum inhibitory concentration (6.5 mg/mL). The crude extracts, therefore, contained a number of compounds that were toxic to *A. thaliana in vivo* and required isolation and purification of the bioactive(s) to allow for a more accurate phytotoxicity study. Investigation into conventional and non-conventional extraction techniques demonstrated that solvent extraction and Soxhlet extraction achieved similar yields of extracts with the same degree of activity compared to ultrasound-assisted extraction.

The structure of the antibacterial compound present in *P. lanosa* could not be identified completely due to the fact that this was a semi-pure fraction, but it was concluded to be a bromophenol through its distinctive constituents such as OH groups, aromatics and the presence of a halogenated compound believed to be a bromine. Antioxidant activity was also exhibited by the extract with phenolic compounds widely reported to be responsible for such activity. Further purification is required to identify the complete structure of the bromophenol bioactive(s). However, activity of the semi-pure fraction was significantly higher compared to its crude extract, demonstrating the increased concentration of the active compounds and the possible antagonistic effects of other compounds present in the *P. lanosa* extract.

In conclusion, this study demonstrated that seaweeds represent a promising source of pesticidal compounds that are active against a range of fungal and bacterial plant pathogens, with potential biopesticide applications. A growing interest in biopesticides signifies a shift in the reliance on synthetic pesticides to a lower impact alternative. This is the first report of seaweed extracts exhibiting activity against *H. annosum s.s.*, and *P. lanosa* against the bacterial pathogen *X. fragariae*.

CHAPTER 1: INTRODUCTION

*Information in this chapter has been published as a mini review in the *Journal of Applied Phycology* (O' Keeffe, E., Hughes, H., McLoughlin, P., Tan, S. P. and McCarthy, N. (2019) Methods of analysis for the *in vitro* and *in vivo* determination of the fungicidal activity of seaweeds: a mini review. doi: 10.1007/s10811-019-01832).

1.1 Introduction

Throughout the ages humans have relied on natural products to cater for their fundamental needs, such as a source of food particularly from plants. Plants have also formed the basis of traditional medicines with the World Health Organisation (WHO) estimating that 65% of the world's population predominantly depended on plant-derived medicines in 1985 (1). This is as a result of plants containing a wide variety of bioactive compounds, which are defined as chemical molecules that are produced by living organisms and exert a biological effect. They have been termed secondary metabolites since they are not directly involved in essential roles such as growth and development (primary metabolites) but instead play important functions in the plants interaction with abiotic and biotic factors (2). Such activities include serving as defence compounds against various pathogens and they have also been known to include UV absorbing compounds, thus preventing serious damage to the plant from sunlight. Humans have exploited some of these metabolites as a source of novel drugs due to their (biological properties including) antimicrobial, antioxidant and anti-inflammatory properties (3).

About 75% of the earth's surface is covered by oceans, that have a broad diversity of marine organisms (4). These marine organisms provide a rich source of bioactive compounds, and it includes seaweeds. Similar to plants, seaweeds produce secondary metabolites as a form of protection against various environmental stresses and these compounds have been found to show antiviral, antiprotozal, antifungal and antibacterial properties (5). Seaweeds have also been reported to exhibit pesticidal properties which will be the focus of this review (6). Many of these compounds are likely to be novel alternatives to existing controls. This is critically important since chemical pesticides have a major impact on biological diversity, including habitat loss and climate change resulting in a need to replace the use of these hazardous chemicals with more ecologically sound alternatives.

The natural drug discovery process generally involves a complete screening of crude natural extracts with appropriate *in vitro* assays for the determination of pesticidal activity followed by bioassay-guided fractionation of the most promising extracts and isolation and purification of the active constituents (7). Once this has been successfully completed, identification of the bioactive compound is required. However, no single extraction, purification and identification method currently exists and this is mainly as a

result of the vast quantity of natural extracts available. This produces a significant amount of trial and error in terms of developing the optimum method for the specific extract type. However this is key to determining the full capacity of the extract of interest as a potential biopesticide.

This literature review provides an insight into the current methods utilised in the extraction, isolation and identification of antimicrobial compounds present in seaweeds, mainly against plant pathogens. The fungal and bacterial plant pathogens that will be assessed within this body of work and a discussion on the current prospects and challenges facing biopesticides is also discussed.

1.2 Seaweeds

1.2.1 Morphology and ecology of seaweeds

Marine marcroalgae, commonly known as seaweeds are macroscopic, multicellular plants that live in either marine or brackish water environments with thousands of species found worldwide (8). Structurally seaweeds consist of three main components, the holdfast, stipe and blade with the whole body of the plant known as the thallus (Figure 1.1).

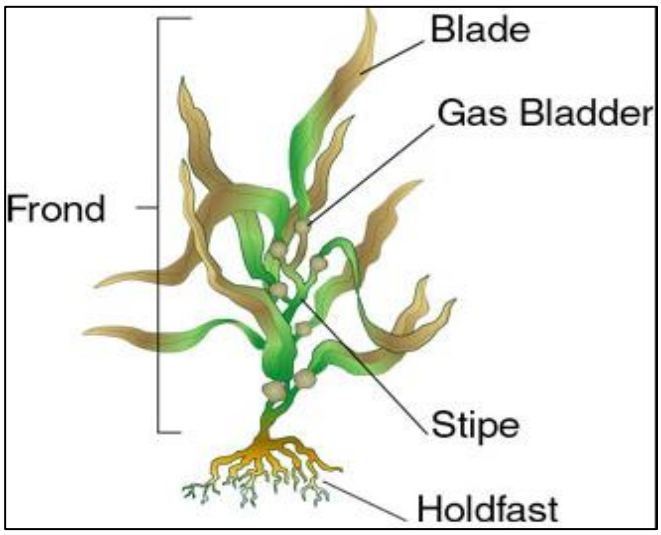


Figure 1.1: The basic structure of seaweed (9).

The holdfast functions as an anchor to keep the seaweed held to the rock or another sturdy object so as to stop it from being swept away by the current. This component can be discoidal, rhizoidal, bulbous or branched depending on the bedrock it is attached to (8). However unlike in a land plant, the holdfast does not act as the primary source for uptake of water and nutrients. The stipe is found between the holdfast and blade and resembles that of the stem of a plant, but its main purpose is to support the blade for photosynthesis and for the absorption of nutrients from the surrounding seawater. The stipe varies in flexibility so as to allow the seaweed to flow freely in the water and not break off (10). The blade exhibits a large surface area so as to receive the maximum amount of sunlight absorption for its main functions, which include photosynthesis and production of reproductive spores as well as the absorption of nutrients. The blade has bubble-like structures commonly known as pneumatocysts which serve the function of keeping the parts of the blade required for photosynthesis afloat in order to efficiently absorb sunlight (10). Therefore, like land plants, seaweeds contain photosynthetic pigments and, with the help of sunlight as well as nutrients present in the algae, can undergo photosynthesis in order to produce food. It is estimated that about 50% of global photosynthesis is contributed to by algae (11).

Two specific environmental requirements that dictate seaweed ecology is the presence of seawater and sufficient light to allow for photosynthesis (12). Additionally, a firm attachment point is required for the holdfast and, as a result of this, seaweeds are most commonly situated in the littoral zone mainly on rocky shores as opposed to on sand or shingle. However, seaweeds can be found in a wide range of locations, including high coastal regions and also in the sub-tidal region in depths where only 0.01% of photosynthetic light reaches (8). Red seaweed species are found at the greatest depths due to their ability to absorb blue light which penetrates the furthest in water, whereas green seaweed is found nearest the surface as it absorbs mostly red light (12, 13). Some seaweed has adapted to live within tidal rock pools, meaning they must be capable of rapidly adjusting to changing temperatures and salinity. Therefore, the type of seaweed found in a specific area depends on environmental factors including temperature, level of light exposure, tides and shore characteristics. Regardless of the species, seaweeds are an important component in marine ecosystems in terms of providing food and shelter for marine life (14).

1.2.2 Classification of seaweeds

Seaweeds are classified into three major groups known as Chlorophyta (green seaweed), Phaeophyta (brown seaweed) and Rhodophyta (red seaweed). These divisions are based on various properties including pigmentation, chemical nature of the photosynthetic storage product, the organisation of the photosynthetic membranes and other morphological features (12). The criteria required to distinguish between the different divisions is based on biochemical, physiological and electron microscopic studies (8).

1.2.2.1 Chlorophyta (green seaweed)

Chlorophyta are green in colour due to the high concentration of the pigments chlorophyll *a* and *b* present, which are in the same proportions as that of higher plants (15). Chlorophyta are mainly found in fresh or marine habitats and are not usually found below a depth of 40 meters (8). However, some green seaweed species have adapted to grow in areas of low salinity such as near river mouths and other fresh water outlets (13). Chlorophyta can exist as unicellular (one cell), multicellular (many cells), colonial (exists in a loose aggregate of cells) or coenocyctic whereby it is composed of one large cell without cross-walls and this cell may be unicellular or multicellular (16). They can range from microscopic to macroscopic with the thalli varying from free filaments to definitely formed shapes, while the portion responsible for photosynthesis may be moderately to highly calcified (8). Their cell walls are thick and stratified with an inner layer of cellulose for support and an outer layer of pectin. The organelle chloroplast found in seaweed, like land plants, serves its function in the formation of starch which is the main food reserve for Chlorophyta as well as some fats and oils. Green seaweed can reproduce both sexually and asexually through the formation of flagellate and sometimes non-flagellate spores. Sexual reproduction can occur in a number of different forms including isogamous, anisogamous or oogamous. Asexual reproduction occurs by zoospores (motile) or by fission (splitting), budding and fragmentation.

1.2.2.2 Phaeophyta (brown seaweed)

Phaeophyta's brown colour is due to the high level of the xanthophyll pigment, fucoxanthin, which masks the other pigments chlorophyll *a* and c, beta-carotene and other xanthophylls (16). It is the most abundant group of seaweed and is exclusively a marine form. They generally exist as large tissue type plants covering rocks, jetties and the bottoms of colder, shallower offshore waters (13). Kelp is the largest species of brown seaweed growing up to 70 metres in length and, as a result of this, they exhibit massive thalli with specialised air bladders in order to remain buoyant in the water (16). Phaeophyta can exist in different forms ranging from a simple free-branched filament to complex differentiated forms. The cell walls are double layered, consisting of an outer layer which is mucilaginous and sticky due to the presence of alginate (long-chained heteropolysaccharide) and an inner layer of cellulose for support (8). The cells themselves exist as uninucleate, composed of one or two nucleoli which are usually large and have a complex network with "little chromatic material" (8). The main carbohydrate reserve in Phaeophyta is laminaran (polysaccharide) arising from the simple sugar in photosynthesis, while starch is absent compared with green seaweed (8). Other food reverses include sugars, higher alcohols and more complex polysaccharides. Similar to the Chlorophyta, the Phaeophyta can reproduce both sexually and asexually with several species having the ability to reproduce vegetatively via fragmentation (17). Sexual reproduction in brown seaweed occurs in the same way as green seaweed either by isogamy, anisogamy or oogamy.

1.2.2.3 Rhodophyta (red seaweed)

The red colour of Rhodophyta is due to the larger volumes of the red phycoblin pigments (phycoerythrin and phycocyanin) that override the green chlorophyll pigment (12). Rhodophyta are considered to be smaller and more delicate in construction compared to brown seaweed but are still abundant and readily seen. They can inhabit intertidal to sub-tidal waters and can even be found at depths of 100 feet or more along the coast, while in the tropics red seaweeds have been found at depths of 875 feet (13). They are almost entirely marine except for a few species and are commonly seen growing on rocks and shells appearing as hard crust-like plants. The main food reserves

include floridean starch and floridoside and, similar to the Phaeophyta division, the presence of true starch is absent (16). The cells are said to be eukaryotic meaning their nucleus and other organelles are enclosed within a membrane similar to green and brown seaweeds. The cell walls themselves are composed of cellulose, agars and carrageenans with the two long-chained polysaccharides agar and carrageenans in widespread commercial use. The cells in Rhodophyta are always uninucleate with the exception of older cells, which exist as multinucleate (8). Rhodophyta can undergo both asexual and sexual reproduction with sexual reproduction being the more common out of the two. Either manner of reproduction produces non-motile spores (non-flagellated) (17). Asexual methods include the discharge of the spores and fragmentation of the algal bodies. Rhodophyta have undergone a more diverse evolution compared to green and brown seaweeds. For instance, the red seaweed *Polysiphonia lanosa* is an epiphyte that grows mainly on the brown seaweed *Ascophyllum nodosum* and benefits from its buoyancy by lifting it closer to the sunlight (12).

The three major classes of seaweed all inhabit Irish waters with over 500 species of seaweed identified in total. Table 1.1 lists a number of species from each group that are not only found in Ireland but also harvested for commercial use (18).

Table 1.1: Seaweed species in Ireland that are commercially harvested (18).

Chlorophyta	**Phaeophyta**	**Rhodophyta**
Ulva lactuca	*Fucus serratus*	*Palmaria palmate*
Enteromorpha intestinalis	*Fucus vesiculosus*	*Chondrus crispus*
Codium fragile	*Ascophyllum nodosum*	*Lithothamnion corallioide*
	Himanthalia elongate	*Porphyra* spp.

1.2.3 Applications of seaweed

The global seaweed market was valued at $4 billion in 2017 and is projected to reach over $9 billion by 2024 with a compound annual growth rate (CAGR) of 12% from 2018 to 2024 (19). This growth is a consequence of seaweeds having numerous applications including use in human and animal food, cosmetics, wastewater treatment, as an agricultural fertiliser, as a biopesticide, in medicinal and industrial uses. Seaweeds are a rich source of bioactive compounds due to their ability to produce a wide variety of secondary metabolites that exhibit a broad spectrum of biological activities such as antiviral, antifungal, antibacterial, cytotoxic and insecticidal activity (20).

1.2.3.1 Food source

Seaweed has been used as a food source for several centuries mainly in China, Japan, and the Republic of Korea (16). The movement of these nationalities to other countries has led to the migration of this custom to other regions. Nori, also known by its scientific name *Porphyra yezoensis,* is probably one of the best-known edible seaweeds and gets its food value from its high protein content (25%-35% dry weight), vitamins and mineral salts, especially iodine (16). Seaweed is regarded as the most nutritionally dense plants on the planet and also contains the most abundant source of minerals in the plant kingdom due to its ability to absorb nutrients from the surrounding ocean (13). As a result of this, the consumption of seaweed has been found to exhibit many health benefits such as a source of dietary fibre (21), high in iron (22), contains antioxidants (23) and aids weight loss (24, 25). Seaweed has a long history of being used as a source of food for livestock, especially during times of food scarcity. However, seaweed nowadays is seen as a valuable alternative to commercial feeds mainly due to its high nutrient content, especially chelated micro-minerals which usually have a higher availability than that of inorganic molecules (26). Seaweeds incorporated into animal feed provides iodine enrichment (27) and improvement on meat composition and shelf life (28).

1.2.3.2 Agriculture fertiliser

Seaweed and its bioactive compounds have been used as a fertiliser in coastal areas throughout the world for many years. This is due to the high amounts of water soluble potash, minerals and trace elements present in seaweeds which are easily absorbed by plants and help control nutrient deficiency (29). Carbohydrates and other organic matter found in seaweed have been shown to change the nature of the soil, as well as improving its moisture retaining capacity. Seaweed also produces plant growth promoting hormones such as auxins, gibberellins and cytokinins (30). Numerous studies have demonstrated the positive effects of seaweeds as a fertiliser. A study was carried out by Babu *et al.* on the use of *Halimeda macroloba* as a seaweed liquid fertiliser (SLF) on the seed germination, growth and protein profiles of *Vigna radiata*. From the results, both seed germination (90%) and seedling growth (shoot length = 11.85 cm and root length = 3.87 cm) were enhanced using a 25% treatment of *Halimeda macroloba* (30). It was noted that effectiveness was dose dependent on SLF with higher concentrations of *Halimeda macroloba* (100%) reducing germination and seedling growth.

A similar study was conducted by Kalaivanan *et al.* on the effect of using the seaweed *Sargassum myriocystum* as an SLF on the germination and growth of *Vigna mungo* (31). The results illustrated that 10% SLF exhibited the best results with 98% germination, high protein content and enhanced seedling growth (shoot length = 21.13 cm and root length 12.19 cm) (31). Selvam *et al.* demonstrated the use of the red seaweed *Hypnea musciformis* as an organic fertiliser on the growth and yield of *Arachis hypogea* (32). It was observed that the 2% concentration of *Hypnea musciformis* as an SLF was the optimum strength for high growth germination. Maximum chlorophyll a and b was observed in 30 day old plants with 1.75 mg/g fresh weight of chlorophyll a and 0.46 mg/g fresh weight of chlorophyll b (32). These chlorophyll concentrations, including total chlorophyll, were generally higher when compared to the control plants (no SLF treatment) (32). Similarly, these plants also exhibited greater total carbohydrates, proteins and lipids in comparison to the control plants. Therefore, from these studies it can be seen that the practice of using seaweeds as bio-fertilisers is a potential alternative to the use of synthetic fertilisers as they enhance germination, growth and crop yield as well as being low cost and eco-friendly.

1.2.3.3 Industrial applications

The main application of seaweed is its use as an industrial gum also known as seaweed phycocolloids and can be classified into three generic groups: alginate from brown seaweed, and agar and carrageenan from red seaweed. These phycocolloids have the particular properties to form gels in aqueous solutions, with carrageenan used as a major texturing ingredient in the food industry and was initially used as a stabilizer for the suspension of cocoa in milk chocolate (33). Similarly, agar is used in the food industry as a gelling and thickening agent, as well as in the science industry in microbiological media and a food source for insect larvae. Alginate is widely used as a stabilizer and an emulsifier and is commonly used in the textile printing industry (16). About 3,000 dry tonnes of *Ascophyllum nodosum* is harvested in Ireland per annum and is exported to Scotland for alginate extraction (16).

1.2.3.4 Cosmetic industry

Seaweeds consist of active components that have been shown to exhibit various cosmetic applications such as anti-wrinkling, whitening of skin, anti-inflammatory and anti-allergy (34). Table 1.2 summarizes the biological activity of brown seaweeds and their potential cosmetic applications.

Table 1.2: Summary of the biological activities of brown seaweed and their corresponding cosmetic applications (34).

Brown seaweed	Active component	Activity	Potential cosmeceutical application
Ecklonia cava	Eckol	Antioxidant	Anit-aging
Ecklonia cava	Phlorotannins	Antioxidant	Anit-aging
Sargassum siliquastru	Fucoxanthin	Antioxidant	Cytoprotective
Fucus vesiculosus & *Fucus serratus*	Polyphenols	Antioxidant	Anit-aging
Ecklonia cava	Dieckol	Antioxidant	Protective against photo-oxidative stress

From the table it can be seen that the majority of the activity is antioxidant, with its main application in anti-aging products. A study was carried out by Ahn *et al.* who found that three purified phlorotannins from the brown seaweed *Ecklonia cava*, namely phloroglucinol, eckol and dieckol, possessed antioxidant activities (34). These findings, therefore, suggest that brown seaweed could potentially be a very good source of natural antioxidants. These bioactives not only fulfill a real cosmetic function but are also added to contribute to the organoleptic properties of the product as well as aiding in the stabilization and preservation of it (35).

1.2.3.5 Medicinal uses

Prior to the 1950s, the use of seaweed as a medicinal application was restricted to traditional medicines. However, in the following years pharmacological properties of many marine bacteria, invertebrates and algae were discovered, with algae being responsible for 35% of the newly found chemicals between 1977 to 1987 (36). Seaweed provides a wide range of bioactive compounds that have been found to exhibit antiviral (37), antimicrobial (5), anticancer (38), anti-inflammatory (39, 40) and anticoagulant activities (41). For instance, Shanmughapriya *et al.* investigated the efficacy of brown, red and green seaweeds against ten human bacterial pathogens and one human fungi pathogen (42). Seven of the species were found to be highly active with *Acrosiphonia orientalis* exhibiting activity against 70% of the tested organisms. Pádu *et al.* also evaluated the bioactive compounds found in brown seaweeds as potential therapeutic agents against breast cancer with its main anticancer mechanism believed to be antioxidant activity (43). The bioactive compounds phloroglucinol, fucoidan and fucoxanthin, therefore, had the ability to quench singlet oxygen, scavenge free radicals and, hence, potentially prevent oxidative stress related diseases such as cancer, cardiovascular and neurodegenerative diseases.

1.2.3.6 Biopesticides

The focus of this research is the potential use of seaweed extracts as biopesticides particularly against fungal and bacterial plant pathogens. This growing interest in seaweeds "as alternatives" is a consequence of the negative environmental and health effects associated with conventional pesticides leading to stringent pesticide regulations and pressure from the public (44). Various studies have shown the broad range of pesticidal activity exhibited by seaweeds from fungicidal (45-47) to larvicidal (48-50). This is a relatively new area with the majority of studies conducted on human pathogens as opposed to plant pathogens. Additionally, not many studies have successfully isolated and identified the specific bioactive compound(s) present in seaweeds that exhibits this activity, as this is not an easy task. Section 1.4 discusses the various steps involved in the assessment and identification of the antimicrobial activity of seaweeds including some guidance on the selection of the optimum techniques with emphasis on

methodologies used to determine the fungicidal and bactericidal activity of seaweed extracts.

1.3 Investigation into the Antimicrobial Compounds in Seaweeds

1.3.1 Collection and processing of seaweeds

The collection and processing of seaweeds is typically carried out according to the same protocol that involves collecting the fresh seaweed from their holdfast at low tide times or Scuba diving for seaweeds located at lower depths. A specific tide time is selected as different seaweed species are found in different tidal conditions. For instance, *Fucus vesiculosus, Fucus serratus, Ascophyllum nodosum, Kappaphycus alvarezii, Caulerpa scalpelliformis, Sargassum polycystum, Gracilaria arcuata, Hypnea pannosa* and *Ulva* spp. are found at low tidal zones (10). Freshly collected seaweeds are then transferred back to the lab with each species being washed with distilled water to remove epiphytes and sand particles (10, 51, 52). This ensures the compounds extracted are from the seaweed rather than the epiphytes and sand.

The next step is to prevent the microbial and enzymatic degradation of these samples during storage by the removal of water. This can be achieved by drying the seaweeds, mainly through shade drying (53, 54), which can take a number of days for completion. Chanthini *et al.* shade dried seaweed species for 10 days at room temperature (44). However, this extended drying period may affect the biological activity of bioactives. Mei Ling *et al.* found that extended drying periods of 3 or 4 days affected the phytochemical content and antioxidant activity of the red algae, *K. alvarezii* (55). This was suspected to be as a result of a leaching effect. Studies have also dried seaweeds in an oven to ensure complete dryness and to speed up the process (52, 56). This method is not without its drawbacks as when high temperatures were employed, for example 80 °C for 24 h for *K. alvarerzii* samples, the phytochemical content and antioxidant activity were negatively affected (55).

Reports have suggested that freeze-drying (lyophilisation) is a superior drying approach with a comparative study completed by Cruces *et al.* finding that the freezing drying of *Lessonia spicata* was the optimum method with regards the extraction of phlorotannins and retention of antioxidant activity compared to silica-dried, oven-dried and air-dried

samples (57). However, freeze-drying is also said to have its disadvantages, mainly through the potential removal of volatile compounds under high vacuum (58). Though most studies report data generated from seaweeds that have been dried, some research has used fresh seaweed samples and, therefore, avoided potential degradation/loss of thermolabile or highly volatile bioactives. This was observed by Kolanjinathan *et al.* where air-dried samples exhibited lower antimicrobial activity compared to fresh samples as a result of the loss of volatile compounds during the drying process (59). However, since these samples are fresh, testing must be completed in a relatively short time period after harvesting to avoid microbial and enzymatic degradation, which may not always be possible. Therefore, there are a number of different drying techniques available where this becomes necessary, with the choice predominantly depending on the stability of the bioactive compounds targeted.

Seaweeds dried or fresh are generally blended to achieve a coarse powder in order to facilitate solvent diffusion into the cells as the smaller particle size increases the surface area of the seaweed exposed to the solvent and hence, promotes optimum extraction of bioactives. Norra *et al.* reported that the smaller particle size of 0.25 mm gave higher total phenolic content compared to the larger particle size of 2.0 mm as a result of enhanced surface area allowing for the greater mass transfer of solutes between phases (60). Some studies including Chanthini *et al.* also sieved the blended seaweed through sieve plates to produce a standardised particle size which ensured more consistent extraction yields (44). Once the seaweeds are processed, they are generally stored under refrigerated conditions at 4 °C or frozen at -20 °C to preserve the samples until further analysis.

1.3.2 Extraction of the bioactive compounds

1.3.2.1 Selection of extracting solvents

The desired antimicrobial components must be extracted and isolated from the seaweed using methodologies that enable the maximum recovery of the target bioactive compounds. There are a number of different extraction methods available but for the extraction to be efficient, it is important to choose a solvent that will penetrate the plant cell and will solubilize the desired compounds (10). Hydrophilic compounds will be

extracted by polar solvents such as water, methanol, ethanol, and acetone, whereas hydrophobic compounds will be extracted by non-polar solvents including hexane, chloroform and dichloromethane. Polarity has a significant effect on the observed activity of generated extracts since it determines how the extract interacts with the functional groups on the surface of the pathogen (61). For instance, Moorthi *et al.* compared the acetone, methanol and chloroform extracts of *Sargassum muticum* for antibacterial activity against foodborne human pathogenic bacteria (62). The acetone extract was found to be the most potent extract with phytochemical analysis of this extract revealing a high composition of flavonoids, alkaloids, sterols, phytosterols, tannins and courmarins. Brown seaweeds including *S. muticum* have been reported as a source of phenolic compounds that have been found to exhibit potent antibacterial activity (62).

However, if the polarity of the antimicrobial bioactives is unknown, a number of different solvents with a range of polarities must be used. This broad spectrum solvent approach is a common method of choice, as the identity of the desired bioactive is typically unknown. A large number of studies have utilised a range of solvents including methanol, ethanol, chloroform, hexane, etc (53, 63, 64). Manivannan *et al.* screened three brown seaweeds for antimicrobial activity. The solvent system consisted of both polar (methanol) and non-polar (petroleum ether) solvents with all generated extracts exhibiting some degree of activity (64). It was reported that the methanol extract of *Turbinaria conoides* showed maximum inhibition against both *Candida albicans* (18.00 ± 2.68 mm) and *Penicillium* sp. (18.00 ± 2.68 mm) while the acetone extract of *Padina gymnospora* exhibited the best antifungal activity against *Cryptococcus neoformans* (23.00 ± 1.78 mm) (64). This indicated that the desired bioactives present also ranged from polar to non-polar in character.

In most cases, extracts generated from organic solvents prove to be more efficient than aqueous extracts (53, 65-67) particularly polar extracts (68, 69). This was suspected to be as a result of the inhibiting compounds being in some part hydrophobic in nature, such as fatty acids (5). Methanol is often reported as the optimum solvent for extracting antimicrobial compounds (63, 70, 71). Kumar *et al.* conducted an antimicrobial screen on 12 different seaweeds against the phytopathogenic bacterium, *Pseudomonas syringae* causing leaf spot disease (72). The seaweeds were extracted in petroleum ether, ethyl acetate, chloroform, methanol and acetone, with the methanol extract of

Sagarssum wightii demonstrating maximum activity with a zone of inhibition of 21.00 ± 0.67 mm compared to the positive control (100 µg tetracycline) of 12.67 ± 0.33 mm. Table 1.3 summarises the solvents used to extract bioactive compounds exhibiting antimicrobial activity (in a variety of seaweed species). According to this table, a vast range of solvents is used ranging from water to dichloromethane, meaning the solvent applied will depend on the type of seaweed and the bioactives required (10). For instance, Gnanambal *et al.* evaluated the insecticidal properties of the seagrass *Halophila stipulacea* against the rice weevil *Sitophilus oryzae* (73). In the study it was found that 100% acetone was the best extracting solvent as it resulted in 100% mortality of the weevils, as opposed to the other solvents, hexane, diethyl ether, toluene, ethyl acetate, water, chloroform and dichloromethane which resulted in lower mortality rates (73).

A mixture of solvents has also been applied for the extraction of antimicrobial compounds from seaweeds to allow for optimum recovery of these desired bioactives. For example, Santhanam *et al.* investigated the antimicrobial activity of seaweed extracts against multi-resistant pathogens (42). The seaweeds were extracted in a number of solvents including ethanol, methanol/toluene (3:1 *v/v*), methanol, and phosphate buffered saline (PBS). The extracts generated from the solvent mixture methanol:toluene (3:1 *v/v*) exhibited the best antimicrobial activity with the minimum inhibitory concentration (MIC)/ minimum bactericidal concentration (MBC) ratio of the extract reported as 0.02 for the seaweed *Acrosiphonia orientalis* against *Pseudomonas aeruginosa* making the active principles a potential bactericicdal agent (42). This potent activity was suspected to be as a result of the presence of lipophilic compounds. Methanol is a highly polar solvent capable of penetrating the cell membrane and extracting out the lipophilic compounds which can be dissolved in low polarity solvents such as toluene and chloroform. These lipophilic compounds extracted from seaweeds have been reported to exhibit antimicrobial activity. Mendiola *et al.* used the supercritical carbon dioxide (CO_2) for 60 min at 40 °C, 400 atm to extract lipids from the microalga *Chaetoceros muelleri* (74). The fatty acids extracted were found to exhibit significant activity against *Escherichia coli* and *Staphylococcus aureus*.

Table 1.3: Antimicrobial activity of seaweed extracts isolated using a range of different solvents with varying polarities (10).

Solvent	Polarity	Seaweed species
Hexane	0	*Amansia multifida*
Diethyl ether	2.8	*Sargassum wightii, Codium adherens, Ulva reticulata, Halimeda tuna, Laurenica majuscule* and *Gracilaria changii*
Dichloromethane	3.1	Number of brown, red and green seaweeds including *Ulva rigida, Chondrus crispus* and *Laurencia* spp.
Butan-1-ol	4.0	*Haliptilon virgatum, Gelidium spinosum, Ceramium diaphanum var. elegans* and *Cystoseira crinite*
Chloroform	4.1	*Stoechospermum marginatum, Ulva lactuca, Sargassum wightii* and *Padina gymnospora*
Ethyl acetate	4.4	*Ulva lactuca, Padina tetrastromatica* and *Gracilaria changii*
Acetone	5.1	*Ascophyllum nodosum, Gracilaria edulis, Hypnea musiformis* and *Ulva reticulata*
Methanol	5.1	Range of red, brown and green seaweeds including *Codium* spp., *Caulerpa* spp., *Sargassum* spp., and *Fucus* spp.
Ethanol	5.2	*Codium adherens, Ulva reticulata* and *Halimeda tuna*
Water	9.0	*Codium decorticatum, Gracilaria crassa, Sargassum wightii, Sargassum binderi* and *Halimeda macroloba*

1.3.2.2 Extraction techniques

The simplest extraction method is solvent extraction and typically involves the addition of dried (blended) seaweed into a specific ratio of solvent which is then stirred for a specific length of time (75-77). The solvent is typically recovered by rotary evaporation at specific temperatures so as to yield dried extracts, as degradation of thermolabile bioactives is possible over an extended period of exposure to the extracting solvent (78). A number of studies that have utilised solvent extraction as their method of choice chose not to remove the solvent via rotary evaporation. Assessment of the antimicrobial potential of these newly generated extracts must be completed within a short period of time so as to avoid the degradation of unstable bioactives. Solvent extraction has the advantages of being cheap and simple, but is also prone to high solvent usage (79). Manikandan *et al.* investigated the antimicrobial activity of seaweeds against multi-drug resistant strains with the seaweeds being extracted using solvent extraction for 24 h and re-concentrated using rotary evaporation with a number of the extracts showing potent antimicrobial activity (80). Water extraction is considered the same methodology to solvent extraction with the water extracts reduced to dryness via evaporation (53, 81). However as water has a relatively high boiling point, rotary evaporation can be a slow process, therefore it is preferable to remove water through lyophilisation (82, 83). Water extraction exhibits all the same benefits as solvent extraction but has the added bonus of being a green extraction technique with most organic solvents being flammable, toxic and requiring specialised disposal which can be costly (84).

Soxhlet extraction is another conventional bioactive isolation technique and follows the principle of refluxing and siphoning to allow the bioactives to be continuously extracted with fresh solvents for a specific length of time, followed by evaporation in order to remove the solvent (53, 63, 64, 85). It is considered more efficient than conventional solvent extraction and generally requires less time and solvent (79). Zakaria *et al.* reported that their Soxhlet extracts exhibited more profound radical scavenging activity compared to the extracts generated from solvent partitioning (liquid-liquid extraction) (86). The main limitation of this technique and similar conventional methods is that it is not suitable for the extraction of thermolabile compounds as the sample is constantly heated (87).

It must be noted that in today's world not only the efficiency of the extraction process to extract the desired bioactives in the highest possible concentration is sought after, but the development of eco-friendly processes is preferred. These developments comply with the Green Chemistry Principles related to extraction which are outlined below (88):

1. Innovation by selection of renewable resources.
2. Use of alternative solvents, mainly water.
3. Reduction of energy consumption using innovative technologies.
4. Production of co-products instead of wastes.
5. Reduction of unit operations, fostering automation of processes.
6. Aim for non-denatured, biodegradable extracts without contaminants.

For this reason, non-conventional extraction techniques are growing in popularity as a result of their green and efficient conditions. Ultrasound-assisted extraction (UAE) is a simple, relatively low cost, efficient alternative to conventional techniques suitable for both small and large phytochemical extractions (89).

Other novel techniques including microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE) can also offer advantages over conventional techniques including lower solvent consumption, shorter extraction time and higher selectivity of specific polarity compounds such as non-polar compounds when carbon dioxide (CO_2) is employed as the extracting solvent in SFE. Such approaches have become popular extraction options for natural products (90-92), including seaweeds (93-95). SFE is considered to be a "green" extraction technique and is based on the use of solvents, mainly CO_2 at temperatures and pressures above its critical point (96). The main drawback of SFE is that CO_2 is non-polar and, therefore, not suitable for the extraction of polar compounds. This low polarity problem can be overcome by the introduction of co-solvents (methanol, ethanol) to change its polarity and in turn increase its solvating power. A major constraint with both MAE and SFE techniques is the initial high start-up cost of the equipment which may not always be justifiable for small companies.

In summary, before a reliable extraction technique is selected, one must consider the cost and time involved and, most critically, the properties of the desired bioactives.

However, if these bioactives are unknown, as in many cases, a simple solvent extraction with short extraction times can be the solution to avoid the loss of unstable compounds as a result of temperature, pressure and sonication. Once the bioactives have been identified a shift to a more efficient extraction technique can be employed, if possible.

1.3.3 In vitro assessment of the antimicrobial properties of the crude extracts

In order to evaluate the antifungal and antibacterial capacity of seaweed extracts, an effective test method must be utilised. Unfortunately there is no single method that is suitable for screening extracts against the various pathogens. This has resulted in a variety of *in vitro* test methods being developed to determine the potential activity of seaweed extracts against plant pathogens with no uniform technique for comparison purposes. The most popular techniques include disk diffusion, well diffusion, broth dilution, and the poisoned food technique. Each method will be discussed, together with their associated advantages and disadvantages.

1.3.3.1 Disk diffusion assay

The disk diffusion assay is one of the most popular methods encountered in the literature (51, 97-99). Since it is the official method used in the majority of clinical laboratories for routine antimicrobial susceptibility testing (100). Disk diffusion exhibits many advantages including; low cost, flexible, allows visibility of growth with a high screening capacity and also utilises low extract and reagent volumes (101). It also has one other advantage over another commonly used method known as well diffusion in that the solvating solvent is evaporated off the sterile paper disks prior to introduction to the inoculated petri dishes and, therefore, toxicity of the organic solvents towards the test organism is avoided. The effectiveness of this method was illustrated by Chanthini *et al.,* who investigated the antifungal activity of six different seaweeds collected along the coast of India against the phytopathogen *Alternaria solani*, responsible for causing early blight disease in tomatoes (44). The seaweeds were extracted by solvent extraction using three solvents; methanol, ethyl acetate and hexane. The antifungal activity was evaluated by disk diffusion assays, with the dried extracts re-dissolved in 5% dimethyl

sulfoxide (DMSO) to give a final concentration of 50 μg of the particular seaweed extract which was then added to the sterile paper disks (44). Of the six seaweeds tested, five produced biocidal activity against *A. solani* (44). However, disk diffusion also has limitations such as diffusion problems, in that the polarity of natural compounds can affect the diffusion process of compounds into the culture medium, with the less polar the compounds the slower their diffusion. Hence, an extract exhibiting good antifungal activity but made up of non-polar compounds, might exhibit a small zone of inhibition in a disk diffusion assay (102).

1.3.3.2 Well diffusion assay

The well diffusion method is also widely used to evaluate the antimicrobial activity of seaweed extracts (65, 103, 104). One of the first studies on the fungicidal activity exhibited by seaweeds was conducted via the well diffusion assay, (105) with the assay proving to be a highly effective method. For example, Rajesh *et al.* focused their study on one seaweed, the green seaweed *Caulerpa scalpelliformis*, against the fungal plant pathogen *Fusarium oxysporum* (63). The seaweed was extracted in three different solvents; hexane, chloroform and methanol using Soxhlet apparatus with agar well diffusion assays employed as the method of determining antifungal activity (63). Well diffusion (similar to disk diffusion) is considered a simple, low cost, rapid method of screening, with a high-throughput sample capacity (10). However, as with all methods it has its limitations specifically around diffusion issues, with the polarity of the solvating solvent affecting the ability of compounds to diffuse through the polar culture medium (10). For these reasons, the use of polar solvents such as water is considered optimum. However, from examination of the studies that employed the well diffusion method, some utilised non-polar solvents including Kausalya *et al.* who investigated the antimicrobial activity of seaweed extracts against a range of pathogens. The chloroform extracts of *Sargassum polycystum* showed maximum activity at 500 mg/mL against the bacterial plant pathogens *Erwinia caratovora* (19 mm) and the fungal plant pathogen *Rhizoctonia solani* (21 mm) (53). Similarly organic solvents can be toxic to the test organism. However, all of these studies included a negative control consisting of the organic solvent to ensure that the solvents did not negatively affect the growth of the pathogen (100).

1.3.3.3 Broth dilution assay

Broth dilution is considered one of the most basic antimicrobial susceptibility testing procedures. The results obtained from broth dilution are generally used to determine the minimum inhibitory concentration (MIC), which is the lowest concentration of seaweed extract that fully inhibits the growth of the test organism in the tubes. The broth dilution method has been standardised by the National Committee for Clinical Laboratory Standards (NCCLS) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (100). Dilution methods have been widely employed in determining the MIC of seaweeds against bacterial pathogens (42, 66, 106, 107) with the majority of these studies first identifying the most susceptible microorganisms using conventional diffusion methods. For instance, Srikong *et al.* investigated the antibacterial activity of *Ulva intestinalis* and *Gracilaria fisheri* against 13 bacterial strains (66). The antibacterial activity of the newly generated extracts was assessed using the disk diffusion assay at 2.5 mg/disk. The MIC of the most susceptible pathogens was determined using the Clinical and Laboratory Standards Institute (CLSI) standardised method with resavurin stain added to the incubated wells in order to quantify the cell viability spectrophotometrically and compare it to the negative control results. The MIC values ranged from 2 – 1.024 μg/mL. This is a major limitation for certain methods such as the poisoned food technique where MICs cannot be accurately defined since it is impossible to quantify the amount of extract that has diffused into the solid media (100). Broth dilution is also highly reproducible and of low cost because of the low volume of reagents and space required, making it suitable for large sample throughput particularly when performed in a 96 well plate. However, broth dilution has some technical constraints such as it is tedious, time consuming and technically demanding (108).

1.3.3.4 Bioautography assay

In the bioautography assay, Thin Layer Chromatography (TLC) is combined with both biological and chemical detection methods. This involves the compounds present in the crude seaweed extract separating out onto the TLC plate according to their polarities. This is one of the simplest methods for the screening of antimicrobial compounds and has been applied in a number of natural product screenings (109, 110). There are three different bioautography approaches that have been used to investigate the antimicrobial

compounds present in natural products including agar diffusion bioautography, direct bioautography and agar overlay bioautography.

Agar diffusion involves the antimicrobial compounds present on the TLC plate diffusing into agar that has been previously inoculated with the tested organism (111). Spraying with detection agents including 2,6-dichlorophenol-indophenol or 2,3,5-tetrazoliumchloride can aid in visualizing this inhibition (111). This is the least used bioauthography technique (112, 113). Chomnawang *et al.* applied this technique in screening Thai medicinal plant extracts for antibacterial activity against the Gram-negative bacterium *Neisseria gonorrhoeae* (114). The methanol extract obtained from the stem of *Cosinium fenestratum* exhibited a zone of inhibition and comparison with an reference sample on the TLC plate identified berberine as the active component. However, the disadvantage of this technique is obtaining complete contact with the agar is difficult and differential diffusion of compounds across the agar particularly in the case of water insoluble samples (113).

In direct bioautography, the developed TLC plate is sprayed with, or dipped into the fungal or bacterial suspension. A large number of other studies have employed direct bioautography in assessing the antimicrobial potential of seaweed extracts (115-119). Rocha *et al.* selected direct bioautography to investigate the potential of new antifungal agents present in the red seaweed, *Centroceras clavulatum* (117). Direct bioautography also exhibits reproducible results against other spore producing fungi including *Aspergillus* and *Penicillium* (100). However this technique is only applicable to organisms that can grow directly on the TLC plate (113).

Overlay bioautography is a hybrid of the other two methods and it is suitable for a wide range of microorganisms (113). It exhibits well-defined areas of inhibition and is not sensitive to contamination. Similar to direct bioautography, zones of inhibition can be visualized with the use of detection reagents such as tetrazolium salts. Overlay bioautography has been widely used in assessing the antimicrobial activity of natural products (120, 121). Tan *et al.* investigated the antibacterial compounds present in the ethyl acetate extracts of *Ulva lactuca* using disk diffusion and overlay bioautography and found that bioautography was the more appropriate method as it separated out the masking compounds from the antibacterial compounds and allowed for a direct assessment of the bands (compounds) responsible for this activity (122).

Therefore, TLC-bioautography regardless of the method, offers a rapid technique for the screening of a large volume of samples for bioactivity with the use of minimum equipment. It also has the added bonus of achieving final clean-up of fractions in order to obtain pure compounds, since the bioactive(s) separate out based on their relative affinities for the stationary phase (TLC plate) and mobile phase (100, 111). However, different mobile phase compositions may need to be investigated in order to obtain the optimum separation which can be a time consuming process.

1.3.3.5 Poisoned food technique

The poisoned food technique is commonly used to determine the antifungal potential of natural products (123-125) including seaweed extracts (47, 126, 127). In the work by Sujatha *et al.* the seaweed extract was incorporated into molten liquid media to produce a desired final concentration before being allowed to solidify in a petri dish (52). The plate was inoculated with a 5 mm disk of an actively growing fungal culture. After incubation, the radial growth of mycelium was measured and compared to that of the control in order to determine the mean inhibition percentage (52). This technique is classified as an agar dilution method which is considered relatively quick and easy and does not involve the use of complicated equipment (128), making this a popular method of choice (46, 47, 129-131). This method suffers from a number of drawbacks including the use of larger volumes of extract compared to other methods. Additionally, hydrophobic extracts can separate out in the agar leading to false negative results, volatile compounds present in the extracts can potentially affect the growth of the fungi and finally the size of inoculum and incubation conditions can affect the rate of growth, hence, percentage inhibition observed (128).

1.3.3.6 Comparison of the various antimicrobial test methods

Table 1.4 summarises the advantages and disadvantages associated with each test method. The factors to consider when deciding on the most appropriate antimicrobial test method to be used include extract solubility properties, microorganism type, cost, labour, etc. These considerations facilitate the most accurate assessment of the antimicrobial capacity of the seaweed extract.

Table 1.4: Summary of the advantages and limitations associated with each *in vitro* antifungal test method used to assess the fungicidal activity of seaweeds.

***In vitro* test method**	**Advantages**	**Limitations**
Disk diffusion	Low cost, simple, flexible, high sample throughput, low extract requirement, avoidance of organic solvents and standardised method.	Possible diffusion issues for non-polar compounds.
Well diffusion	Low cost, simple, flexible high sample throughput, low extract and consumable requirement and a standardised method.	Possible diffusion issues for non-polar compounds. Extract must be soluble in non-toxic solvents such as water.
Broth dilution assay	Low cost, highly reproducible, high sample throughput and low extract requirements. Determination of MIC and a standardised method.	Tedious, time consuming and technically demanding. Risk of errors in sample preparation and extract must be soluble in non-toxic solvents such as water.
Bioautography	Simple, rapid, reproducible results for spore producing fungi. High sample throughput, clean-up step and minimum equipment required.	Time consuming and tedious process in optimising mobile phase leading to potential high use of organic solvents.
Poisoned food technique	Widely used antifungal test method, suitable for wide range of fungi, quick, simple and avoids the use of complicated equipment.	Possible diffusion issues for non-polar compounds. Extract must be soluble in non-toxic solvents such as water. Large volume of extract required and size of inoculum can give variable results.

1.3.4 Investigation into the efficacy of the most potent extracts

From completion of the initial antimicrobial screen, the most promising extracts will have been recognized. The next step is to determine the efficacy of these extracts as potential biopesticides. There are a number of tests that can be conducted to achieve this namely; minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC)/minimum fungicidal concentration (MFC), antibiofilm activity and toxicity tests.

1.3.4.1 Minimum inhibitory concentration and minimum bactericidal concentration/minimum fungicidal concentration

MIC is essentially the broth dilution assay as described in section 1.3.3.3 and, as mentioned previously, it is the lowest concentration of the antimicrobial agent required to inhibit the visible growth of the microorganism after overnight incubation with a reduction of more than 80% of bacterial growth considered valid (132). It is generally reported as 50% (MIC_{50}) or 90% (MIC_{90}) inhibitory for natural products against the negative control (133, 134). MIC assays are typically conducted in clinical scenarios as a form of resistance surveillance and allows for a more quantitative result compared to the disk diffusion assay (135). These results give a good indication into the susceptibility of the microorganism to the antimicrobial agent and, hence, aids in ensuring that the correct dosage is administered i.e. prevents under-dosing whilst also minimizing application costs by avoiding the use of excessively high doses (136). Therefore, determination of the MIC values of seaweed extracts is commonly completed in the latter stages of an antimicrobial screen against phytopathogens (70, 72, 137).

For example, Deepa *et al.* investigated the antimicrobial properties of the red seaweed *Kappaphycus alvarezii* against fungal and bacterial plant pathogens (138). The ethanol extract proved to be the most potent and, therefore, was selected to determine its MIC against *Fusarium oxysporum*, *Aspergillus oryzae*, *Penicillium chrysogenum*, *Phytophthoracapsici*, *Colletotrichumgloeosporoides*, *Geotrichumcandidum*, *Xanthomonas punicae* and *Ralstonia solanacearum* which was found to be 30 μg/mL, 50 μg/mL, 30 μg/mL, 30 μg/mL, 30 μg/mL, 30 μg/mL, 20 μg/mL and 20 μg/mL, respectively. However, the MICs of different antimicrobial agents for a particular

organism are not directly comparable (139), therefore the MIC's are required to be completed on all promising extracts.

However, this concentration may only be bacteriostatic/fungistatic i.e. only inhibit the growth of the pathogen and once the antimicrobial compound is removed the pathogen will start to grow again. Therefore, a simple test known as the MBC or MFC is regarded as the most commonly used method to estimate the minimum bactericidal or fungicidal concentration of an antimicrobial agent (100). In this assay, sub-culturing of the MIC samples is conducted onto fresh media plates which are incubated for a specific length of time. The MBC or MFC endpoint is defined as the lowest concentration that kills >99% of the final inoculum (100). Saleh *et al.* investigated the antimicrobial potential of the three seaweed species *Ulva lactuca*, *Dilophus spiralis* and *Jania rubens* against selected pathogens (140). The MIC of the ethanol extract of *D. spiralis* against the Gram-positive bacteria *S. pyogenes* was found to be 26.7 μg/mL compared to the corresponding MBC value of 66.7 μg/mL. Frequently the MBC is greater than the MIC, since a higher concentration of the antimicrobial compound is typically required to completely eliminate the bacteria (141).

Concentration studies are also a very good indicator of the optimum treatment protocol because if the extract is found to be concentration dependent, then increasing the dose rather than the application frequency on the plant is appropriate, provided the phytotoxicity level is not reached (136). Cox *et al.* reported a concentration dependent response by the hydrophilic extracts from the brown seaweed *Himanthalia elongate* against the two foodborne bacterium *Salmonella abony* and *Listeria monocytogenes* (142). The antibacterial activity was found to decline as the extract concentration was decreased, which is expected since there are less bioactive compounds present.

1.3.4.2 Causes of biofilm formation and problems associated

Microorganisms attach to a surface and can potentially develop into biofilms. Biofilms are an assemblage of microbial cells that are irreversibly surface bound (not easily removed) (143). Biofilms are enclosed within an extracellular matrix primarily composed of polysaccharide but non-cellular materials may also be present such as; clay particles, mineral crystals of blood components depending on the environment. Typically 5 – 35% of the actual biofilm volume corresponds to the microorganisms with

the remaining composed of the extracellular matrix (144). Biofilms are capable of forming on a variety of biotic and abiotic surfaces including living tissue, medical devices, water piping systems or natural aquatic systems with Van Leeuwenhoek first detecting biofilms on the surface of teeth as sticky plaque (143). Biofilm formation requires a specific set of signaling known as quorum sensing between the microorganism and transcription of certain genes (144). Although it is regarded as a complex process, it can be broken down into a few common steps;

(1) Initial contact: The microbial cells attach to the surface via their appendages like pilli and flagella and/or physical forces e.g. van der Waal's forces with studies finding that microorganisms typically attach to hydrophobic surfaces such as Teflon and other plastics rather than hydrophilic surfaces e.g. metals. Formation of the extracellular polymeric substances (EPS) also occurs at this attachment stage.

(2) Micro-colony formation: Once attachment has been achieved, a process of multiplication and division of the microbial cells is initiated by a particular signaling within the EPS. This leads to micro-colonies consisting of many types of micro-communities which coordinate with one another such as for the exchange of substrates, distribution of metabolic products and removal of these metabolic end-products.

(3) Maturation and formation of the biofilm architecture: At this stage of formation, the microbial cells are able to communicate with one another through auto-inducer signals and facilitate quorum sensing.

(4) Detachment of the biofilm: The microbial cells within the biofilm perform further multiplication and dispersion in order to convert from sessile into motile forms. During the detachment process, the microbial communities produce different saccharolytic enzymes that help to release the surface of the microbes and allow them to enter new areas for colonization. This ability for microbes to transfer to new sites aids in the spreading of infection.

Biofilms develop for a multitude of reasons with the main incentives including; protection from the host, i.e. defence system, to allow movement to a new nutrient rich area and utilisation of cooperative benefits of living in a micro-community. Planktonic cultures are a form of *in vitro* artifact making biofilms the default mode of growth (145). It is estimated that 60 – 80% of microbal infections in the body are caused by biofilm-forming bacteria (146). These bacteria cause persistant tissue and foreign body

infections and are resistant to treatmennt from antimicrobial agents. Bacteria protected by biofilms are estimated to be up to 1000 times more resistant than their plantkonic counterparts which severly complicates treatment methods (147). There are a number of mechanisms resulting in this phenotypic resistance including antibacterial agent permeation, efflux pump systems, varying environments within the biofilm and the presence of radical scavengers within the biofilm (148). A variation in nutrients and oxygen can cause different metabolic states within the bacteria depending on their depth inside the biofilm which can affect their susceptibility to antibacterial agents. Furthermore, compounds present in the matrix can limit the diffusion of the antibacterial agents eventually binding to the antibacterial compound, thus reducing this free drug concentration. The efflux pump systems in biofilms facilitate bacterial survival under extreme conditions, such as the presence of antimicrobial agents (149). When the bacteria are dispersed from their biofilm state, antibiotic sensitivity is usually rapidly restored indicating an adaptive resistance mechanism rather than a genetic alteration (150).

This discussion on biofilms and their formation is mainly in terms of bacterial biofilms but it should also be noted that a diverse range of fungal species are capable of forming biofilms in a similar manner. They are also causing great concern to the healthcare industry with many of the medically important fungi exhibiting this property including *Candida*, *Cryptococcus*, *Aspergillus* and *Pneumocystis* (151). Similar to biofilm forming bacteria, these fungal species are more resistant to antifungal drugs compared to their planktonic counterparts by up to 1000-fold (152). Contributing factors include structural complexity, presence of an extracellular matrix, biofilm associated up-regulation efflux pump genes and metabolic heterogeneity intrinsic to biofilms with quorum sensing also believed to play a pivotal role (151). Fungi form biofilms for a myriad of reasons including as a form of survival i.e. against antifungal agents.

Bacterial and fungal phytopathogens can also form biofilms which makes them less susceptible to heat, cold and UV light. The major effects of these biofilm-based infections are the increased resistance to conventional pesticides and avoidance of the hosts defence system (153). The effect of biofilm forming pathogens and their role in plant diseases has been previously reported. For example, the pathogenicity of *Xylella fastidiosa* which is responsible for causing Pierce's disease in grapes and citrus fruits has been attributed to its ability to form extensive biofilms leading to a blockage of the

plant's vasculature (154). Peiqian *et al.* investigated the biofilm formation by the fungal pathogen *Fusarium oxysporum* f. sp. *cucumerinum* and found that *F. oxysporum* f. sp. *cucumerinum* was capable of forming biofilms under suitable conditions such as temperature, pH and carbon source (155). Susceptibility tests on these biofilms reported that biofilms possess survival advantages over their planktonic counterparts in harsh environments and more pressingly resistance against biocides which may explain the resistance of *F. oxysporum* f. sp. *cucumerinum* against antifungal drugs. This illustrates that biofilm forming plant pathogens are a major concern but are not as widely studied as medically important pathogens. Consequently, few options are currently available for controlling these pathogens, with chemical pesticides still dominating.

There are however more green strategies available particularly with increasing reports of resistance against these chemical pesticides and this is further heightened by stricter regulations and mounting pressure from the public on concerns with respect to human and environmental health (153). This has stimulated the search for alternative methods. Seaweeds are a potential substitute and have been reported to exhibit antibiofilm activity (156-158) making them an effective biopesticide against even biofilm forming phytopathogens. Studies have also investigated the sub-lethal concentration of natural products such as zosteric acid, a secondary metabolite isolated from the seagrass *Zostera marina* (159). This bioactive was capable of affecting biofilm thickness and morphology and, therefore, prevented biofilm formation. The pathogen was still metabolically active, but as mentioned previously, once a pathogen is dispersed from their biofilm state resistance usually diminishes (159). This would allow current control methods to then kill this pathogen without the excessive use of antimicrobial agents.

The most commonly used method for investigating the antibiofilm activity of antimicrobial agents is based on the static model using 96-well microtiter plates, thereby quantifying microbial biomass or metabolic activity using crystal violet (160, 161). This method is easy to perform but poses inaccuracies since it cannot distinguish between dead and living organisms within the biofilm unless complemented with colony counts.

Biofilm assay's can also give insight into the potential mode of action of the seaweed extract i.e. does it effect biofilm formation and/or disrupt the established biofilm. Antimicrobial action generally falls into one of four mechanisms:

(1) Inhibitors of cell wall synthesis: The cell wall is a critical structure necessary for the life and survival of bacteria and therefore drugs that target and inhibit the synthesis of this cell wall can kill bacterial organisms (162). This is often the mode of action of β-lactam agents such as penicillin which binds and inhibits the enzyme transpeptidase which is involved in the restructuring of the cell wall (162). The inhibition of this particular enzyme prevents the reformation of the peptide bonds making the cell wall weak with such a loss in integrity causing the bacteria to leak out its cellular contents and perish.

(2) Disruption of cell membrane: The cell membrane is an essential barrier for microbes to regulate intra- and extracellular flow of substances (162). Disruption or damage to this membrane could lead to a leakage of important solutes essential for the cell's survival. The antibiotic Polymyxin B binds to the cell membrane altering its structure, making it more permeable with an increase in water uptake leading to cell death.

(3) Inhibitors of protein biosynthesis: Protein synthesis is a vital process needed for the multiplication and survival of bacterial cells. This biosynthesis is catalysed by ribosomes and cytoplasmic factors. The bacterial 70S ribosome is composed of two ribonucleoprotein subunits, the 30S and 50S subunits with antimicrobials inhibiting protein synthesis by targeting the 30S or 50S subunit of the bacterial ribosome (162). This activity results in the disruption of the cellular metabolism of the bacteria leading to death of the organism or inhibiting of its growth and multiplication. The antibiotic chloramphenicol works by inhibiting the 50S subunit (162).

(4) Inhibitors of DNA replication: DNA and RNA are key to the replication of all living organisms including bacteria and fungi. Some antibiotics mode of action involves binding to compounds that are involved in the process of DNA or RNA synthesis, which causes normal cell processes to be, altered which will ultimately effect bacterial multiplication and survival (162). The antibiotic quinolones inhibits the enzyme bacterial DNA gyrase which is involved in the maintenance of the superhelical structure of DNA leading to cell death (162).

1.3.4.3 Toxicity testing

Toxicity testing is paramount in the screening of newly developed drugs to determine the drugs safety and whether it exhibits any adverse effects. Since the seaweeds are intended for use as biopesticides, their safety to humans and the environment must be assessed prior to their introduction to the field. *In vitro* cytotoxicity assays have become an integral part of drug discovery as it is a convenient, cost effective method for characterising the toxic potential of novel compounds on mammalian cells prior to clinical trials (163). Certain studies investigating the antimicrobial activity of seaweeds have also conducted cytotoxicity studies. For example, Baliano *et al*. studied the potential of the brown seaweed *Padina gymnospora* for its ability to improve wound healing *in vitro* (164). The antibacterial potential of the methanol extracts were assessed using the broth dilution assay against *S. aureus* that is commonly responsible for wound infections. In order to determine the safety of this extract for human use, an *in vitro* cytotoxicity study was conducted on two cell lines; fibroblasts (L929) macrophage cell lines and human ovarian carcinoma cell lines (OVCAR-3) at a concentration range of 12 - 110 μg/mL. No cytotoxic effect was detected in both cell lines until the maximum concentration (110 μg/mL) was reached (164).

The most commly used method to evaulate the cytotoxic effect of drugs is the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay which principally determines cell viability through the determination of the mitochondrial function of cells (165). The yellow MTT agent enters the cells and passes into the mictochondria where it is reduced to an insoluble purple coloured formazan product by the NAD(P)H-dependent cellular oxidoreductase enzymes. This level of formazan formed can be measured spectrophotometrically but these enzymes are only present in metabolically active cells. Therefore, these results are compared to the negative control (untreated cells) in order to determine whether the presence of this drug, e.g. the seaweed extract, has had an effect on the viability of the cells. Some studies that have investigated the safety of seaweed extracts have conducted it through both cellular cytotoxicity and acute oral toxicity, such as in mice (166, 167). Toxicogenomics analysis can be used in toxicity studies including genetics, genome sequence analysis, gene expression profiling, proteomics, metabolomics to investigate the adverse effects of environmental and pharmaceutical chemicals on human health

and the environment (168). Therefore such a genome technology can be used if toxicity by a seaweed extract is observed in order to identify the possble source of toxicity and provide insight into gene-environment interactions and the response of biologic pathways.

Although it is important to determine any potential adverse effects exhibited on humans and animals by the seaweeds, the intended use of these bioactives is as biopesticides meaning they will be applied directly to the environment. Therefore, it is critical to establish their toxicity on plants through phytotoxicity studies. Phytotoxicity is defined as a delay in seed germination, inhibition of plant growth or any adverse effect caused by a particular substance (phytotoxin) or growing conditions, with such effects depending on the uptake, the amounts reaching the site of toxic action and the toxicity exhibited at cell level (169, 170). This type of testing is typically conducted to assess potential toxic effects of pesticides on higher plants (171). Plants that exhibit phytotoxic effects from pesticide applications can demonstrate some distinctive symptoms including abnormal growth, chlorosis and leaf distortion (172). In order to establish this, the phytotoxic study must be conducted on healthy, well-established plants to separate injuries caused to plants from the pesticide rather than pathogen damage, nutrient deficiency, temperature effects, etc.

These studies have also been conducted on natural products which were investigated as potential biopesticides. Tariq *et al.* completed phytotoxicty studies of the essential oil from *Calamus* which was intended for use as a biopesticide against agricultural pests on the cotton crop, mango tree and coconut plants (173). The essential oil was found to have a phytotoxic effect on the cotton crop at a dose of 1.5 litre/acre, due to the cotton's sensitive thin leaves. The two hard leaf crops the mango tree and coconut plant, exhibited no phytotoxic effect at 2 litre/acre and additionally, produced strong control (70-90%) of mango hoppers and rhinoceros beetles on coconut plants (173). The use of parts of the plant such as root hairs to study toxicity is also possible and can be completed within a laboratory setting (174).

This relative toxicity assessment can also be completed *in vitro* as an initial indication into the potential phytotoxicity effects prior to field trials. Plant cells and tissue culture are *in vitro* test methods that can manipulate cells that have been isolated from a particular plant to allow examination into the physiology and biochemical effects of the

test substance, e.g. herbicide/seaweed extract at the cellular level (175). These methods have the added advantage of monitoring the effects of the substance under controlled conditions and allow the substance to reach the cellular target site which would not be possible with whole plant studies. The majority of studies are performed on undifferentiated callus or cell suspension cultures (175). Harms investigated the phytotoxic effect of pesticides and xenobiotics using both cell suspension cultures and whole plants (176). The cell cultures allowed for rapid evidence of the ecotoxicological behaviours of certain chemicals in plants. The study found that chemical uptake depends on both the plant species and on the physico-chemical properties of the tested chemical.

Although these tests can be a tedious time consuming process, they are essential to determine the efficacy of the extracts with the most promising ones selected for purification followed by elucidation of the antimicrobial bioactive(s).

1.3.5 Separation and purification of crude extracts

1.3.5.1 Solvent partitioning

Once the extraction step is complete, the crude extract must undergo further purification in order to obtain only the desired bioactives. This is especially necessary when the bioactives are going to be used in human applications such as in cosmetic and pharmaceutical products, mainly due to the possibility of the potential negative effects of the unknown bioactives on the user. Nonetheless, it is important for commercialization purposes to have a product with consistent efficacy, which will involve determining the stability of the particular bioactives regardless of its intended application (177). There are many techniques available for the separation and purification of crude extracts, with chromatographic methods the most commonly utilised. An alternative strategy to this is solvent partitioning of the crude extracts. The compounds are separated out depending on their relative solubilities in two specific solvents usually a polar and non-polar solvent. For example, the use of the solvent hexane can remove non-polar compounds namely lipids, oils, waxes and chlorophyll pigments, and polar solvents such as water remove sugars, amino acids and polar glycosides (10). The use of chloroform/dichloromethane can remove slightly less polar

compounds namely terpenes, with ethyl acetate/diethyl ether reported to be capable of solubilizing phenolics and halogenated compounds, and finally alcohols such as butanol which remove mainly polar glycosides namely polyphenols (10). Table 1.5 demonstrates the typical chemical classes that will be extracted in various solvents.

Table 1.5: Solvent polarity chart of the typical chemical compounds and their compatible solvents (178).

Group	Formula	Suitable solvents
Alkanes	R-H	Petroleum ether, hexane
Aromatics	Ar-H	Toluene
Ethers	R-O-R	Diethyl ether
Alkyl halides	R-X	Tricholomethane, chloroform
Esters	R-COOR	Ethyl acetate
Aldehydes and ketones	R-CO-H/R-CO-R	Acetone, methyl ethyl ketone
Amines	$R\text{-}NH_2$	Pyridine, triethylamine
Alcohols	R-OH	Methanol, ethanol, butanol, isopropyl alcohol
Amides	$R\text{-}COHN_2$	Dimethyformamide
Carboxylic acid	R-COOH	Ethanoic acid

Solvent partitioning has been used in a number of studies as a clean-up step for crude seaweed extracts (179, 180). For example, in the work by Cox *et al.*, the crude extract of *H. elongata* obtained from sequential extraction was subjected to solvent partitioning in a biphasic solvent system of water and ethyl acetate (1:1 *v/v*) (142). The aqueous extract was found to posses higher extraction yield, phenolic content and antioxidant activity compared to the ethyl acetate extract. This fractionated aqueous extract was also found to exhibit potent antimicrobial activity. Solvent partitioning is often a preliminary purification step with these active fractions in most studies subjected to further purification in order to isolate the active compounds.

1.3.5.2 Chromatographic techniques

Chromatographic techniques are the most commonly utilised isolation methods, with separation based on the compounds absorption to a solid phase which is typically silica. This absorption is influenced by the physical and chemical properties of the compounds (10). Therefore, the selected chromatographic technique and mobile phase will depend on the nature of the compounds of interest in the crude or semi-pure extract. Thin Layer Chromatography (TLC) is a type of planar chromatography in which the solid phase known as the stationary phase is present on a plane as opposed to in a column. This technique is widely used in the field of phytochemicals and biochemistry as a method of separation and characterization of components present in a mixture. The principle of TLC is that the sample is applied directly on to the stationary phase and the plate is placed in a suitable mobile phase (181). The mobile phase is drawn up through the particles on the plate via capillary action and as the mobile phase moves over the sample, the sample will either remain with the solid phase or dissolve up in the mobile phase and migrate up the plate.

Chromatographic separation depends on a number of factors; the physical properties of the individual compounds, i.e. their molecular structure, the stationary phase and the mobile phase composition (181). As mentioned previously, the most commonly used stationary phase is silica-based (normal phase separation) and, therefore, polar compounds will have a strong interaction with the stationary phase (like-for-like). Consequently, the less polar compounds will move higher up the plate but if the mobile phase is changed a to more polar mixture it will be more capable of dispersing the

compounds from the silica binding sites, hence, migrate further up the plate. Therefore, compounds are separated out according to their different polarities. The advantages of applying TLC as a separation technique are: easy to use, low cost, rapid, high reproducibility and requires minimum equipment (181). As with the majority of chromatography, there are two types of TLC known as analytical and preparative. Analytical TLC is mainly used to determine the purity of the mixture and identify the desired bioactives, whereas preparative TLC is used to isolate the compounds for further use. Numerous studies on natural products have utilised TLC as an isolation technique (182, 183). Tan *et al.* employed both types of TLC for separating the antibacterial components present in the crude ethyl acetate extract of the green seaweed *Ulva lactuca* (122). As mentioned previously, TLC can be used as a characterization technique and this typically involves staining developed plates with specialized stains as a preliminary phytochemical screening (184).

Column chromatography is also a widely applied chromatographic technique used to separate a mixture into their individual components. Similar to TLC, it consists of two phases: one mobile phase and one contiguous stationary phase where the stationary phase, typically silica-based, is packed into the tube or column (185). The sample mixture is added to the top of the column and with the aid of the mobile phase is moved through by "dispersing" the compounds from the binding sites. The rate at which compounds migrate through the column depends on the different degrees of adhesion to the silica binding sites. In a similar manner to TLC this rate depends on the compounds, stationary phase and mobile phase. Each band that is eluted from the column is collected as a purified fraction that can be used for further analysis. Column chromatography has been utilised as one of the top isolation methods for the separation of bioactives in seaweeds (69, 186, 187).

Preparative high performance liquid chromatography (HPLC) has also been utilised in the isolation and purification of products (188) but is not as popular (a purification method) compared to the other chromatographic techniques for purifying natural products, since the equipment is expensive and has high mobile phase throughput (189). Additionally prior to scaling up to preparative HPLC, the sample is typically analysed on an analytical HPLC method to allow for method development with reduced extract and solvent consumption before the optimum chromatographic conditions are directly transferred to preparative HPLC (188). Wei *et al.* successfully isolated and purified four

isomeric bioactive saponin compounds from the seeds of *Aesculus chinensis* by preparatory HPLC using a gradient mobile phase solvent system of methanol-water-acetic acid completed in a 120 min one step separation (190).

Overall, chromatographic techniques are effective methods in isolating antimicrobial compounds present in seaweeds including polysaccharides (191), lipids (192, 193), diisooctyl phthalate (187) and halogenated compounds (bromophenols) (194). Regardless of the selected method, careful consideration of the stationary phase and mobile phase is necessary for the successful isolation of the compound of interest. There have been other methods applied including solid phase extraction (SPE), precipitation and membrane dialysis for the purification of crude seaweed extracts (195).

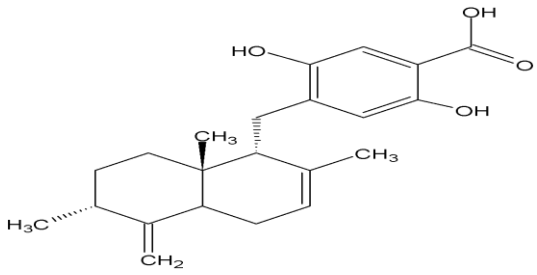
1.3.6 Identification and structural elucidation of purified antimicrobial compounds

The next step is the structural elucidation of these purified antimicrobial compound(s). This can be a tedious time consuming process but can be easier if the compound has been previously identified, as this allows for comparison and confirmation with databases, such as the National Institute of Standards and Technology (NIST) database (196). However, if the compound is novel and has not yet been previously reported, it means complete spectroscopic analysis is required. Table 1.6 outlines the numerous novel antimicrobial compounds that have been isolated from seaweeds over the last number of years (197).

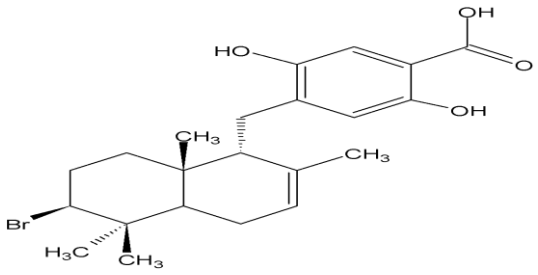
Table 1.6: Novel antimicrobial compounds isolated from seaweeds.

Seaweed	Antimicrobial compound	Ref
Peyssonnelia sp.	Peyssonoic acid A and B	(198)
Laurencia sp.	Tiomanene acetylmajapolene (A and B)	(199)
Polysiphonia lanosa	3-Dibromobenzaldehy-4-5-disulfate dipotassium salt 5-Bromo-3,4-dihydroxybenzaldehyde	(200)
Dictyopteris zonarioides	Zonarene (conjugated diene member)	(201)
Sargassum macrocarpum	Sargafuran	(202)
Laurencia mariannensis	10-Hydroxy kahukuene B	(203)

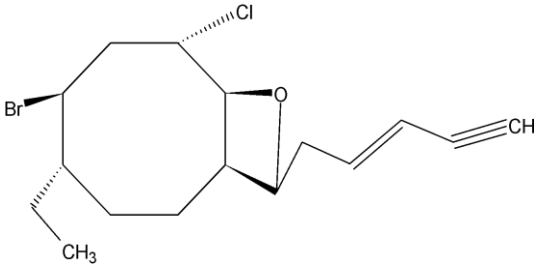
Figure 1.2 demonstrates the structure of each of the antimicrobial compounds outlined in Table 1.6.



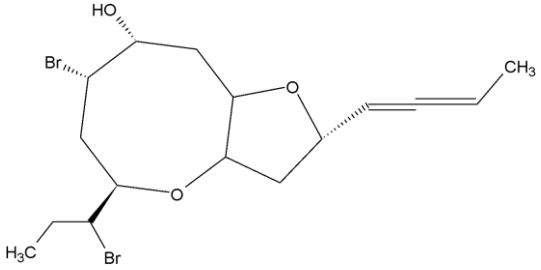
Peyssonoic acid A



Peyssonoic acid B



Tiomanene acetylmajapolene A



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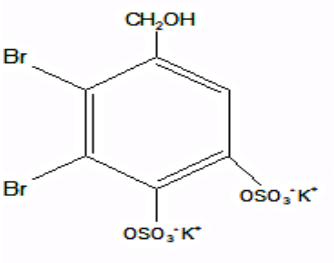
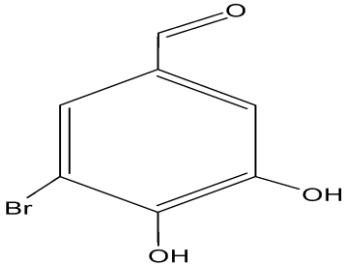
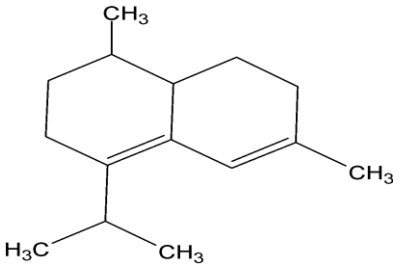
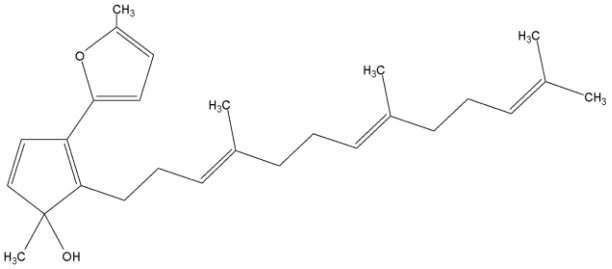




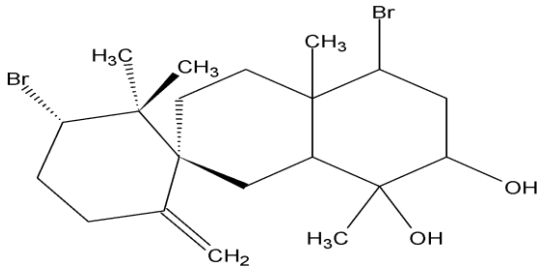
3-Dibromobenzaldehy-4-5-disulfate dipotassium salt/5-Bromo-3,4-dihydroxybenzaldehyde



Zonarene



Sargafuran



10-Hydroxy kahukuene B

Figure 1.2: Structures of the antimicrobial compounds isolated from the seaweeds in Table 1.6.

Determination of the structure of these compounds can involve a range of spectroscopic techniques including ultraviolet-visible (UV-visible), infrared (IR), nuclear magnetic resonance (NMR) and mass spectroscopy. Numerous studies have used the spectrums produced from either one or more of these techniques for structural clarification on their compound of interest (118, 198) or identification of the chemical constituents in a particular seaweed extract (104). UV-visible spectroscopy can be used to identify certain classes of compounds in both a pure and crude extract. IR spectroscopy is related to the vibrational changes that happen within a molecule when it is exposed to infrared radiation. Different bonds such as C-C, C=C, C-O, C=O, O-H, and N-H exhibit distinct vibrational frequencies (197). Therefore, if the antimicrobial compound is subject to IR analysis and contains such bonds they will be identified by their characteristic frequency absorption bands observed in the resultant IR spectrum. Fourier transform infrared spectroscopy (FTIR) is commonly used in the identification of bioactives in natural compounds (197). FTIR is a high-resolution analytical technique for identifying the chemical constituents and elucidating structural components. FTIR offers a rapid and non-destructive form of investigative analysis to fingerprint natural extracts.

NMR is essentially related to the magnetic properties of certain atomic nuclei; namely the nucleus of the hydrogen atom, the proton and the carbon and an isotope of the carbon (197). Based on the differences between the various magnetic nuclei a picture of what position these nuclei are in within the molecule can be produced. Furthermore, it will demonstrate which atoms are present in neighboring groups and ultimately the amount of atoms that are present in each of these environments. Mass spectroscopy can also be used in compound identification and involves molecules being bombarded by either electrons or chemically within the mass spectroscopy system (197). This results in the ions becoming charged (highly energetic) which are separated according to their specific mass-to-charge ratio (*m/z*). This produces a mass spectrum, which is a plot of the relative abundance of a fragment ion against the ratio of *m/z* of these ions. Therefore, mass spectroscopy can be used to determine the molecular mass of the antimicrobial compound with high accuracy and from piecing the fragment pattern together, the molecular formula can also be predicted (197). Mass spectroscopy is typically coupled with a separation technique such as liquid chromatography-mass spectroscopy (LC-MS) or gas chromatography-mass spectroscopy (GC-MS). X-ray

crystallography can be conducted if the other spectroscopic analysis proves inconclusive, since x-ray crystallography provides detailed atomic information and a 3-dimensional image of the compound of interest.

As mentioned previously, UV-visible spectroscopy can provide information on certain functionalities within the compound. For example, unsaturated organic compounds can exhibit a wide absorption range between both the UV and visible wavelength regions, but the larger conjugated compounds will shift to longer wavelengths into the visible light region (204). Carotene exhibits this trait due to its unsaturated structure which consists of a system of extensively conjugated pi-electrons which explains its distinct orange colour (204). Saturated compounds on the other hand only have sigma bonds, which are very stable and hard to break compared to the weaker pi bonds present in unsaturated systems. Therefore, saturated compounds are less reactive and therefore exhibit a shorter wavelength generally in the range of 150 -250 nm (λ).

A compound of interest can be subjected to IR spectroscopy in order to determine particular bonds that produce these characteristic absorption bands. For instance, an O-H stretch for alcohol typically produces a distinctive broad mountain peak around 3400 cm^{-1}, N-H stretch corresponding to amines occur between 3000 – 3700 cm^{-1} but are weaker and narrower, carbonyl stretch (C=O) produces the most distinguishing bands and can be found at 1640-1820 cm^{-1} as a very strong peak. Benzene rings produce distinctive peaks between 680 – 900 cm^{-1} depending on its substitution pattern and hydrocarbons show IR peaks between 2800 – 3000 cm^{-1} due to C-H stretching vibrations (205).

Rajauria *et al.* used the spectroscopic techniques, UV-visible and FTIR, to characterize the active compound fucoxanthin present in *H. elongata* (118). The crude extract was purified using preparative TLC with UV-visible analysis of the purified compound identified three lambda max's (λmax) (331, 446 and 468 nm) (118). Figure 1.3 illustrates the chemical formula of fucoxanthin. The FTIR spectrum revealed characteristic functional groups such as OH group, C=O, C-H stretch and CH_2 stretch (118). Since these spectroscopic techniques provide only information on the characteristic functional groups present in the compound, it is not conclusive conformity. Therefore, comparison with a commercially available standard is required as completed by Rajauria *et al.*, which confirmed that the active compound was

fucoxanthin (118). This may not always be possible, particularly if dealing with a novel compound.

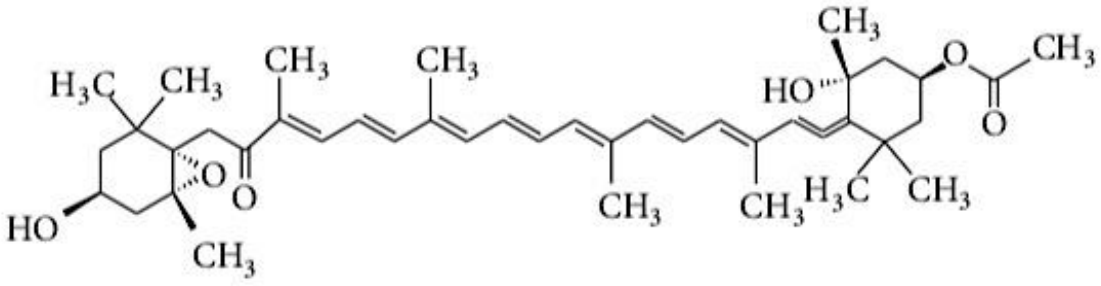


Figure 1.3: Chemical structure of fucoxanthin (formula $C_{42}H_{58}O_6$) (118).

GC-MS has also been utilised as a method for structural elucidation and allows identification and quantitation of the compounds simultaneously. The first thing to examine on the resultant mass spectrum is the parent ion followed by the largest peaks in order to determine the fragments generated. Typical fragmentation ions and their corresponding *m/z* are: CH_3 = 15 *m/z*, H_2O (water/aldehyde/ketone) = 18 *m/z*, CH_2OH^+ (alcohol/ether) = 31 *m/z*, CH_3-OCO^+ (ester) = 59 *m/z*, $C_6H_5^+$ (aromatic) = 77 *m/z* and $C_6H_5O^+$ (phenol) = 93 *m/z*. These ions may appear as peaks on the spectra i.e. fragmentation ions or the loss of these ions from the molecule (206) and by piecing these together the molecular formula of the compound can identified. GC-MS is one of the most widely used spectroscopic techniques to identify components present in seaweed extracts (141, 207-209). This is important to note that for a compound to be suitable for GC-MS analysis, it must posses sufficient volatility. LC-MS can be used, particularly if the compound is thermally unstable. Chia *et al.* investigated the antioxidant and cytotoxic activities of three seaweed species (210). The purified extracts were subject to LC-MS analysis and revealed the presence of bioactive alkaloids namely camtothecin, lycodine and pesudopelletierine.

Once the basic structure is known, NMR can be used to confirm molecular identity (211). NMR can provide information on the relationship between the hydrogen and carbon atoms with the chemical shifts indicative of the functionalities present in the molecule. In ^{1}H NMR, chemical shifts of common functional groups include: alcohol =

0.5-5 ppm, aromatic = 7.3 ppm, phenol 4-7 ppm, amide = 5-9 ppm and carboxylic acid = 10-13 ppm (212). Each peak shown in the ^{13}C NMR data represents a carbon atom in a different environment within a molecule with typical ^{13}C chemical shifts as follows: ether/alcohol/ester = 60-80 ppm, alkene = 120-160 ppm, aromatic = 125-170 ppm and aldehydes/ketones = >200 ppm (213). More in depth information can be obtained by the other techniques available in NMR including 2-dimensional correlation spectroscopy (2D COSY), distortionless enhancement by polarization transfer (DEPT) and nuclear overhauser effect spectroscopy (NOESY). Studies have utilised NMR as an effective identification technique for bioactives present in seaweeds.

Rodrigues *et al.* characterized both *Sargassum muticum* and *Osmundea pinnatifida* extracts and confirmed the presence of important polysaccharides for example fucoidans in *S. muticum* extracts and agarans as sulfated polysaccharides in *O. pinnatifida* extracts (214). This was achieved through the help of ^{1}H NMR analysis with the spectrums for both extracts relatively similar revealing distinct peaks including alkylic hydrogen atoms, carbonyl, aliphatic C-H directly bound to an oxygen atom and aromatic hydrogen atoms. FTIR-ATR analysis of the extracts further supported this data with the detection of sulfate ester groups which is a characteristic component in fucoidan and other sulfated polysaccharides. Barros *et al.* completed structural characterization of polysaccharides obtained from the red seaweed *Gracilaria caudate* (215). High performance size exclusion chromatography found that the polysaccharide fraction consisted of a high molecular weight polysaccharide. Chemical analysis of this fraction by NMR spectroscopy revealed a signal from the α anomeric proton at δ 5.13 was assigned to 3,6-α-L-anhydrogalactose (LA) while the signal at δ 4.56 was attributed to β-D-galactose (G) linked to LA.

Overall, it can be seen that there are a wide range of spectroscopic techniques currently available with typically a number of these techniques combined in the identification of a bioactive compound. For instance, El Shouny *et al.* identified the antibacterial compound present in the *U. lactuca* extract as an aromatic ester derivative named di-isooctylphthalate through UV, IR, GC-MS and ^{1}H NMR analysis of the purified sample (187). Once the compounds have been identified, its efficacy must be tested utilising MIC, MBC and its toxicity profile.

1.4 Biopesticide Properties of Seaweed Extracts

Economic losses due to pathogenic infections are estimated at $40 billion annually in the United States alone (216). In order to minimize this disease-induced damage to crops, chemically-based substances are typically applied, but due to their toxic and biologically active nature, they are of serious concern to the environment, especially when used on food products where toxic residues can remain. These synthetic pesticides are generally non-targeted, meaning they can exhibit harmful effects on surrounding organisms (217). They have also been found to remain in the environment for a very long duration after being introduced, which poses added health risks and aids in the development of pest resistance to them (73). However, marine plants, animals and microbes are known to contain a variety of novel bioactive compounds with over 12,000 chemicals discovered (73). Seaweeds are a rich source of these bioactive secondary metabolites, with recent studies demonstrating the potential pesticidal properties of a variety of seaweed extracts, as discussed below.

1.4.1 Antimicrobial activity of seaweed extracts against plant pathogens

Many plant pathogens are spreading to new regions mainly due to the increased movement of people and products, while global warming is allowing disease vectors to expand their ranges (218). In order to control this influx, the spraying of fungicides and bactericides has become the main protocol. These pesticides are generally of a chemical nature, but due to recent measures on restricting the use of synthetic pesticides, the use of safer more environmentally friendly alternatives are being considered, such as the use of natural compounds. Marine algae are being considered due to their potential as a source of novel bioactive compounds that could aid in protecting plants against pathogenic microbes (219).

Puglisi *et al.* investigated the potential antifungal activity of the green alga *Penicillus capitatus* against the marine pathogen *Lindra thallasiae* (220). Crude aqueous and ethanol extracts of the seaweed were shown to exhibit potent inhibition of the fungal pathogen with 100% and 97% growth inhibition, respectively. The authors also isolated two new triterpene sulphate esters known as capisterones A and B from the crude ethanol extracts using bioassay-guided fractionation. The identification involved a

number of spectroscopic techniques including, IR, and ^{13}C, ^{1}H, 1D, 2D COSY, DEPT NMR. Figure 1.4 illustrates the chemical structure of the triterpene sulphate esters that was found to be solely responsible for the antifungal activity possessed by *Penicillus capitatu*.

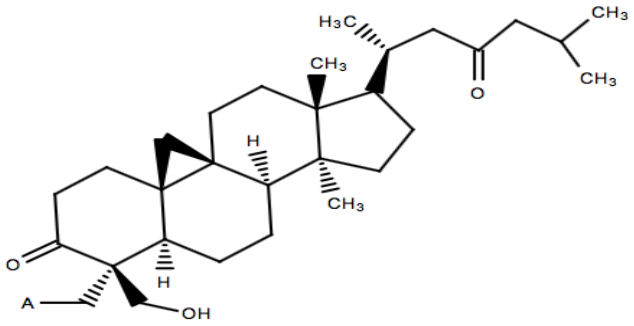


Figure 1.4: Chemical structure of the triterpene sulphate esters (A = NaO_3SO) isolated from *Penicillus capitatus* through bioassay-guided fractionation (220).

The fungicidal potential of seaweeds have been evaluated on a wide variety of fungal plant pathogens including *Alternaria radicina* (44), *Alternaria solani* (44), *Fasarium oxysporum* (63), *Phytophthora cinnamomi* (219), *Rhizoctonia solani* and *Macrophomina phaseolina* (52). Jayaraj *et al.* conducted a study on the fungicidal activity of the commercial seaweed spray formulation containing the aqueous extract of the brown seaweed *Ascophyllum nodosum* (0.2%) on carrot plants that had been inoculated with the fungal pathogens *Alternaria radicina* and *Botrytis cinerea* (221). Control plants were sprayed with water whilst other plants were sprayed with salicylic acid which has been reported to induce systemic acquired resistance in plants. It was found that the application of the seaweed extract and salicylic acid to the infected plants resulted in a reduction in the disease after 10 days with additional applications of seaweed extract on day 10 and 20 leading to a further reduction, with both *Alternaria radicina* and *Botrytis cinerea* reduced by up to 57% and 53.5% respectively (221). The seaweed treated plants were found to show less of both diseases compared to salicylic acid and the control. Molecular analysis determined that the seaweed sprayed plants exhibited an accumulation of defence gene transcripts at higher levels with increased

enzyme defence activity as well as phenolics and phytoalexins, providing evidence of the potential induced resistance of the carrot plants as a result of treatment by seaweed bioactives (221).

Peres *et al.* investigated the antifungal activity of ten seaweed species against the plant pathogen *Colletotrichum lagenarium* that mainly effects a wide range of economically important plants namely cucumbers, pumpkin and eggplant (51). The seaweeds were extracted with ethanol and the antifungal activity was assessed using direct bioautography assays. *Stypopodium zonale*, *Laurencia dendroidea*, *Pelvetia canaliculata*, *Sargassum muticum*, *Ascophyllum nodosum* and *Fucus spiralis* extracts were found to significantly inhibit this pathogen. The red seaweed *L. dendroidea*, was selected for further analysis and was purified using column chromatography. GC-MS analysis on the fractions revealed the terpenes neophytadiene, cartilagineol, obtusol elatol and ethyl hexadecanoate which may be responsible for this fungicidal activity against this important agricultural pathogen (51).

The bactericidal activity of seaweeds against plant pathogens has also been explored by researchers with Kulik one of the first researchers to investigate the possible use of cyanobacteria and algae against plant pathogenic diseases (222). Similar to the fungicidal activity, seaweeds have also been evaluated against a range of bacterial plant pathogens such as *Xanthomonas* sp. (70), *Clavibacter michiganensis* subsp. sepedonicus (223), *Pseudomonas syringae* (72), *Xanthomonas punicae* and *Ralstonia solanacearum* (138). From examination of the mentioned pathogens, it can be seen that the majority of these are Gram-negative bacteria demonstrating the potent nature of seaweed extracts since these type of bacteria are considered much harder to kill because of their protective outer membrane (224). For example, Ibraheem *et al.* evaluated the antimicrobial activity of crude methanol extracts from the brown seaweed species *Sargassum latifolium*, *Hydroclathrus clathratus* and *Padina gymospora* against soil borne pathogens (137). All of the extracts exhibited some degree of activity against the two Gram-negative bacterial pathogens. The *P. gymospora* extracts demonstrated the strongest activity with inhibition zones of 18.3 and 16.3 mm against *Ralstonia solanacearum* and *Pectobacterium carotovora,* respectively (137). Phytochemical analysis of the extract showed a high concentration of saturated fatty acids. Paulert *et al*. also reported this potent antibacterial activity against the two Gram-negative plant pathogens *Xanthomonas campestris* and *Erwinia carotovora* from the green seaweed,

Ulva fasciata (70). The methanol extracts exhibited activity against both pathogens with the highest activity against *E. carotovora* with a MIC of 1 mg/mL (70).

All of these studies demonstrate that compounds found within seaweeds exhibit potent fungicidal and bactericidal activity and would be a potential alternative to the chemical pesticides currently in use. However, detection of this antimicrobial potential from seaweeds is not enough for commercialization purposes, with few authors definitively identifying such bioactive compounds and the majority of researchers highlighting the requirement for further purification. Such studies generally only indicate a number of key compounds which may be responsible for such activity, including phenol compounds whose mode of action is believed to lie in their ability to disrupt the cytoplasmic membrane of the pest, causing cell leakage (225). Steroids such as terpenes composed of a specific ring structure of four cycloalkane rings are potent compounds that interact with sterols in the pest's membranes (225). A number of studies have however reported specific compounds responsible for antimicrobial activity in seaweeds (220, 226). Nonetheless, this trend supports the importance around the development of effective isolation and identification techniques to ensure complete elucidation of the compound of interest.

1.4.2 Insecticidal and larvicidal activity of seaweed extracts

In addition to the antifungal and antibacterial activity, seaweeds were found to contain insecticidal and larvicidal compounds. This has recently become a popular concept, especially due to their non-toxic nature when used in agricultural situations. Numerous studies on the insecticidal potential of seaweeds has been carried out against a range of pests: the tomato moth *Tuta absolute (Povolny),* the greenbug, *Schizaphis graminum* (227), the mosquito larvae, *Culex pipiens*, the cotton leafworm, *Spodoptera littoralis* (228) and a nematodes *Meloidogyne javanica* (229). Asha *et al.* investigated the insecticidal activity of *Ulva fasciata* Delile and *Ulva lactuca* Linnaeus extracts against *Dysdercus cingulatus* (230). *Dysdercus cingulatus* is considered a serious cotton pest that infests cotton from a seedling through to harvesting by sucking on the developing cottons bolls and ripe cotton seeds and transmitting fungi. The bioactives were extracted by Soxhlet extraction using polar (methanol and water) and non-polar (chloroform and hexane) solvents (230). Different concentrations of the extracts were fed to five healthy

insects in an artificial mix for 96 h continuously with mortality recorded every 24 h. *U. fasciata* and *U. lactuca* extract activity was found to be dose dependent, with the methanol extracts for both exhibiting the highest mortality. It was found that the seaweed extracts entered the alimentary canal while feeding and caused mortality. It was also observed that the extracts reduced the relative growth rate, adult longevity, fertility and hatchability of the *Dysdercus cingulatus* (230). The management of this pest (*Dysdercus cingulatus*) by the use of marine algae was also conducted by Sahayaraj *et al.* who also reported this potent insecticidal activity by the tested crude extracts from *Caulerpa veravalensis, Caulerpa scalpelliformis, Padina pavonica, Sargassum wightii, Ulva fasciata* and *Ulva lactuca* (231).

Larvicide is a type of insecticide that is specifically targeted towards interrupting the larvae life stage of an insect. The majority of these studies were evaluated on the potential larvicidal activity of seaweed extracts against mosquito larvae (232-234). Mosquitos play a major role in the transmission of some deadly diseases including dengue, malaria, yellow fever and filariasis and are among one of the greatest health problems worldwide (235). Since larval stages of mosquitoes are entirely aquatic, the treatment with algal based larvicides during their breeding habitat is much more efficient and safer than interrupting the adult stage, especially since this stage occurs in human settings and can easily overcome remedial controls (236). Ali *et al.* investigated the mosquito larvicidal efficacy of ethanol extracts of seaweed against *Aedes aegypti, Culex quinquefasciatus* and *Anopheles stephensi* vectors (235). The larvae were placed in a bowl containing distilled water and plant extract for 24 h after which the mortality rate was recorded. Out of the nine seaweeds tested, *Caulerpa racemosa* was found to be the most potent against 4^{th} instar larvae of *Aedes aegypti, Culex quinquefasciatus* and *Anopheles stephensi* with LC_{50} (lethal concentration required to kill 50% of population) values of 0.055 μg/mL, 0.067 μg/mL and 0.066 μg/mL, respectively (235). Therefore, these studies support the evidence that seaweeds consist of a multitude of bioactives with a range of biological activity with their potential to be developed into eco-friendly insecticides.

1.4.3 Herbicidal activity of seaweed extracts

Herbicides are a specific type of pesticide that are used to kill unwanted vegetation and are commonly used in agriculture and forestry for the control of weeds. These are generally synthetic herbicides, mainly because they are faster and less expensive than manually removing these invasive species. The ideal herbicide would be only effective against the target pest and not against non-target species including humans, but unfortunately this is not the case. The overuse of such pesticides has resulted in negative effects to the health of humans and animals as well as the surrounding environment. The production of chemical pesticides now accounts for about 2% of the total world chemical market (237). Furthermore, as herbicides are generally sprayed on agricultural land, the potential for pesticide residues to remain in the plant products is always a concern. However, work related to the herbicidal activity in seaweeds is niche compared to the search for new antimicrobial and insecticide compounds. Andras *et al.* conducted a study on seaweed allelopathy against coral with four seaweeds applied against the coral *Poritesrus.* The red alga *Phacelocarpus neurymenioides* was shown to exhibit the highest allelopathic activity against the coral and through bioassay-guided fractionation, it was found that the already characterized antibacterial metabolite, neurymenolide A was the main allelopathic agent (238). Another red seaweed *Plocamium brasiliense* was evaluated for its potential allelopathic effects on seed germination, radicle elongation and hypocotyl development of the weeds *Mimosa pudica* L. and *Senna obtusifolia* (239). The dichloromethane extract showed the maximum inhibition of seed germination for both weeds at 35% and 14%, radical germination (52% and 41.7%) and hypocotyl development (17.1% and 25.5%), respectively (239). These results illustrate the potential use of the red seaweed *Phacelocarpus neurymenioides* and *Plocamium brasiliense* as herbicides against problematic species due to their production of allelopathy substances.

1.5 Applications of Biopesticides

1.5.1 Factors contributing to the emergence of plant pathogens

The economic impact arising from the spread of pests and diseases in plants is a major worldwide issue (240). For example, ash dieback caused by the fungus *Hymenoscyphus pseudoalbidus* is developing rapidly across Ireland. Ash is one of the most important native tree species in Ireland accounting for 3.8% of the entire forest estate with a high sale value in the hurley market (241). The loss in potential revenue as result of this disease is forecast to be around €800 million for farmers and landowners. Although, overall, the Irish landscapes are relatively healthy with only a few serious pests threatening individual species mainly due to Irelands island status and strict plant health regulations (242). The Irish Department of Agriculture, Food and the Marine (DAFM) regulates the movement of plants and plants products into and within Europe (243). This comprises of checks at the border on plants and plant products and, therefore, such goods are imported at an approved Border Inspection Post such as Dublin airport and port (244). Customs detain and inform the DAFM who will advise the correct procedure to follow and once all checks have been successfully completed and are complaint with the DAFM regulations a Clearance Certificate is issued, allowing customs to release the consignments for entry into free circulation (244). But if the consignments are non-compliant the next course of action is either re-export or destruction of consignments as instructed by the DAFM. Plant and plant products intended for export also require checks in order to obtain their export certification of Irish grown produce (243). These, regulations therefore help to minimise the potential introduction of plant pathogens into Ireland and within Europe.

However, with the increasing movement of plant materials and wood products between countries such as logs, timber pallets, packing cases etc., together with the growing movement of people, the likelihood of the spread of more potentially damaging pests and diseases is increasing. Additionally, even with these regulations in place, the international plant trade, which is estimated to be worth US $40 – 80 billion annually (245), has been tightly linked to biological invasions (246), with more than half of the emerging infectious plant diseases in the last few decades originating from plant trade (247). Over 2400 microorganisms exist in seeds of 383 genera of plants worldwide with an estimated one third of plant viruses being seed borne (245).

Another key factor in the introduction of new plant pathogens is climate change with increasing temperatures allowing the establishment of pathogens into new geographical areas (where they have the opportunity to come into contact with new potential hosts). For example, forests on the West coast of North America are becoming susceptible to invasion from the oomycete *Phytophthora ramorum* which is responsible for sudden oak death, currently causing high mortality rates across Europe (248). Climate change predictions indicate trees and other plants may be put under increased stress, making them more susceptible to infection from native pests and diseases (249), even from species that are usually considered harmless. The endophytic fungi *Biscogniauxia mediterranea* (De Not.) Kuntze can cause charcoal disease on drought-stressed cork oaks (*Quercus* spp.) and other hardwood hosts (250). Additionally, changes in temperature and moisture availability may have a negative impact on the population of certain species, leading to an epidemic increase of certain pests (249).

The impact of invasive species does not only affect biodiversity but also has impacts on key sectors such as forestry, agriculture, tourism and construction. The estimated combined annual cost of invasive species such as the Japanese knotweed (*Fallopia japonica*) to economies of the UK and Ireland is €2.3 billion (251). Therefore, it is clear that controlling plant pathogens is important, especially in the initial stages of invasion. Once a pathogen is established, there are very few treatments that can help a diseased tree to recover (252). This not only impacts the region's biodiversity, but also increases the costs associated with long-term control and management, making prevention key.

Although invasion from non-native pests and diseases can have a devastating effect on biodiversity and economically important industries already establised, pathogens can be extremely problematic especially when they form resistance against their current control methods. Here in Ireland, a number of native pests are causing havoc to the forestry industry namely the Large Pine Weevil (*Hylobius abietis*), ash dieback disease (*Hymenoscyphus pseudoalbidus*), root rot fungi (*Armillaria mellea and Heterobasidion annosum*) and some weed species such as the *Rhododendron ponticum*.

In section 1.5.2 a discussion on the plant pathogens to be investigated within this body of work will be conducted and includes two native root rot fungi and nine quarantine bacterial pathogens.

1.5.2 Economically problematic pathogens

1.5.2.1 *Armillaria* spp.

Armillaria spp. is amongst the best-known and most destructive pathogens of forest trees causing root and collar rot on trees. They are found worldwide and have broad host ranges infecting mainly woody species such as *Abies*, *Picea*, *Pinus*, *Pseudotsuga* (253) resulting in serious losses in managed natural forests and plantation sites consisting of non-native tree species (254). Most of the species have a saprophytic lifestyle and are natural components of the forest ecosystems contributing to the decomposition of organic materials (254). They only become problematic pathogens when trees become stressed, for example prolonged drought or attack by other fungi, making them susceptible (255). The fungus exists as rhizomorphs and vegetative mycelium in the dead wood of tree stumps and roots, with research showing the fungus surviving on the trunk of a tree that was killed several years earlier (256). In late autumn, these rhizomorphs may form mushrooms that have the ability to release millions of basidiospores which are then carried by the wind to another dead stump or the injured bark of a living tree.

Under the correct moisture and temperature conditions, the basidiospores germinate and produce a mycelium that initially infects the bark before spreading to the sapwood and cambial regions of the tree (256). While on the sapwood, the mycelium forms white mats followed by the formation of rhizomorphs which have been found to grow for a distance of up to three metres through the soil causing root and collar rot on trees. This is the principal method of tree-to-tree invasion of the fungus, as these rhizomorphs move through the soil to adjacent healthy trees or direct root contact. Infection arises once the mycelium comes into contact with and adheres to a susceptible hosts roots in the manner known as gelatinous secretion. In this process, the rhizomorphs penetrate the root by a sequence of mechanical pressure and enzymes that partially digest the root's cell walls and continues to grow into the root tissue between the cells (256).

The early symptoms of *Armillaria* infection is the appearance of yellowish-brown water soaked lesions on the cortex at the root collar (257). The cortex gradually becomes darker and eventually rots. The phloem tissues readily flake off leading to the inner tissues such as the xylem becoming exposed and turning brown before rotting. The final stage is the growth of white filamentous mycelium into the infected tissues with the

formation of clustered yellowish umbrella-shaped sprocarps on the rotten roots (257). Figure 1.5 demonstrates these signs of infection by the sub-species *A. mellea* (Vahl) P. Kumm.

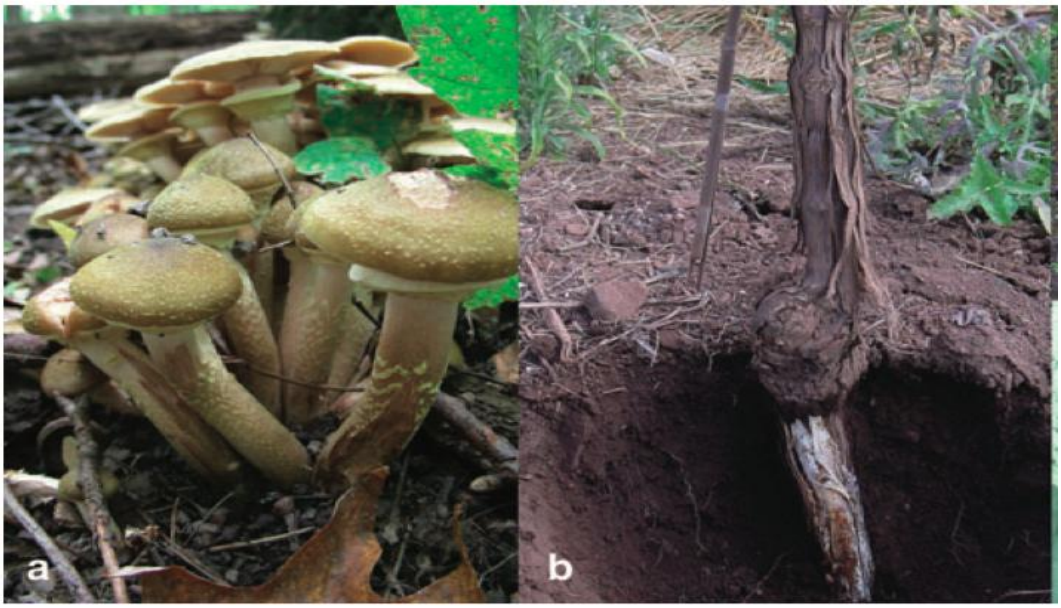


Figure 1.5: Signs of *Amrillaria* infection (a) basidiopscarps of *A. mellea* in a mixed hardwood forest (b) mycelial fans found beneath the bark at the root collar of *Vitis vinifera* (grapevine) infected with *A. mellea* (253).

Infection of the root system does not immediately show symptoms of infection to be observed on the aerial part of the tree. The above ground symptoms of a *Armillaria* infection includes yellowing of leaves, reduced terminal growth, dieback of twigs and branches, chlorosis of needles, crown thinning and premature fall coloration (256). However, it is not uncommon for the disease to be very advanced without showing any obvious symptoms.

There is currently no fungicide available that will kill *Armillaria* (258). Control of *Armillaria* has been mainly focused on pre-plant eradication through soil fumigation, but due to increased regulation as a result of fumigates being toxic, biocides that threaten worker safety, depletion of the ozone layer and negatively impact on non-target species this will have to change (253). There has been a shift to new environmentally friendly methods including the use of biological control agents such as *Trichoderma*

species and depending on the particular isolate of *Trichoderma* used, control may be achieved through competition (259). Asef *et al.* investigated the antagonistic effects of *Trichoderma* species as biocontrol against *A. mellea* infection (260). Disks of *T. virens* and *T. harzianum* were placed onto cultures of *Armillaria* and it was found that both *Trichoderma* isolates colonized *Armillaria* colonies within 5-7 days. Volatile compounds present in *Trichoderma* isolates inhibited the growth of *Armillaria* colonies and rhizomorphs formation. Mechanisms of action were investigated through light and scanning electron microscopy and these included penetration of *Trichoderma* hyphae in rhizomorphs, degeneration and lysis of rhizomorph tissue and discharge of *Armillaria* rhizomorph content (260).

Armillaria consists of more than 40 morphologically distinct species (253) with significant differences in pathogenicity observed among species with *A. ostoyae* (Romagn.) Herink and *A. mellea* (Vahl) P. Kumm species found to be strongly pathogenic (261). The subspecies *A. mellea (*Vahl) P. Kumm has been recorded in Ireland on *Fraxinus* (Ash), *Quercus* (Oak), *Pinus sylvestris* (Scot's pine) and *Picea sitchensis* (Sitka spruce) as parasitic (262, 263). Identification of *A. mellea* was achieved by microscopical examination, digital imaging and secondary morphological characteristics such as clamp connections (262). *A. mellea* has been reported on hardwood forest trees, amenity trees, orchard trees and grapevines. This disease, therefore, poses a threat to the timber industry as well as affecting recreational areas. For this reason, the subspecies *A. mellea (*Vahl) P. Kumm was selected as one of the fungal pathogens to be tested in Chapter 2.

1.5.2.2 Heterobasidion annosum

Another root rot fungi causing devastation to the forestry industry is a complex of the genus *Heterobasidion* known as *Heterobasidion annosum* (Fr.) Bref. *sensu lato* (*s.I.*) and is regarded as one of the most destructive fungal pathogens on conifers worldwide, especially in Europe, causing annual losses of over US$1030 million (264). The complex consists of five different species: *H. annosum sensu stricto* (*s.s.*), *H. parviporum*, *H. abietinum*, *H. irregulare* and *H. occidentale*. These species can be differentiated from one another on the basis of partial reproductive isolation and morphology examination and can also be characterised by distinct host preferences (265). For instance, *H. annosum s.s.* mainly infects pines particulary Scots pine (*Pinus sylvertris* L.) and other conifer species. *H. parviporum* is strictly associated with Norway spruce (*Picea abies* (L.) Karst.), *H. abietinum* mainly attack Silver fir (*Abies alba* Mill.), *H. irregulare* typically infects pines, Junipers (*Juniperus* spp.), and Incense cedar (*Calocedrus decurrens* (Torr.) Florin), whereas *H. occidentale* exhibits a broader host range and can be found on the genera *Abies*, *Picea*, *Tsuga*, *Pseudotsuga*, and *Sequoiadendron* (265). *H. annosum s.s.*, is widely distributed in Ireland affecting the economically important Sitka species which is the most common species in Ireland, occupying 52.4% of the entire forest landscape (266). For this reason *H. annosum s.s.* was the second fungal pathogen to be investigated in this thesis.

Heterobasidion annosum s.s is known as a white-rot fungus that causes a destructive root rot disease found throughout the Northern Hemisphere. *H. annosum s.s.* is a necrotrophic pathogen (265) and as mentioned previously, attacks many coniferous species. Conifer trees inhabit the earth to a greater extent than any other plant group, with the timber production from conifers being one of the largest industries in Europe. It is estimated to contribute €100 billion net export income annually, which accounts for US$ 370 billion on a global scale (267). Therefore, infection from *H. annosum s.s.* can have a devastating impact on the forestry industry, with economic losses attributable to this pathogen in Europe alone currently standing at €800 million annually through tree mortality, decay of valuable timber and increased risk of wind-throw as shown in Figure 1.6 (267, 268).

Figure 1.6: Damage caused by *H. annosum* on trees in Sękocin Stary, Poland intended for the timber industry (damage marked with red circle to aid in visualization).

Similar to *Armillaria mellea, H. annosum* is also a basidiomycete fungus. Its fruiting bodies, known as basidiocarps, are formed from button shaped pads of mycelium and it is these basidiocarps that produce the sexual spores (basidiospores) needed for *Heterobasidion annosum* to reproduce (269). In summer, the basidiocarps release these basidiospores which have been found to be the primary infective propagule as they are carried long distances by the wind before falling on and infecting freshly cut tree stumps. The fungus initially colonizes the stump before migrating into the host's roots via mycelium (269). Within a stand of trees, *H. annosum* can move short distances from the roots of infected stumps to healthy trees through root grafts. Therefore, regardless of the host, infection biology of *H. annosum s.s.* is accomplished in two manners: (a) by spores (primary infections) and (b) by growth of the mycelium from infected roots to healthy roots through contact (secondary infections) (270).

Wood decay caused by *H. annosum s.s.* can be present in trees without showing any external symptoms, with infection only identified after the tree is wind-thrown or after harvesting (265, 271, 272). It has been reported that up to two-thirds of the root system could be dead before any obvious symptoms appear on the rest of the tree (273). Possible symptoms of *H. annosum s.s.* infection in trees are resin exudation from the

stem or roots, reduced crown density, chlorosis and swelling of the butt (272). The result of such fungal infections is tree mortality, leading to the development of gaps in the forest canopy which affect the light, moisture and temperature of the forest and in turn change the habitat for a diverse range of plants and animals, as well as greatly affecting productivity (267). Management strategies can help in controlling this disease, with preventative control measures being one of the most effective ways to manage *H. annosum s.s.* Since *H. annosum s.s.* is almost impossible to get rid of once on site, prophylactic application with control products is absolutely key.

Other strategies include stump removal, but this is expensive and requires relatively level ground for the machines to work on leading to the removal of soil, including valuable nutrients (274, 275). Logging-in periods when spore infection is low is also a common approach in Scandinavia, but this is not always possible in temperate climates like Ireland and the UK, where the pathogen sporulates all year round (267). Therefore, freshly cut stumps are immediately treated with the Plant Protection Product known as urea or the biological agent *Phlebiopsis gigantea* (Fr.) Jülich to prevent their infection by the airborne basidiospores (276-278). Urea is one of the most commonly used chemical agents at its EU approved concentration of 37% w/v; this high concentration also helps reduce the chances of the delivery equipment freezing and maintains the active process of hydrolysis throughout the weeks when stumps remain susceptible to infection (279). Urea is a corrosive substance that damages and reduces the lifetime of the application equipment on harvesters and this dose poses a small risk of increased nitrification of the soil and is mildly phytotoxic to herbaceous plants (279, 280).

1.5.2.3 Quarantine bacterial plant pathogens

Nine quarantine bacterial plant pathogens were supplied for this study by the DAFM which are causing devastating effects on economically important crops and plants. The DAFM has a mission to lead the sustainable development of a competitive, consumer focused agri-food sector and to contribute to a vibrant rural economy and society. Table 1.7 compiles information on each pathogen. These pathogens were tested for susceptibility to seaweed extracts in Chapter 4 with the hope of finding a new environmentally friendly control method. It must be noted that symptoms can differ between hosts with treatments depending on specific country regulations.

Table 1.7: The general plant hosts, symptoms and control strategies of the nine bacterial plant pathogens supplied by the DAFM to be tested in Chapter 4.

Pathogen	Susceptible plant species	Symptoms of infection	Control strategies	Ref
Xanthomonas arboricola pv. *Pruni*	Prunus species such as peach, apricot, cherry and plum.	Lesions on leaves, twigs, fruit and steam cankers.	Preventive applications of copper-based bactericides. Use of disease resistant plants and infected plants destroyed.	(281, 282)
Xanthomonas hyacinthi (Wakker)	*Hyacinthus orientalis, Scilla tubergeniana, Eucomis autumnalis* and *Puschkinia scilloides*.	Infected bulbs produce, yellow discoloured vascular tissues and surrounding parenchymas. If the plant manages to develop leaves will turn black and wither.	Bulb heat treatment, treatment with alkyl dimethyl benzyl ammonium chloride (if permitted) on leaves showing disease symptoms and infected bulbs and plants are destroyed.	(283)
Clavibacter michiganensis subsp. *nebraskensis* (Vidaver and Mandel)	Maize - Goss wilt (*Zea mays* L.).	Foliar blight lesions and vascular wilt symptoms, e.g. internal orange discoloration of the vascular bundles and by the external water-soaked and slimy appearance of the stalk.	Crop rotation, use of disease resistant plants and infected plants destroyed (no chemical treatments labeled for maize against this pathogen).	(284, 285)
Clavibacter michiganensis subsp. *michiganensis*	Main host of economic importance is tomatoes - Bacterial canker disease *(Solanum lycopersicum*) but reported to infect other *Lycopersicon* spp. and *Capsicum annuum, Solanum douglasii, S. niigrum* and *S. triflorum*.	Under glass house conditions: Wilting on leaves and eventually the whole plant desiccation. In the field, extremely dry leaflet edges observed and the plant slowly desiccates.	Healthy seeds acid extracted. Chemical treatments such as copper hydroxide to reduce incidence. Chemical activation of the hosts defence system e.g. salicylic acid treatment. Use of biocontrol agents e.g. *Bacillus subtilis* and infected plants destroyed.	(286, 287)
Clavibacter michiganensis subsp. *tessellarius* (Carlson and Vidaver)	Wheat - bacterial mosaic *(Triticum aestivum*).	Leaf freckles and leaf spots.	Infected plants destroyed and development of more resistant genotypes.	(288)
Xanthomonas fragariae Kennedy and King emend. Van den Mooter and Swings	Cultivated strawberries (*Fragaria ananassa*) main host of concern.	Angular leaf spot.	Use of healthy plant materials and avoidance of conditions favoring disease. Treatment with copper containing products and infected plants destroyed.	(289)
Xanthomonas campestris (Pammel) Dowson pathovar *uppalii*	Vegetable Brassica crops such as broccoli, cabbage, chinese cabbage, cauliflower, brussel sprouts and a number of other cruciferous crops, ornamentals and weeds.	V-shaped yellow lesions starting from the leaf margins and blackening of the veins commonly known as black rot.	Use of pathogen-free planting material, crop rotation and the elimination of other potential inoculum sources such as destruction of infected plants. Seed treatments including hot water, antibiotics, sodium hypochlorite, hydrogen peroxide but not fully effective.	(290)
Ralstonia sp.	Wide host range of over 200 species including model plant Arabidopsis thaliana and important crops such as potato & tomato.	Wilting and discoloration of the leaves, dark streaking in the vascular tissue of the infected stems.	Use of pathogen-free planting material. No effective biological or chemical control available. Infected plants must be destroyed.	(291)
Erwinia amylovora (Burrill)	Maloideae sub-family of Rosaceae such as apples, pears and ornamental Rosaceae species.	Infects all host tissues with flowers, leaves, shoots and fruits dark coloured or blackish.	Integrated management: pruning, tree nutrition, use of disease resistant plants and infected plants destroyed. Chemical controls: spraying plants with streptomycin in North America or in Europe testing the use of flumequine a biological control.	(292, 293)

1.5.3 Current control strategies

The past few decades has witnessed a considerable increase in global food production in order to meet the needs of the growing population. Pesticides, including plant protection products and biocides, have had a major impact on achieving this as well as other innovative technologies. Therefore, it is not unexpected that the European Union (EU) have reported increased sales of pesticides between 2002 to 2008 (294) with Figure 1.7 illustrating the total pesticide sales in the EU with herbicides, fungicides and bactericides representing the largest share. These products consist of active substances that help the grower control various pests and diseases leading to increased yields and greater quality of produce. The major benefits of pesticide use include: improved shelf life, reduced employment costs such as a reduced need to weed areas, invasive species control and garden plant protection (294). These pesticides have also been readily applied in the forestry industry to protect from valuable timber loss.

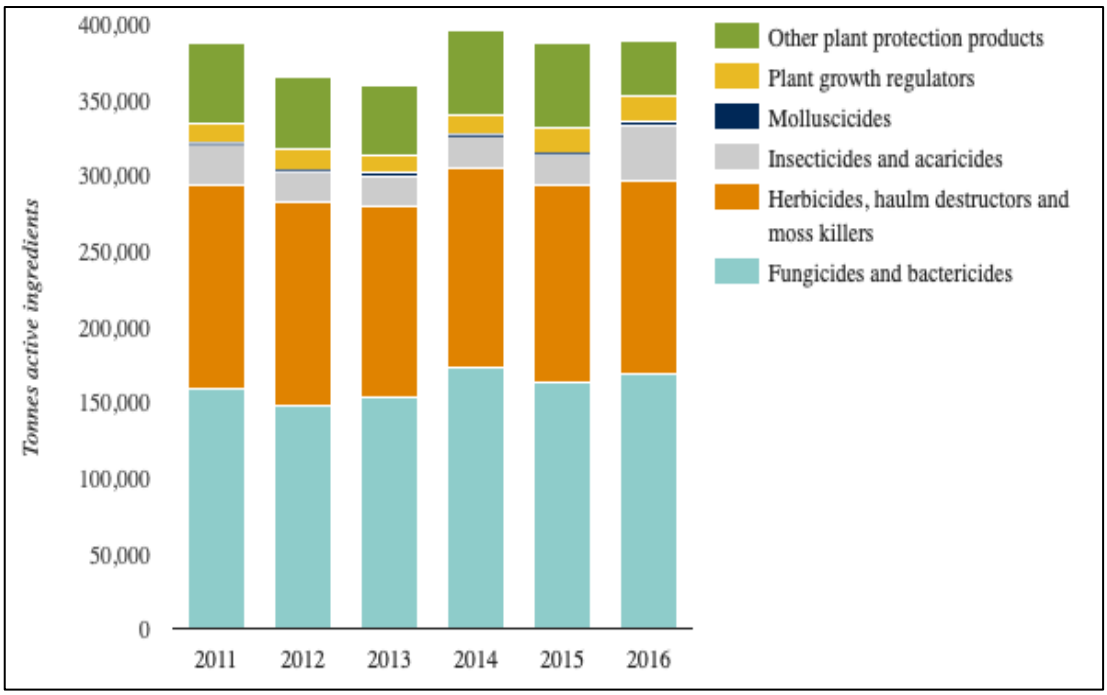


Figure 1.7: Total sales of pesticides (in tonnes of active ingredients) in the EU over the period 2011-2016, including the specific pesticide groups (295).

However, excessive input of these chemical pesticides in both of these industries has had a concomitant impact on the environment. Many studies demonstrate that the indiscriminate and excessive use of synthetic pesticides poses serious risks to the environment through water contamination and soil contamination (296) and has also resulted in the development of resistance among pests (297). Pesticide resistance is a genetically based statistically significant increase in the ability of a pest to tolerate a pesticide and is one of the major reasons for a shift in chemical-based controls to non-chemical alternatives (298). Worldwide, it has been estimated that over 500 species of pests have developed some level of pesticide resistance (299). For instance, glyphosate is one of the most widely used herbicide but its use is currently being threatened by the occurrence of a number of glyphosate-resistant weed species (300). It has been reported that 38 weed species including *Lolium rigidum* have now evolved resistance to glyphosate (300).

Additionally, the large-scale application of chemical agents can have an impact on non-target species (301). Woodcock *et al.* completed a study on the effect of neonicotinoid seed treatment on the environment and found that such treatment had a negative impact on the interannual reproductive potentials of both wild and managed bees, including honey bees, across certain countries including Hungary, Germany and the United Kingdom (302). More pressingly, chemical pesticides have had a negative effect on human health (303, 304), particularly when the degree of exposure exceeds that of safety levels. Farmers applying pesticides can be directly exposed as can bystanders in close proximity to the application site (294). Residues from pesticides can be found in a variety of foods and beverages but in the majority of cases do not exceed the legislatively determined safe levels (304). Although the consumption of food with excessive residues may cause acute and/or chronic health effects such as nausea, abdominal pain with prolonged intake potentially leading tp damage to the nervous system or other organs (305).

Some commercially available synthetic pesticides include the insecticide chlorpyrifos which is a form of organophosphate pesticide. Organophosphate pesticides work by affecting the nervous system through the disruption of enzymes that regulate acetylcholine, a neurotransmitter (306). However, their effect on insects is similar to that exhibited on exposed humans. Another common chemical pesticide is the fungicide chlorothalonil, which is used against grey mould, early and light blights and on various

agricultural crops (307). Chlorothalonil's mode of action is as a multi-site inhibitor affecting various enzymes and other metabolic processes in the fungi (307). It inhibits spore germination and is also toxic to fungal membranes. Although it is of low toxicity to birds and bees, it is highly toxic to aquatic life and may cause allergic reactions to humans (307).

The Irish Department of Agriculture have reported that 3,135 tonnes of pesticides were used in Ireland in 2016 which included herbicides, fungicides, insecticides and other industrial chemicals. This was an increase in 14 tonnes to pervious years and 219 tonnes compared to 2013 consumption. This is as consequence of increased food demand and controlling of pests with new and reregistered pesticides constantly been authorised for use with Teasgasc detailing any change to pesticide information including up-to-date information on deregistered chemicals, off-labels, emergency use authorisations and plant protection products (PPP's). For instance, the fungicide fandango has been registered as a new off-label for use on onion and shollat against downy mildew (*Peronospora destructor*) in September 2019. This demonstrates the significant increases in the use of pesticides in Ireland with the EPA's drinking water report in 2016 revealing that pesticides are one of the top three causes of concern for the water quality in Irish public water supplies.

Individual EU countries and the European Commission (EC) have a long history of controlling pesticides through multiple country-specific programmes with pesticide polices first introduced in 1979 (294). In 2006, the European Commission implemented a thematic strategy on the sustainable use of pesticides within all member states. This strategy was to promote the sustainable use of pesticides in agriculture and mindfulness around the health of the public and environment (308). The measures include national action plans, training for professional users and distributors, certification and control of application equipment, protection of the aquatic environment, proper handling and storage of chemicals and their packaging, restricting or banning the use of pesticides in certain areas (309) and are outlined in various regulations and directives including regulation (EC) 1107/2009 concerning the authorisation of plant protection products on the market, Directive 2009/128/EC on sustainable pesticide, and regulation (EC) 1185/2009 on the collection of pesticide usage data (308). Outside of this thematic strategy is the waste framework directives 2006/12/EC and the directive on hazardous waste 91/689/EEC constituting regulations surrounding the safe disposal of pesticides

such as unused or expired products (294). Agricultural pesticides are primarily controlled in the EU by legislation regarding the authorisation of PPP on the market through risk assessments ensuring PPP do not pose a threat to human and animal health and the environment under the correct usage guidelines (309). In addition this legislation details the maximum pesticide residue levels to be present in food and feed in order to reduce the exposure of pesticides to the consumer. In Ireland, the Pesticide Control Service of the DAFM is responsible for implementing the regulatory controls around PPP and biocidal products (309).

The future goals are establishment of quantitative reduction targets and the introduction of tax schemes. Ireland and many other European countries have also adopted Sustainable Forest Management (SFM) policies which include reducing pesticide use (310). Countries also seek certification from agencies such as the Forest Stewardship Council (FSC) to certify that forests are sustainably managed. It is under these certification bodies that agreements have been reached in relation to chemical usage (311).

1.5.4 Biopesticides as alternatives: current prospects and challenges

In order to achieve the objectives mentioned in section 1.5.3, a sustainable low risk alternative product must be developed and authorised with plant extracts looking like an attractive alternative due to their potential low adverse impacts on animal and human health (312). Plant derived bioactives appear to be biodegradable resulting in a reduced environmental impact (313, 314). This is because plant compounds have been known to break down rapidly into non-toxic residues that have reduced environmental longevity, hence, reducing their effect on the surrounding ecosystem (315). Furthermore, plant biodiversity has provided a great source of biologically active compounds for use in crop protection (316). These biodegradable pesticides are known as biopesticides and as such are derived from natural products including seaweeds, which as mentioned previously have been reported to exhibit a wide range of biological activities, including pesticidal. This has led to extensive research into new molecules that are non-synthetic and biodegradable with reduced toxicity with some very promising results reported (317-319).

In spite of their positive prospects, the biopesticide market has a relatively small market share accounting for only 2.5 % of the total world pesticide market (320). In the American market more than 200 biopesticide products are available compared to only 60 analogous products in the EU (321). This is as a result of the EU applying the same regulations when assessing synthetic active substances requiring new provisions in the current legislation and preparation of new registration guidelines, which in turn increases the complexity of EU-based biopesticide regulations (320). Additionally, biopesticides have been reported to exhibit a short shelf life and field persistence leading to repetitive applications required for the effective eradication of a pest (322). This increases the costs of using biopesticides as an integrated pest management strategy. Additionally, the specificity exhibited by biopesticides is considered an advantage in reducing toxicity to non-target species. On the contrary, it typically narrows the range of activity such as the fungus *Verticillium lecanii* against cereal aphids, meaning one may require the use of a number of different biopesticide products or application in conjunction with conventional agrochemicals (323). Nonetheless, the challenges and the constraints experienced by natural products is outweighed by the environmetal benefits they produce through a reduction in synthetic pesticide usage and discovery of novel compounds effective against a range of problematic pathogens.

1.6 Summary

Recently, there has been a massive shift in the use of chemicals to low risk alternatives as a way to reduce human's impact on the environment. This has been as a consequence of the emergence of resistance towards these controls and the negative impact chemicals have on the environment, coupled with more stringent EU regulations. Seaweeds have become an interesting focus as a potential source for alternatives due to their ability to produce a broad spectrum of chemically active secondary metabolites. These secondary metabolites can possess antimicrobial, antioxidant, anti-inflammatory, and antifungal properties. Seaweeds have been reported for their pesticidal activity against a variety of pathogens including insects and bacterial plant pathogens. Few authors however have definitively identified such bioactive compounds with the majority of researchers highlighting the requirement for further purification. The isolation and identification of the bioactive compounds is important since crude extracts contain a multitude of compounds, some of which may act as antagonists towards active compounds leading to reduced efficacy. Isolation of these compounds would also allow a thorough insight and understanding into the mode of action which is necessary for commercialisation purposes. However, the true potential of a seaweed-derived biopesticide is the completion of field trials in order to assess their effectiveness in real life scenarios and impact on non-target species. This is highly important since the environment will be exposed to the novel biopesticides, which could have a negative impact on health, whether it be human, biodiversity or environmental. These compounds may also be active against human pathogens with seaweed-derived antibiotics an area of huge potential.

1.7 Objectives of this Research

This study aims to investigate the potential of seaweed extracts as biopesticides against a range of fungal and bacterial pathogens. Specific objectives of this research include;

1. To screen a range of extracts from seaweed species collected from the South-East coast of Ireland against two problematic root rot fungi currently active in Irish forests.
2. To compare and determine the optimum *in vitro* antifungal test method followed by the development and standardisation of an *ex vivo* method for investigating the protectant activity of the most promising seaweed extracts with comparison to commercially available plant protection products.
3. To screen these seaweed species against a range of quarantine plant bacterial pathogens in order to determine their bactericidal potential, followed by efficacy and toxicity tests on the most promising extracts.
4. To compare conventional and non-conventional extraction methods in terms of extraction yield and antibacterial activity as well as the effect of extraction time and feed:solvent ratio on these outputs.
5. To isolate and identify the antibacterial compounds from the seaweed extracts.
6. To assess the activity of the purified extract for enhanced activity or for the presence of synergistic properties between the compounds.

CHAPTER 2: ANTIFUNGAL SCREENING OF SEAWEEDS COLLECTED ALONG THE SOUTH-EAST COAST OF IRELAND

2.1 Introduction

In response to the stricter use of synthetic pesticides and the emergence of resistance against these controls, the need to find a low risk alternative that is safe to both humans and the surrounding environment is growing. In addition to this, pesticidal compounds must be of equal or greater potency to that of existing chemical-based pesticides to make them a viable substitute (324). The ocean contains a large diversity of organisms that could potentially contain a vast untapped resource of bioactives. These marine organisms include seaweeds which have the ability to produce a great variety of secondary metabolites characterized by a wide spectrum of biological activities (44). Antimicrobial compounds are believed to be produced by seaweed as a form of protection against the organisms that call the ocean their home and these compounds also aid in protecting against ecological pressures such as competition for food and space and unfavourable environmental conditions (325, 326).

The antimicrobial activity of seaweed extracts was first reported by Pratt *et al.* in 1951 when several species of marine seaweed collected from the central coast of California exhibited antibacterial activity against *Staphyococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* (327). Since then, a vast amount of studies on the antimicrobial properties of seaweeds and seaweed extracts have been conducted against different organisms. These studies have included investigating the antifungal activity of seaweed extracts. For example, Ara *et al.*, in one of the earliest studies, evaluated the effect of the brown seaweed species *Sargassum* spp., both alone and in combination with the microbial antagonists, *Pseudomonas aeruginosa, Bradyrhizobium japonicum* and *Paecilomyces lilacinus,* against the plant pathogens *Macrophomina phaseolina* and *Fusarium solani* (328). These pathogens are responsible for root rot disease in sunflowers. From the results, it was found that *Sargassum* spp., in combination with biocontrol agents, showed better control of the two fungal pathogens and improved plant growth (when used together) as opposed to when used individually (328). Thus the use of seaweeds with compatible biocontrol agents seemed promising.

However, a more interesting finding was discovered by Sultana *et al.* who conducted a similar study on the fungicidal potential of the red seaweed *Solieria robusta* against three common fungal pathogens *Macrophomina phaseolina, Rhizoctonia solani* and *Fusarium solani* (329). The seaweed was applied both individually and in combination

with chemical fertilisers, urea and potash and the conventional pesticide known as Topsin-M. Overall it was found that the seaweed *S. robusta* was not only as effective as the two chemical fertilisers in enhancing plant growth, but had more or less equivalent effects in suppressing the fungal pathogens as the pesticide Topsin-M. This further indicated the capability of seaweed to be developed as a more environmentally safe protocol for the management of seed-borne fungal diseases (329). This was also reported by Barreto *et al.* who investigated the fungicidal activity of ethanol extracts from selected seaweeds against two phytopathogens; *Verlicillium* sp. and *Rhizoctonia solani* (54). The extracts from *Cauferpa filiformis*, *Ulva rigida*, *Zonaria tournefortii*, *Gelidium abottiorum*, *Osmundaria serrata* and *Hypnea spicifera* inhibited fungal growth by more than 50%. Pandithurai *et al.* evaluated the antifungal activity of various solvent extracts from the brown seaweed *Spatoglossum asperum* against fungal pathogens. It was found that the methanol extracts were more effective against dermatophytic fungi including *Candida albicans* (57.14%) and *C. tropicalis* (54.75%) compared to the other solvent extracts (77), demonstrating the antifungal potential of seaweeds.

Seaweeds all over the globe have been found to exhibit pesticidal properties including species of red, green and brown seaweeds harvested in Chili (219), India (63), Brazil (81) and South Africa (116). For instance, antifungal activity was observed with seaweeds extracts from Brazil (81) and South Africa (116) against *Candida albicans*. In addition, the green seaweed *Penicillus capitatus* harvested in The Bahamas was found to demonstrate potent antifungal activity against the marine pathogen *Lindra thallasiae* (220).

It is clearly evident that seaweeds contain secondary metabolites that exhibit antimicrobial activity. However, the production of these metabolites is influenced by a number of factors; geographical location, environmental factors such as nutrient availability, climate, season, light intensity and physiological factors including variation in growth phases and damage from herbivore grazing (330-332). For example, Manilal *et al.* evaluated the effect of seasonality on the antimicrobial potential of red seaweed collected from the South-West coast of India against a variety of shrimp, human and phytopathogens, including four species of fungal plant pathogens (333). The study found that a difference in the spectrum of inhibition was observed at different seasons with maximum antimicrobial activity achieved during December to January before it

began to gradually reduce (333). This study suggests that different or greater quantities of specific compounds might be produced in seaweeds in different seasons.

Seaweeds contain a wide variety of antifungal compounds that range in polarities requiring a selection of solvents to extract them from the biomass. In order to assess the optimum solvent with regards to achieving the highest antifungal activity possible, solvent selection studies must be conducted in conjunction with bioactive tests. This requirement led to the main objective in this chapter, which was to screen four different seaweed species collected from the South-East coast of Ireland for fungicidal properties. The seaweeds were extracted in a number of different solvents, in order to determine which seaweed exhibited the best activity. The solvents water, methanol, ethanol and acetone were selected on the basis of previous studies that reported antifungal activity from extracts generated from these specific extracting solvents (44, 64, 219, 334). The study conducted by Manivannan *et al.* found that the methanol extract of *Turbinaria conoides* showed maximum inhibition against both *Candida albicans* (18.00 ± 2.68 mm) and *Penicillium* sp. (18.00 ± 2.68 mm) while the acetone extract of *Padina gymnospora* exhibited the best antifungal activity against *Cryptococcus neoformans* (23.00 ± 1.78 mm) (64).

Similar studies by Govindasamy *et al.* investigated the antimicrobial activity of three seaweeds species *Gracilaria corticata* (red seaweed), *Padina tetrastromatica* (brown seaweed) and *Halimeda macroloba* (green seaweed) collected from the Mandapam coast against human pathogens (335). The acetone extract of *G. corticata* exhibited an inhibition zone of 15 ± 0.89 mm against *C. albicans*. Kubanek *et al.* also reported antifungal activity from the acetone extract of the brown seaweed *Lobophora variegata* against marine fungi (336). Whilst Tüney *et al.* who screened 11 seaweed extracts against a number of different plant pathogens found that the ethanol extract of *Dictyota linearis* exhibited strong antifungal activity against *Candida* sp. compared to the methanol, diethyl ether and acetone extracts (67). There are numerous other studies illustrating the effectiveness of ethanol extracts as fungicides (131, 334, 337). The final solvent selected was water, although it is used less than organic solvents. Jiménez *et al.* demonstrated the effectiveness of water as an extracting solvent when the aqueous extracts from various seaweed species prevented the growth of *Phytophthora cinnamomi* (219). Water also has the added bonus of being a green solvent and it is

widely available and easily deposited, making it highly desirable in industries, specifically in forestry applications.

From examination of the four solvents used, it can be seen that the polarity range is small with all four being considered polar solvents. However studies on the chemical composition of seaweeds have shown that seaweeds consist of a large amount of polar constituents with carbohydrates accounting for almost 24-44%, followed by lipids at 6-23% (338). Therefore it was envisaged that the use of these solvents would guarantee higher yields of bioactives and in addition to this, carbohydrates particularly polysaccharides, have been shown to exhibit antimicrobial activity. This was reported by Vijayabaskar *et al.* who demonstrated that a sulphated polysaccharide from the brown seaweed, *Sargassum swartzii* inhibited the growth of both Gram-positive and Gram-negative bacteria namely *Bacillus subtilis* and *Escherichia coli*, respectively (339). Kantachumpoo *et al.* also investigated the components and antimicrobial activity of polysaccharides extracted from brown seaweeds, with certain polysaccharides exhibiting strong antifungal activity against *Candida albicans* (340).

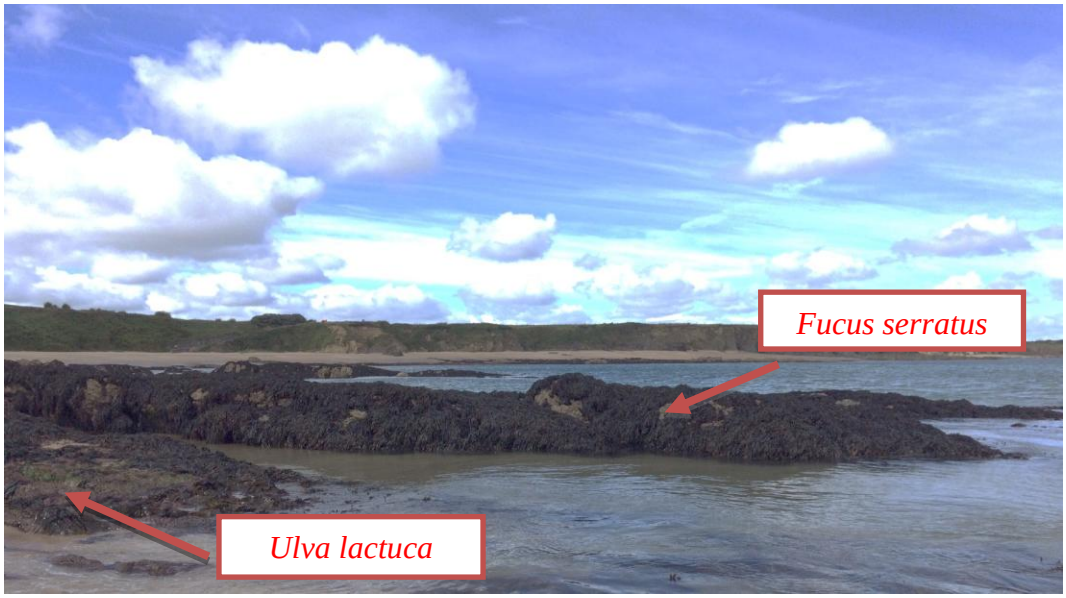
Since the desired bioactives are unknown, a simple solvent extraction technique was selected so as to avoid the loss of unstable compounds as a result of temperature, pressure and sonication. A relatively low concentration extract (1:50 *w/v*) was produced to ensure all of the bioactive compounds were extracted. These newly generated extracts were then tested against two plant pathogens known as *Armillaria mellea* (Vahl) P. Kumm and *Heterobasidion annosum sensu stricto* (Fries) Brefed with Chapter 1 section 1.5.2.1 and 1.5.2.2, respectively describing their problematic nature to the forestry industry and the need to find alternative control means. Therefore, the key objective of this study was to determine the potential of seaweed extracts as a novel source of biopesticides against these problematic plant pathogens.

2.2 Experimental Procedure

2.2.1 Seaweed collection and processing

The seaweeds were harvested from two locations (beach and harbour) in Baginbun bay, Fethard-on-Sea, Co. Wexford, Ireland, Figure 2.1, in September 2015 (52°11’53.68’’N, 6°49’34.64’’W), except for the green seaweed species which was collected in July 2016 due to its low availability at the initial visit in September 2015.

(a)



(b)

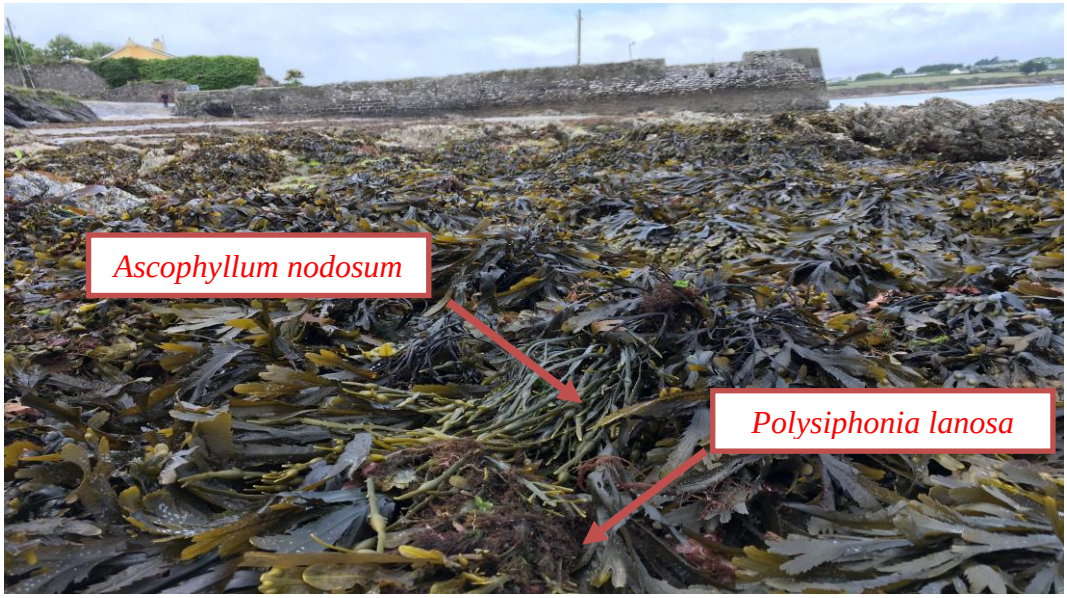


Figure 2.1: Site locations of seaweed harvesting: (a) Baginbun bay beach and (b) Baginbun bay harbour, Fethard-on-Sea, Co. Wexford, Ireland.

The species are indigenous to the South-East coast of Ireland and were identified as *Fucus serratus* (brown seaweed), *Polysiphonia lanosa* (red seaweed), *Ascophyllum nodosum* (brown seaweed) and *Ulva lactuca* (green seaweed), Figure 2.2. *F. serratus* and *U. lactuca* were collected at the beach in Baginbun bay, whilst *P. lanosa* and *A. nodosum* were collected at the harbour. Seaweeds were collected at low tide, transported in a cooler box and processed in the laboratory within 6 h of harvesting.

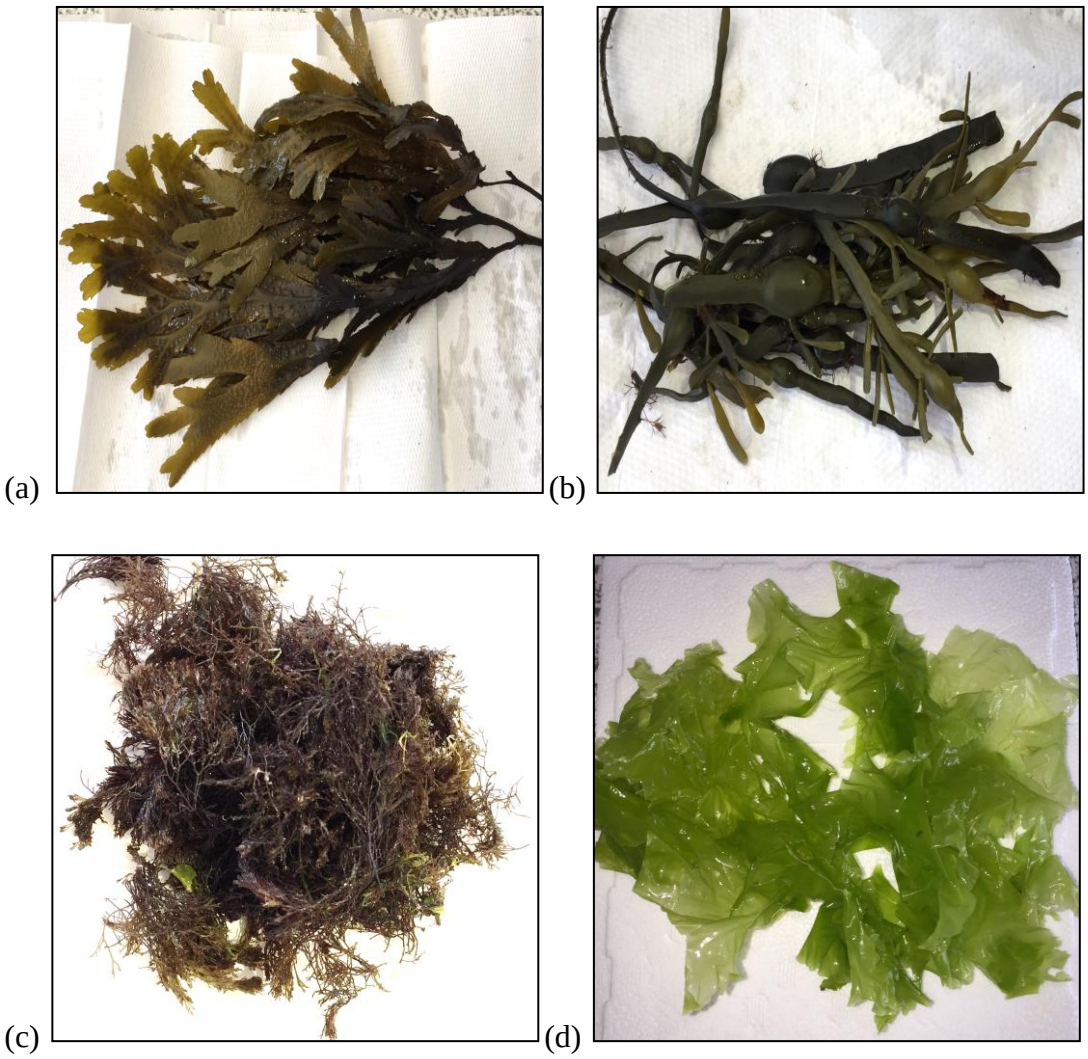


Figure 2.2: Fresh seaweeds harvested from Baginbun Bay, Wexford, Ireland. Brown seaweeds: (a) *F. serratus* and (b) *A. nodosum*. Red seaweed: (c) *P. lanosa*. Green seaweed: (d) *U. lactuca*.

The processing of the seaweeds involved a number of different steps. The first step comprised of washing the seaweeds thoroughly with deionised water (SG Water, Germany) to remove any macroscopic epiphytes and sand particles before freezing at -20 °C. Once the seaweeds were fully frozen, they were freeze-dried (VirTis, SP Scientific, PA, USA) until a constant weight was obtained. The seaweeds were then powdered in an electric blender and sieved to approximately 850 μm. The seaweed powders were stored in polyethylene bags under a nitrogen atmosphere at -20 °C until required for further analysis.

Water content analysis for all four seaweed species was performed by retaining a small amount of seaweed prior to processing. Approximately 5 g of fresh seaweed was weighed into glass petri dishes which were then dried over a 5 day period in an oven at 100 °C. After this time, they were removed and allowed to cool to room temperature for a day in a desiccator prior to weighing. The experiment was repeated in triplicate with the desiccator employed to ensure no water was absorbed by the seaweed during the cooling down process which could affect the results.

2.2.2 Production of crude seaweed extracts

All solvents used in this screening study were of HPLC grade and purchased from Fisher Scientific, Dublin, Ireland except for ethanol which was purchased from Lennox Laboratory Supplies Ltd, Dublin, Ireland. The seaweed powders were extracted in four different solvents of increasing polarity; acetone (99.8%), ethanol (96%), methanol (99.8%) and deionised water. Approximately 1 g of the seaweed powder was extracted with 50 mL of the solvents (1:50 *w/v*) and stirred with either a magnetic stirrer or in an automatic-shaker (C25 Incubator Shaker, classic series; New Brunswick Scientific, Edison, NJ, USA) for 2 h and 24 h at room temperature. The solid seaweed was removed from the solvent containing the bioactives by filtering under a vacuum with Whatman No. 1 filter paper (11 μm pore size, Whatman, Kent, UK). The solvent was rotary evaporated under vacuum (Biddy heated water bath, Heidolph Laborota 4000 motor unit condenser) at temperatures no higher than 30 °C, to yield crude dried extracts. In order to transfer the extracts into pre-weighed amber bottles, they were re-dissolved in a minimum quantity of their respective extracting solvent. This solvent was then removed in a nitrogen concentrator and the dried extracts were once again stored at

-20 °C until further analysis. This extraction process was applied for all solvents except for the aqueous extracts where rotary evaporation was not possible. Instead after the extraction period, the samples were centrifuged at 4500 revolutions per minute (RPM) for 4 min after which a solid pellet of the residue was formed and the solvent containing the bioactives was decanted. The water was then removed through freeze-drying and the dried extracts were transferred to pre-weighed amber bottles. All extractions were carried out in triplicate. The % yield for each extraction was calculated using Equation 2.1:

Equation 2.1: $$\% \text{ yield} = \frac{\textbf{weight of dried extract}}{\textbf{Initial weight of dry seaweed}} \times 100\%$$

2.2.3 Stocking of the fungal species

Pure cultures of the two fungal pathogens *Armillaria mellea* (Vahl) P. Kumm and *Heterobasidion annosum sensu stricto* (Fries) Brefed were supplied by Coillte labs in Newtownmoutkennedy, Wicklow. *A. mellea* was obtained from small sections of rotted wood pieces that were surface sterilised in a 2% *w/v* sodium hypochlorite solution and washed in several changes of sterile deionised water, blotted dry on filter paper and transferred to a general growth medium. Malt extract agar (Biolab, Lennox laboratory supplies Ltd, Dublin, Ireland), was found to produce the best aerial growth of mycelium and for this reason *A. mellea* was maintained on malt extract agar at 20 °C and sub-cultured every 2 weeks to fresh medium.

The *H. annosum* culture was obtained from the fruiting bracket on an old tree stump and was isolated on the selective medium Kuhlman Hendrix (KHM) and transferred to a general growth medium. Similar to *A. mellea*, the *H. annosum* cultures were maintained on malt extract agar at 20 °C and sub-cultured every 2 weeks to fresh medium. In order to prevent the induction of pleomorphism in the fungi from continuous sub-culturing, each fungal species underwent distilled water stasis (341). This is the storage of metabolically inactive fungi under sterile water, with the viability of some fungi for up to 20 years reported for this method (341). In this method 3 - 5 mL of sterile water was added to sterile screw cab tubes. A moistened swab was rolled over a young culture (7

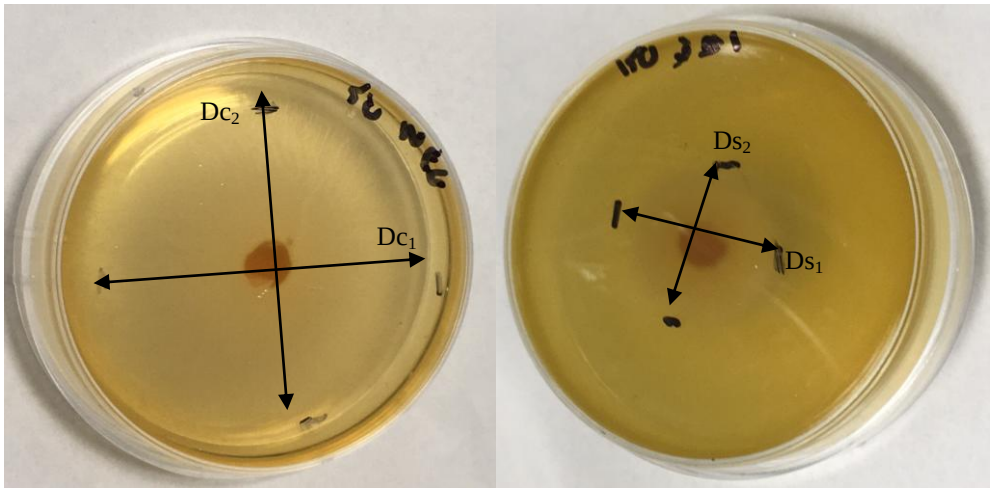
to 14 days old) to collect spores or conidia and then swirled in the sterile water. The cap was tightened and the suspension was stored upright in a refrigerator at 4 °C.

Health and Safety: It must be noted that since these fungal strains are regarded as problematic pathogens in forestry all work was conducted within a Class II laminar flow cabinet. Benches and surfaces were sterilised with 70% ethanol before and after laboratory work to avoid cross contamination. All consumables including plates, spreaders, pipette tips, etc and the cultures themselves used were autoclaved under the waste cycle (30 min at 121 °C) and disposed of in the general waste bin.

2.2.4 Fungicidal activity of the crude extracts

The antifungal activity of the extracts in this study were determined by the poisoned food technique as described by Sujatha *et al.* (52). The dried extracts were weighed out into 2 mL eppendorf tubes at a concentration of 25 mg and were re-dissolved in 200 μL of ethanol. The individual extracts (200 μL) were added to 5 mL of malt extract agar before being poured into sterile petri dishes to give a final concentration of 5 mg/mL. The medium, consisting of just 200 μL of ethanol, served as the negative control, while the medium containing 50 μg/mL (342) of the antifungal agent cycloheximide (Lennox laboratory supplies Ltd, Dublin, Ireland) served as the positive control. All processes were conducted under aseptic conditions.

A fungal disk (*H. annosum* and *A. mellea*) of 5 mm in diameter was removed from 7 to 14 day old cultures, with the help of a sterile glass pipette and transferred aseptically onto both the malt extract agar previously supplemented with the seaweed extracts and the untreated control plates. The inoculated plates were incubated at 20 °C and colony diameters were measured in mm in two perpendicular directions after 7 days of incubation for *H. annosum* and 14 days for *A. mellea* due to the different growth rates of the two fungi, as shown in Figure 2.3.



Negative control **Extract plate**

Figure 2.3: Determination of the antifungal potential of the methanol extract of *U. lactuca* at 5 mg/mL against *H. annosum* compared to the negative control. Ds = diameter of colony on sample plate and Dc = diameter of colony on control plate.

The antifungal effect of the crude extracts against *A. mellea* and *H. annosum* was estimated using Equation 2.2 (100):

Equation 2.2: $$\text{Inhibition (\%)} = \frac{(Dc - Ds)}{Dc} \times 100$$

Where Dc is the averaged diameter of growth of the negative control and Ds is the averaged diameter of growth in the plate containing the extract. To ensure consistency between the growth rates of the fungi on plates, all mycelial disks were removed from the same culture plate and maintained under the same growth conditions on the same day. After incubation, the plates were examined for any discrepancies in their growth pattern as a result of stress. The antifungal assays were all performed in triplicate and repeated in duplicate.

2.2.5 Statistical analysis

Data obtained for the water content of the seaweeds and dose response studies of *U. lactuca* were performed with repeated measures using one-way ANOVA followed by a post-hoc analysis using Tukey's multiple comparison tests. These analyses were performed using Minitab 17 Statistical Software using a 5% statistical significant level (ρ<0.05). The results were said to be statistically different if ρ<0.05 and were designated with different superscripts. In addition, the data obtained for the antifungal activity of the four seaweeds species in various solvents at two set time-points (2 h and 24 h), also underwent statistical analysis in order to identify whether extraction time affected yield and activity. The activity between a seaweed after 2 h and 24 h extractions was once again said to be statistically different if ρ<0.05. All experiments were performed in triplicate and presented as the mean ± standard deviation (SD), unless otherwise stated.

2.3 Results and Discussion

2.3.1 Water content analysis of the four seaweed species

Table 2.1 demonstrates the average water content for each seaweed species with the brown seaweeds *F. serratus* and *A. nodosum* found to contain the lowest water content of 72.9 ± 2.15% and 60.8 ± 2.97%, respectively. The red seaweed species *P. lanosa* exhibited the highest water content of 84.8 ± 0.7%. This was not significantly different to *U. lactuca* (green seaweed), which was found to have a water content of 81.1 ± 0.21%.

Table 2.1: Water content of the seaweed species.

Seaweed	Water content (%)
F. serratus	72.9 ± 2.15^{a}
A. nodosum	60.8 ± 2.97^{b}
P. lanosa	84.8 ± 0.70^{c}
U. lactuca	81.1 ± 0.21^{c}

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

It is estimated that seaweeds consist of 80-90% water making it the most abundant component in seaweeds followed by carbohydrates, minerals and lipids (343). In general water content can be highly variable between seaweed species as shown in Table 2.1. Ahmad *et al.* also reported this when the water content of 15 fresh seaweed samples was determined, which included all three phylum (344). The moisture content ranged between 75.95% to 96.03% with the red seaweed species (96.03%) found to have the highest water content followed by the green seaweeds (90.8 - 92.00%) and finally the brown seaweed species (83.51 – 86.86%) which supports the data obtained in this study for each phylum (344).

The variation that exists between the results of the same seaweed phylum in this study (Table 2.1) and Ahmad *et al.* is most likely as a result of the different geographical

locations, seasonal variations and the fact that these were different seaweed species to the ones investigated here. Ahmad *et al.* collected the seaweed samples from Sabah, Malaysia which is classified as a tropical climate compared to Ireland which has a temperate oceanic climate, with this difference in temperatures being shown to affect the pigments and fatty acids produced by seaweeds (345). For example, Becker *et al.* observed a higher percentage of saturated fatty acids produced by the red seaweed *Palmaria decipiens* when grown under higher temperatures (346). In addition to this, the salinity in the habitat can affect the composition of seaweed with some seaweeds demonstrating an increase or decrease of specific ionic compounds in order to compensate for this osmotic stress (345). Kakinuma *et al.* found that low salinity levels resulted in the accumulation of pigments in the green seaweed *Ulva pertusa* meaning geographical location effects the water content in seaweeds regardless of species (347).

However, Tan *et al.* also completed water content analysis on the same four seaweed species at the same geographical location in September 2009 (348). This was the same season (autumn) that *F. serratus, A. nodosum* and *P. lanosa* were harvested, meaning seasonal variations between the results were avoided. Therefore, it was not surprising when similar water content values were observed for these three seaweeds (*F. serratus* = 76.3 ± 0.5%, *A. nodosum* = 65.8 ± 1.0% and *P. lanosa* = 84.8 ± 0.7%). Although *U. lactuca* was harvested in July (summer) it can be seen that the water content for both seasons were quite similar with Tan *et al.* reporting a moisture content of 80.3 ± 0.3% in autumn 2009 compared to the 81.1 ± 0.21% reported in this study for summer 2016 (348). This similarity in water content between these two specific seasons was also observed by Khairy *et al.* who investigated the variations in moisture content of a number of different seaweed species including *U. lactuca* over three seasons' spring, summer and autumn in 2010 (349). Khairy *et al.* reported little difference between the summer and autumn seasons with the moisture content of *U. lactuca* in summer found to be 85.43 ± 0.5% and 85.20 ± 0.75% in autumn (349).

All of this information aided in ensuring that the seaweeds were completely dried following freeze-drying by giving an indication of the volume of water to be removed from each seaweed species before being considered dry. This has a major implication from a commercial perspective since fresh seaweeds are perishable in nature and, therefore, require immediate preservation with the drying of seaweeds being regarded as a critical step in the processing of seaweeds (350). Dried seaweeds reduce the costs of

transport and allow for better preservation of the seaweeds during storage without microbial decomposition of the bioactive compounds (351). It also aids in the mixing of seaweed with other products such as animal feeds (352). This determination of water content also has the added bonus of allowing for a more accurate estimation of the time required to dry each seaweed species and hence, avoided the time consuming process of drying and re-weighing until a constant weight is achieved, which is always a factor in industry.

2.3.2 Optimisation of the solvent extraction method

Magnetic stirring is commonly used as the method for solvent extraction. However, an investigation into the use of an automatic-shaker was explored. This approach had the benefits of being able to run more samples and also allowed the accurate control of temperature and RPM which was set at 19 °C and 200 RPM for the entire screen study. In order to evaluate the effect different shaking mechanisms had on the yield and antifungal activity, an initial experiment was conducted where flasks of *F. serratus* in the solvent methanol were subjected to either magnetic-stirring or automatic shaking for a set time of 2 h with Table 2.2 illustrating the % yields obtained.

Table 2.2: The extraction yields for *F. serratus* in methanol by magnetic stirring and automatic shaking.

Experimental conditions	Average % yield
F.serratus – stirring for 2 h	15.6 ± 1.16^{a}
F.serratus – shaking for 2 h	11.6 ± 1.63^{b}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different in terms of % yield ($\rho<0.05$, $\rho=0.04$; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

From Table 2.2, it can be seen that magnetic stirring for 2 h gives slightly better results with an average yield of 15.6 ± 1.16% compared to automatic shaking for the same length of time which produced an average yield of 11.6 ± 1.63%. Although variation exists between the triplicate runs, this is most likely due to loss in yields when transferring between containers and as a result of variations that exists within natural products. Even though the magnetc stirring was shown to achieve a higher percentage yield compared to automatic shaking, the main factor that determined what method would be applied for the rest of the study was whether there were differences in antifungal activity exhibited by either extraction method. Table 2.3 demonstrates the effect on the growth of *H. annosum* when exposed to stirring or shaking methanol extracts of *F. serratus.*

Table 2.3: Effects of *F. serratus* extracts generated from either stirring or shaking at a concentration of 5 mg/mL against *Heterobasidion annosum* using the poisoned food technique.

Extraction conditions (time & solvent)	***Fucus serratus* (mm)**	**[a]Positive control (mm)**	**[b]Negative control (mm)**
2 h, Methanol (stirred)	43.5 ± 1.32[a]	0	36
2 h, Methanol (shaken)	43.0 ± 1.47[a]	0	38

[a]Positive control was cycloheximide solution (50 µg/mL); [b]Negative control was 200 µL of ethanol; Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different (ρ<0.05; One-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test).

Examination of Table 2.3 illustrates that there was no difference in activity between both stirring methods (ρ>0.05) but unfortunately the results showed that the methanol extracts of *F. serratus* promoted the growth of this problematic plant pathogen when compared to that of the negative control, which had no extract incorporated into its media. This observed growth promoting activity was suspected to be either the effects

of nutrients present in the extracts or a hormetic response as described in section 2.3.4. Nonetheless, solvent extraction incorporating the use of magnetic stirring was found to be the superior mechanism for achieving optimum yields and for this reason, it was decided to apply solvent extraction under continuous magnetic stirring for the rest of the study.

2.3.3 Variation in extraction yield using different extracting solvents

The seaweeds were extracted in four solvents of slightly different polarities (water, methanol, ethanol and acetone) under the previously optimised solvent extraction technique and with the extraction yields presented in Table 2.4. As predicted, different species and solvents yielded different amounts of crude extracts with water resulting in the highest yield for all seaweeds compared to the other solvents. This can be explained by the fact that seaweeds consist of a large amount of polar constituents including carbohydrates, which can account for almost 20% of the dry weight followed by proteins and lipids, which are slightly more non-polar (349, 353). Methanol was found to produce the second highest yield followed by ethanol and acetone based on their relative polarities. For instance, the 24 h methanol extract of *F. serratus* had an average yield of 21.8 ± 3.29% compared to the ethanol extract which was almost three times less than that with a yield of 7.1 ± 0.42%. Acetone produced the lowest yield, with the 2 h acetone extract of *P. lanosa* having an average yield of just 0.3 ± 0.12%. Since at least 50 mg of dried extract was required for each antifungal test, both the ethanol and acetone extracts of *P. lanosa* and *U. lactuca* had to be excluded from the study as too low a yield was obtained even after 24 h of stirring and increasing the time beyond this could potentially lead to degradation of the bioactive(s).

Table 2.4: Extraction yields obtained for the four seaweed species in solvents of decreasing polarity.

Solvent	Time (h)	Average % yield			
		F. serratus	***A. nodosum***	***P. lanosa***	***U. lactuca***
Water	2 h	34.8 ± 8.17^{a}	23.6 ± 1.86^{bc}	14.2 ± 1.18^{c}	$*18.5 \pm 0.67^{bc}$
	24 h	40.1 ± 3.29^{d}	25.7 ± 1.39^{e}	16.6 ± 1.02^{e}	$*26.8 \pm 1.91^{e}$
Methanol	2 h	$*15.6 \pm 1.16^{f}$	$*12.8 \pm 0.25^{g}$	$*2.7 \pm 0.15^{hi}$	$*1.9 \pm 0.03^{i}$
	24 h	$*21.8 \pm 3.29^{j}$	$*20.5 \pm 6.88^{k}$	$*5.7 \pm 1.20^{l}$	$*4.4 \pm 0.17^{l}$
Ethanol	2 h	6.5 ± 0.59^{m}	12.9 ± 0.36^{n}	0.6 ± 0.10^{o}	0.7 ± 0.65^{o}
	24 h	7.1 ± 0.42^{p}	18.8 ± 6.34^{q}	0.7 ± 0.02^{p}	1.0 ± 1.04^{p}
Acetone	2 h	5.4 ± 1.06^{r}	9.4 ± 0.19^{s}	0.3 ± 0.12^{t}	0.6 ± 0.03^{t}
	24 h	7.5 ± 0.99^{u}	9.6 ± 0.64^{v}	0.4 ± 0.04^{w}	0.6 ± 0.04^{w}

Data (n=3) are presented as the mean ± SD; (*Statistically significant difference between extraction times $\rho<0.05$). Data that do not share a common superscript are statistically different in terms of yield at that specific extraction time and solvent depending on seaweed species ($\rho<0.05$; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

Schmid *et al.* also reported this effect of polarity for the lipid extraction of fatty acids from two seaweed species (*Palmaria palmata* and *Laminaria digitata*) using varying solvents (354). The extraction efficiency in terms of yield of total fatty acids was found to be chloroform/methanol>ethanol>hexane>ethanol/hexane for both species, with polarity seen as a limiting factor. The polar solvent methanol was easily able to extract the polar compounds, while the neutral lipids were further extracted by the non-polar solvent chloroform (354).

Statistical analysis of the seaweeds under the same extraction conditions found that even between seaweeds of the same phylum, variation in yields still existed. For instance, the 24 h extraction of *F. serratus* and *A. nodosum* (both brown seaweeds) in water produced average yields of 40.1 ± 3.29% and 25.7 ± 1.39%, respectively. This is a relatively large difference in yields and demonstrates that each seaweed, regardless of species, contains different polarity compounds, with *F. serratus* containing a higher level of water-soluble compounds. This difference in yields between seaweed species was also reported by Matanjun *et al*. who investigated the antioxidant activity of eight edible seaweeds under the same extraction conditions (355). Once again a major difference in yields was seen between the same division of seaweeds with the Phaeophyta species *Dictyota dichotoma* and *Sargassum polycystum* demonstrating extraction yields of 40.33% *w/w* and 4.05% *w/w*, respectively (355). These extractions were carried out in methanol meaning *D. dichotoma* has a high level (40.33%) of relatively polar constituents compared to *S. polycystum*.

From examination of Table 2.4, it can also be seen that the % yield of the crude extracts generated from the seaweeds after both 2 h and 24 h extraction times differed from one another with the 24 h extraction times yielding higher masses of potential bioactives. This was due to the longer extraction periods allowing more bioactives to be extracted into the liquid phase. For example, the 24 h methanol extracts of *F. serratus* had an average % yield of 21.8 ± 3.29%, whereas the 2 h extraction period only produced an average yield of 15.6 ± 1.16%. This difference was illustrated statistically by a ρ value of 0.007 (ρ<0.05) between the two extraction periods (356). Yaich *et al.* also demonstrated this in their study which investigated the effect of extraction conditions including time on the yield, chemical composition and purity of the polysaccharide ulvan extracted from the green seaweed *Ulva lactuca* (357). The powdered seaweed was extracted in hydrochloric acid (60 g of seaweed in 1L of HCl) for 1 h, 2 h and 3 h at a constant pH and temperature. As predicted, as time increased so did yield, with a 1 h extraction found to have a yield of 2.71% *dw/dw* while after 3 h this increased to 3.30% (357). This was further supported by Ahmad *et al.* who employed a Soxhlet extraction technique for extracting chemical compounds from the medicinal plant, *Herba leonuri* (358). In their study, the extraction times investigated were 6 h, 9 h and 12 h and once again the results indicated that the mass yield increased with increasing length of

extraction period. For instance, the mass yield for a 6 h methanol extraction was 12.54%, while after a 12 h extraction, the yield was found to be 14.18% (358).

Although this project and numerous other studies have demonstrated that longer extraction periods resulted in greater yields, since the seaweed and solvent were in contact with each other for a longer degree of time, it does not mean that activity would therefore be greater for these extracts compared to those obtained at shorter times. This is mainly due to the fact that the specific bioactives may have been extracted within the first 2 h with the remaining hours only extracting unrequired compounds. A more important factor was that the majority of the extractions were carried out in organic solvents, such as methanol and acetone, which could possibly breakdown the compounds rendering them inactive if left exposed to these solvents for an extended period of time (359, 360).

Mokrani *et al.* investigated the effect of extraction times on phenolic content and antioxidant capacity of peach fruit (361). The solvent extraction time was varied between 30 to 450 min at a fixed temperature of 25 ^{o}C. After 180 min, phenolic content remained unchanged and this is based on Fick's second law of diffusion (361). This means that after a certain duration (180 min in this study) final equilibrium is achieved between the solvent and sample and hence, the rate of extraction of compounds decelerates (361). Therefore optimum antioxidant activity was found after a period of 180 min. This was also reported by Maisuthisakul *et al.* who found that after 3 h of extraction, yields from kradonbok leaves no longer increased, while after 6 h antioxidant activity decreased (EC_{50} increased) most likely as a result of the oxidation of the phenolic compounds (362). Similarly Rosell *et al.* demonstrated this effect on seaweeds when several brown seaweeds were extracted over a 24 h, 48 h and 72 h period before being tested for antimicrobial activity (363). The 72 h acetone extract of *Nereocystis luetkeana* showed reduced activity against *Staphylococcus aureus* compared to the 48 h extract meaning activity had weakened (or degraded) after such an extended period of time in an organic solvent. Therefore, the antifungal activity of both the 2 h and 24 h extracts were tested to confirm whether this degradation effect had occurred with results presented in section 2.3.4.

2.3.4 Antifungal activity of crude seaweed extracts

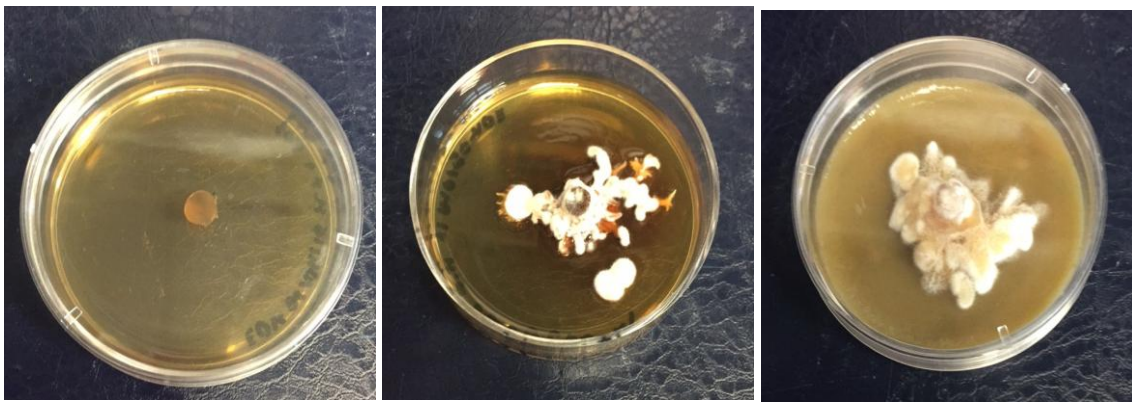
The newly generated seaweed extracts were investigated for their antifungal activity against two problematic plant pathogens currently active in Irish forests using the poisoned food technique at an extract concentration of 5 mg/mL. *A. mellea* was the first of the fungal species to be tested and is one of the most potent killers and decayers of deciduous and coniferous trees (364). Unfortunately from examining Table 2.5, it can be seen that none of the extracts generated were active against *A. mellea*.

Table 2.5: Antifungal activity assessment of crude seaweed extracts generated from water, ethanol, methanol and acetone using the poisoned food technique against *A. mellea* at 5 mg/mL.

Solvent	**Time (h)**	**Effect on the mycelial growth of *A. mellea***					
		F. serratus	***A. nodosum***	***P. lanosa***	***U. lactuca***	**[a]Positive control**	**[b]Negative control**
Water	2 h	++	+	+	+	-	+
	24 h	++	+	+	+	-	+
Methanol	2 h	+	+	+	+	-	+
	24 h	+	+	+	+	-	+
Ethanol	2 h	+	+	N/A	N/A	-	+
	24 h	+	+	N/A	N/A	-	+
Acetone	2 h	+	+	N/A	N/A	-	+
	24 h	+	+	N/A	N/A	-	+

[a]Positive control was cycloheximide solution (50 µg/mL); [b]Negative control was 200 µL of ethanol; Effect on fungal growth reported as; ++ indicates promoted growth; + no effect on growth; - no growth; N/A too low a yield to test. Data presented as n=3.

This method (poisoned food technique) of antifungal testing for this particular fungus is qualitative rather than quantitative meaning after 2 weeks of incubation it could only be determined whether the extract had fully inhibited the growth of the fungi or not. Figure 2.4 demonstrates the growth pattern of *A. mellea*. It can be seen that its rhizoid grew out from the point of inoculation at various lengths and directions and, therefore, it was impossible to determine whether the extract had affected its growth negatively as it couldn't be directly compared to the negative control. Therefore, it could not be confirmed whether any of the crude extracts exhibited any/some degree of antifungal activity against *A. mellea* without the need for another test method to be utilised.



Positive control **Negative control** **Acetone extract of *A. nodosum***

Figure 2.4: Growth of the root rot fungus *A. mellea* from the crude acetone extract generated from *A. nodosum* collected in September 2015 via the poisoned food technique at 5 mg/mL. Positive control: 50 μg/mL cycloheximide; Negative control: 200 μL of ethanol. Data (n=3).

However, this poisoned food technique did identify the promotion of growth illustrated by the water extract of *F. serratus* as shown in Figure 2.5.

Positive control **Negative control** **Water extract of *F. serratus***

Figure 2.5: Growth promoting activity of the crude water extract generated from *F. serratus* collected in September 2015 against *A. mellea* using the poisoned food technique at 5 mg/mL. Positive control: 50 µg/mL cycloheximide; Negative control: 200 µL of ethanol. Data (n=3).

This is not surprising since seaweeds have been used for many years as an organic fertiliser in agriculture with a number of other studies reporting that when seaweeds were used as a soil additive in crop protection systems, not only was plant growth improved, but there was also a promotion in the growth of beneficial soil microbes, indicating that seaweeds can potentially affect soil health (365). For instance, Wang *et al.* who evaluated the effects of seaweed fertiliser on the growth of apple seedlings under replant conditions found that plant height and dry weight significantly increased as well as the activities of the soil enzymes (366). But most interestingly, examination of the soil fungal communities found that soil treated with 40 g/kg of seaweed fertiliser had the most fungal diversity (366). Numerous studies have also observed this trend of increased growth of soil microbes in conjunction with seaweed treatment (367-369). In addition to this, it was specifically the crude extracts of *F. serratus* that promoted such growth. *F. serratus* is part of the brown seaweed family which are known to be rich in polyuronides such as alginates and fucoidans (370). These compounds have been shown to affect soil properties, such as improved ability to retain moisture and hence encourage the growth of beneficial fungi (370). Ishii *et al.* found that alginate oligosaccharides extracted mainly from brown seaweeds stimulated hyphal growth and

elongation of arbuscular mycorrhizal fungi which aid a plant in the uptake of soil mineral nutrients (371).

This could also be a hormetic response which is a biological effect to environmental stresses in the form of a biphasic dose-response relationships i.e., low-dose stimulation and high-dose inhibition (372). Barreto *et al.* also described this effect when they tested low concentrations of the red seaweed *Hypnea spicifera* and observed an increase in the growth rate of the plant pathogen *Rhizoctonia solani* compared with a negative control and suspected it was such a biological response (54). This growth promoting activity for this particular seaweed species was already observed in section 2.3.2 by the methanol extracts against *H. annosum*.

Although this wasn't an expected result, it did demonstrate the potential use of seaweeds to promote the growth of certain microbes for example in biocontrol agents. In the forestry industry, the use of biocontrols is becoming increasingly popular specifically against invasive species. For instance, the fungal species known as *Trichoderma* spp. is currently being investigated as an antagonist against *A. mellea* (259). However, the level of control exhibited by *Trichoderma* spp. depends on a number of factors including the growth of the antagonist. This factor can be overcome by the use of seaweeds in order to increase the growth rate, particularly before contact with *A. mellea*. This is a novel concept and allows enhanced biocontrol activity without the use of chemicals.

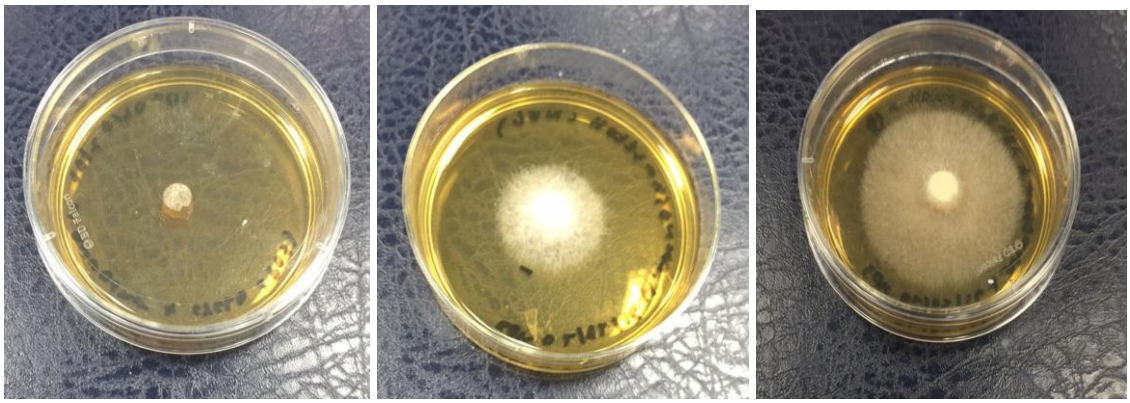
The antifungal potential of the crude seaweed extracts were also tested against *H. annosum*, one of the most destructive diseases of conifers in northern temperate regions of the world. Table 2.6 demonstrates the results obtained against this problematic pathogen using the poisoned food technique. Once again the ethanol and acetone extracts for *P. lanosa* and *U. lactuca* species demonstrated too low a yield to be tested and, therefore, were not tested for activity against *H. annosum*.

Table 2.6: Effect of crude seaweed extracts generated from water, ethanol, methanol and acetone on the mycelia growth of *H. annosum* at 5 mg/mL using the poisoned food technique compared to the negative control.

Solvent	Time (h)	Percentage inhibition/promotion of *H. annosum* (%)				Control (mm)	
		F. serratus	***A. nodosum***	***P. lanosa***	***U. lactuca***	**Positive control[a]**	**Negative control[b]**
Water	2 h	-12.4 ± 4.3	0 ± 0	+12.8 ± 1.0	0 ± 0	0	36.8 ± 5.9
	24 h	-9.7 ± 5.5	0 ± 0	+6.4 ± 1.8	0 ± 0	0	36.8 ± 5.9
Methanol	2 h	+28.4 ± 1.5	+10.4 ± 2.4	+100.0 ± 2.6	-45.4 ± 7.5	0	30.8 ± 6.4
	24 h	+31.6 ± 0	+5.0 ± 3.2	+84.2 ± 3.3	-50.0 ± 9.2	0	30.8± 6.4
Ethanol	2 h	-21.2 ± 14.7	*+16.7 ± 3.0	N/A	N/A	0	16.5 ± 5.1
	24 h	-4.2 ± 3.7	*-2.2 ± 7.5	N/A	N/A	0	16.5 ± 5.1
Acetone	2 h	*+67.3 ± 6.7	+34.7 ± 10.4	N/A	N/A	0	46 ± 1.6
	24 h	*+14.5 ± 3.7	+70.0 ± 11.7	N/A	N/A	0	46 ± 1.6

[a]Positive control was cycloheximide solution (50 µg/mL); [b]Negative control was 200 µL of ethanol; Results reported as; - percentage inhibition; + percentage promotion; N/A yield was too low to test; Data (n=3) are presented as the mean ± SD; (*Statistically significant ρ<0.05). The percentage inhibition/promotion was calculated using Equation 2.2 (100).

The results found that the seaweed extracts exhibited two different effects on the mycelia growth of *H. annosum*, including promotion and inhibition, with these effects varying between seaweed species, extraction solvent and even extraction period. Some extracts exhibited no activity against *H. annosum*. For example, the water extracts of *A. nodosum* and *U. lactuca* demonstrated 0% inhibition. The majority of the methanol extracts produced growth promoting activities, with the 2 h methanol extract of *P. lanosa* demonstrating over a 100 ± 2.6% increase in growth compared to the negative control. As with the screen on *A. mellea*, this was suspected to be as a result of either nutrient supply by the extract itself or the hormetic response, further supporting investigations into the concept of integrating extracts with biocontrols such as the antagonist fungi *Phlebiopsis gigantea* currently used against *H. annosum* (373). Figure 2.6 illustrates the differences in growth between the negative control and media supplemented with the methanol extract of *P. lanosa*.

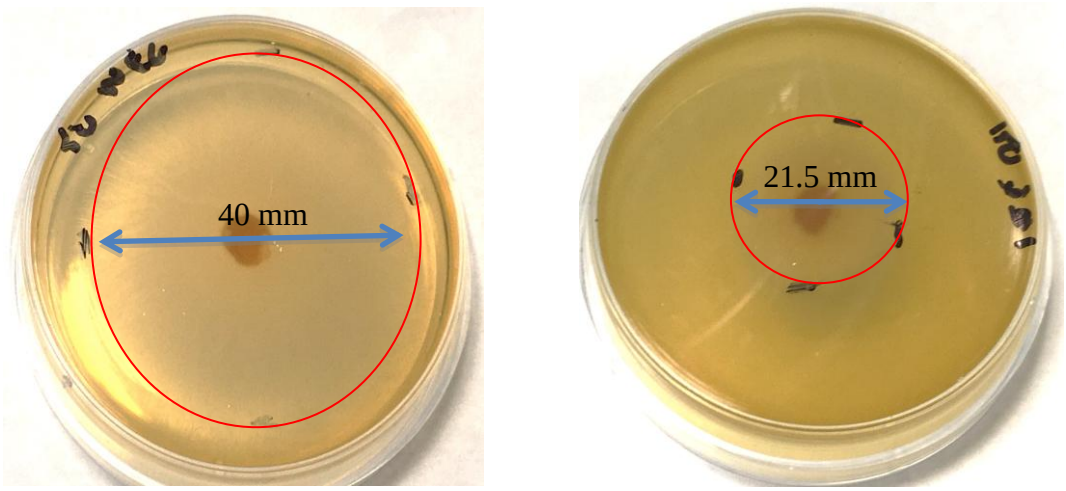


Positive control **Negative control** **Methanol extract of *P. lanosa***

Figure 2.6: Growth promotional activity of the crude methanol extract generated from *P. lanosa* collected in September 2015 against *H. annosum* using the poisoned food technique at 5 mg/mL. Positive control: 50 μg/mL cycloheximide; Negative control: 200 μL of ethanol. Data (n=3).

Interestingly, it can be seen that the solvent methanol is responsible for extracting these growth promoting compound(s) in both the red and brown seaweed species.

However, the highest percentage of inhibition was also exhibited by this solvent but on extracts generated from the green seaweed *U. lactuca* with the 24 h generated extract producing a 50 ± 9.2% inhibition against *H. annosum* compared to the negative control, Figure 2.7. This, therefore, means that although the bioactives of the same polarity were being extracted from all three seaweed species, it does not mean that the same activity is exhibited between species.



Negative control **Methanol extract of *U. lactuca***

Figure 2.7: Antifungal activity of the crude methanol extract generated from *U. lactuca* collected in July 2016 against *H. annosum* using the poisoned food technique at 5 mg/mL. Negative control: 200 µL of ethanol; (Red circle added to aid in the visualisation of the mycelia growth of *H. annosum*). Data (n=3).

This antifungal activity by *U. lactuca* was not unexpected. As mentioned previously, *U. lactuca* could not be attained at the initial collection timepoint of September 2015 due to its low availability. However, as part of a previous project, *U. lactuca* that had been extracted with ethyl acetate in May 2015 was available and was, therefore, extracted with water, methanol, ethanol and acetone via solvent extraction and tested as part of an initial proof-of-concept until fresh *U. lactuca* was available. The methanol extract after 24 h of extraction indicated some degree of antifungal activity with % inhibition of 35.1 ± 3.7% compared to the negative control against *H. annosum* at 5 mg/mL.

Arising from these initial results, fresh *U. lactuca* was collected in July 2016 when the species was in abundance in order to avoid total removal of this species from a section of the Irish coastline. The fresh *U. lactuca* was also extracted with ethyl acetate to see whether the ethyl acetate extract would exhibit the same if not, better activity as the methanol extract. The ethyl acetate extract of the fresh *U. lactuca* exhibited an inhibition of 9.1 ± 2.20% confirming that ethyl acetate was not as efficient as methanol in extracting the desired bioactive(s) and explained why the previously extracted *U. lactuca* had reduced activity compared to the fresh *U. lactuca*. Since both harvest seasons were in summer (May 2015 and July 2016) and at the same geographical location it meant that such variations could not be responsible for this difference in activity. This difference in activity may also be explained by the fact that pre-extracted *U. lactuca* was stored in a freezer for over a year. Vlachos *et al.* reported both an increase and decrease in antibacterial activity after 3 years of storage depending on seaweed type (374). For example, the antibacterial activity of *Zonaria subarticulata* was found to increase with increasing storage time, whilst *Sargassum incisifolium* exhibited a decrease in activity.

The extracting solvent methanol exhibited the strongest degree of inhibition against *H. annosum* it further demonstrated the major influence polarity has on of the level of activity observed for specific seaweeds. This is supported by Abbassy *et al.* who also investigated the fungicidal activity of the green seaweed *U. lactuca* which was extracted in a number of different solvents including acetone, chloroform, ethanol, methanol and petroleum ether (228). The methanol extract was found to be the most potent against the three phytopathogenic fungi (*Aspergillius niger*, *Penicillium digitatum* and *Rhizoctonia solani*). This is possibly due to methanol being a polar solvent and, as mentioned previously, seaweeds have a high level of polar constituents such as carbohydrates (22-44%) which have been reported as exhibiting antifungal activity (339, 340). Compositional analysis of *U. lactuca* found that it has a high carbohydrate content of 58% while a relatively low lipid content (non-polar) of 1.22% (375). Similarly Selim *et al.* also reported the methanol extract of *U. lactuca* as having the strongest fungicidal activity compared to the ethanol, methylene chloride, chloroform and hexane extracts (376). The effects of slight changes in composition of the extracting solvent was demonstrated by O'Sullivan *et al.* on the three brown seaweeds *A. nodosum*, *F. vesiculosus* and *F. serratus* who found that four solvents (100% water, 60% ethanol,

80% ethanol and 60% methanol) of relatively similar composition illustrated different activity with 80% ethanol exhibiting the greatest antioxidant potential particularly the *A. nodosum* extract (377).

These studies strongly support the results obtained in this screen where the methanol extracts demonstrated the highest degree of activity. In addition to this, it was a green seaweed phylum, specifically *U. lactuca*, that exhibited the strongest antifungal activity compared to the red and brown species. This was also observed by Aruna *et al.* who evaluated the antifungal activity of all three seaweed species green, red and brown (85). The methanolic extract of *U. lactuca*, showed the maximum activity including a 56 mm zone of inhibition against *A. niger* at an extract concentration of 200 mg (85). This theory was further supported by Barot *et al.* who investigated the activity of *U. lactuca* (green), *Sargassum tenerrimum* (brown) and *Laurenica obtusa* (red) against *Penicillium janthinellum* and *Aspergillus niger* (378). The seaweeds were extracted in solvents of increasing polarity: hexane, ethyl acetate, chloroform and methanol (1:10 *w/v*). The antifungal efficacy of each extract was determined using the agar plate method and once again *U. lactuca* demonstrated the highest activity, specifically the methanol extracts, which not only exhibited the largest inhibition zones, but also the maximum crude extract yields (53.46%) (378). These studies further support the great potential of *U. lactuca* to be used as a biopesticide. Additionally, this was the first study to test seaweed extracts against this particular pathogen, *H. annosum*.

2.3.5 Dose response effect of the U. lactuca extract

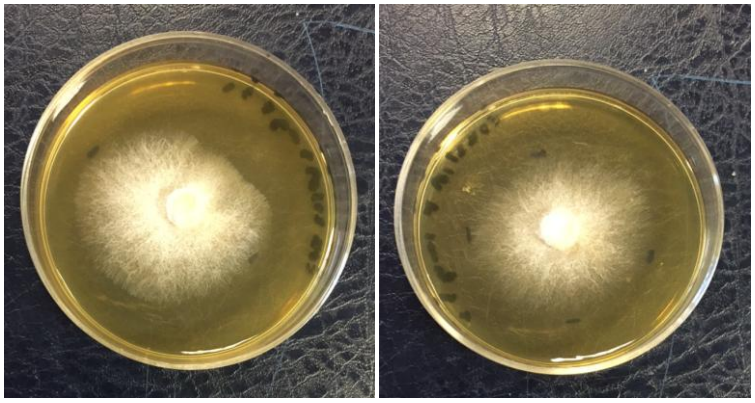
Since the methanol extract of *U. lactuca* collected in July 2016 exhibited the strongest degree of antifungal activity, a concentration study was completed on the extract in the concentration range of 1, 3, 5, 7 and 10 mg/mL in order to determine whether activity was dose dependent as presented in Table 2.7.

Table 2.7: Concentration study of the methanol extract of *U. lactuca* using the poisoned food technique. Concentrations tested: 1, 3, 5, 7 and 10 mg/mL.

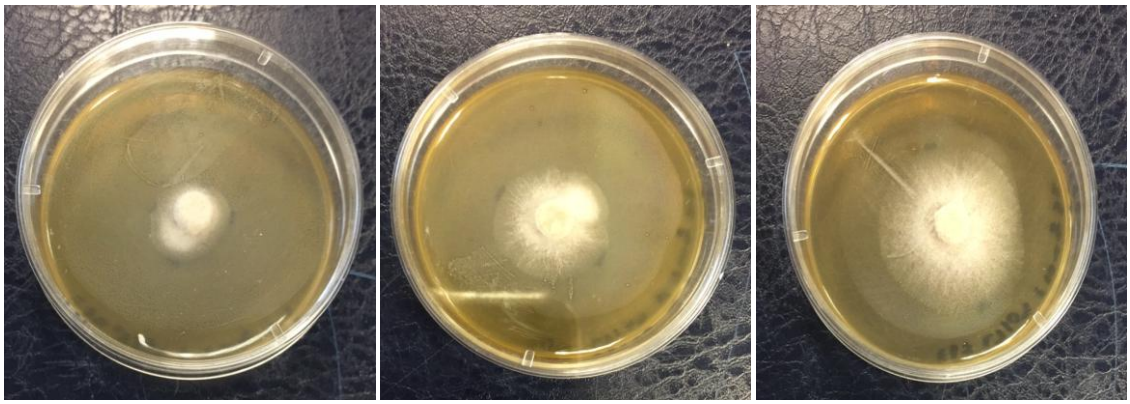
Extract concentration (mg/mL)	**Percentage inhibition of *H. annosum* (%)**
1	27.0 ± 1.00^{a}
3	31.0 ± 1.00^{b}
5	47.0 ± 1.00^{c}
7	43.3 ± 0.58^{d}
10	40.3 ± 1.53^{e}

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

From examination of Table 2.7 it can be seen that the extract illustrated a dose-response effect with an increase in concentration resulting in higher activity, but only up to a concentration of 5 mg/mL, with Figure 2.8 giving a visual representation of this dose-response effect and resultant drop in activity. This trend is most likely due to the fact that 200 µL of ethanol was unable to solvate all of the bioactives and increasing the volume of ethanol above 200 µL negatively effected fungal growth.



1 mg/mL **3 mg/mL**



5 mg/mL **7 mg/mL** **10 mg/mL**

Figure 2.8: The dose-response effect of *U. lactuca* at 1, 3, 5, 7 and 10 mg/mL against *H. annosum* using the poisoned food technique. Data (n=3).

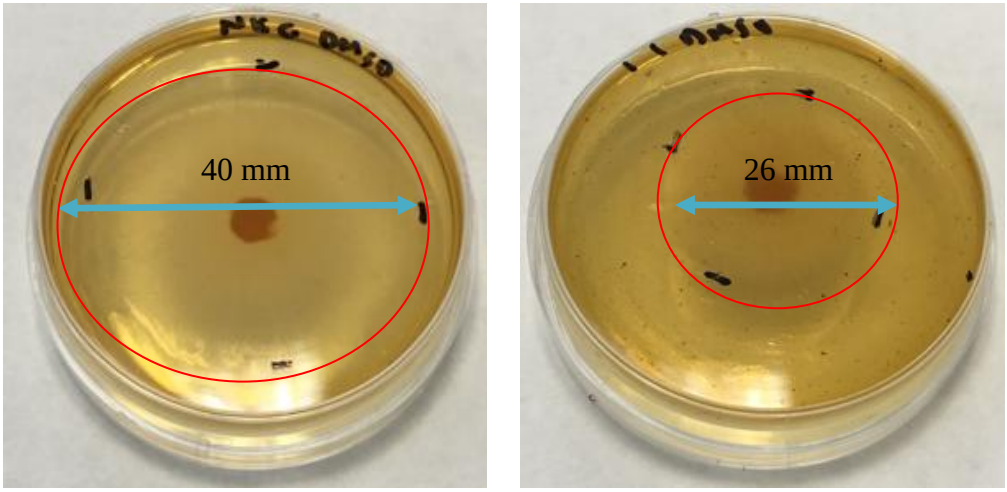
A dose response effect was also observed in a number of other studies including Aruna *et al*. who investigated the antifungal activity of seaweed extracts against a number of fungal pathogens (85). The seaweed extracts were tested at three different concentrations 50, 100 and 200 mg and it was found that 200 mg produced the strongest activity, specifically for the aqueous extract of *U. lactuca* which produced an inhibition zone of 56 mm (85). This was further supported by Sujatha *et al.* who evaluated the antifungal activity of red, green and brown seaweeds through the poisoned food technique (52). The crude extracts were tested at three different dose levels of 10%, 20% and 30% concentrations. Once again seaweed efficacy was strongest at 30% with the brown seaweed *Sargassum myricocystum* reducing the mycelial growth of *Rhizoctonia solani* and *Macrophomina phaseolina* by 61.44% and 58.53%, respectively, compared to the untreated plates (52).

2.3.6 Determination of the optimum solvating solvents for the U. lactuca extract

Ethanol has been solely applied as the solvating solvent throughout this antifungal screen study. However, an investigation into the use of different solvating solvents was performed in order to determine whether activity could be further improved using different solvents and without affecting the tested fungi. The first solvent selected was dimethyl sulfoxide (DMSO), a polar aprotic solvent, meaning it had the ability to dissolve both polar and nonpolar compounds. In addition to this, DMSO has been used in a number of studies as the solvating solvent for dried seaweed extracts, generally at a concentration of 5% or below in bioactivity testing due to its toxicity (85). For instance, Chanthini *et al.* investigated the antifungal activity of six seaweed extracts against the phytopathogen, *Alternaria solani* (44). Five percent aqueous DMSO was used to solvate the dried extracts before being tested via the disk diffusion method with strong activity demonstrated by a number of extracts. Similarly, Rajesh *et al.* prepared dried seaweed extracts in DMSO at various concentrations (0.05%, 0.1%, 0.2%, 0.4% and 0.8%) for antimicrobial bioassays (agar well diffusion) and found that the methanolic extract of *Caulerpa scalpelliformis* inhibited the growth of *Fusarium oxysporum* with an inhibition zone of 12.66 ± 0.33 mm and a MIC of 8 mg/mL (63). Although in both of these studies the impact of DMSO on the growth of the fungal species wasn't disclosed.

The effect of DMSO on the growth of fungi has been previously investigated since DMSO is commonly used in antifungal susceptibility assays for water insoluble agents. Randhawa *et al.* conducted a study on the effect of DMSO on the growth of dermatophytes in a concentration range of 0.125 to 10%. Not surprisingly, the 10% concentration fully inhibited the growth of all fungi with a dose-related inhibitory effect between 5 and 1.25%, while below 1% the effect was variable among species with *Microsporum canis* being significantly inhibited down as far as 0.25%. Similarly Hazen *et al.* investigated this effect on water-soluble drugs against *Candida* species in the presence and absence of DMSO. A concentration range of 0.125 to 4% (*v/v*) was used with some isolates still demonstrating reduced growth at 0.125% concentration of DMSO. Rodriguez-Tudela *et al.* reported 2% DMSO as significantly effecting the growth of *Candida* species by the broth dilution method, whereas a 1% concentration or below had an insignificant effect. In this project, 200 µL of 5% aqueous DMSO was

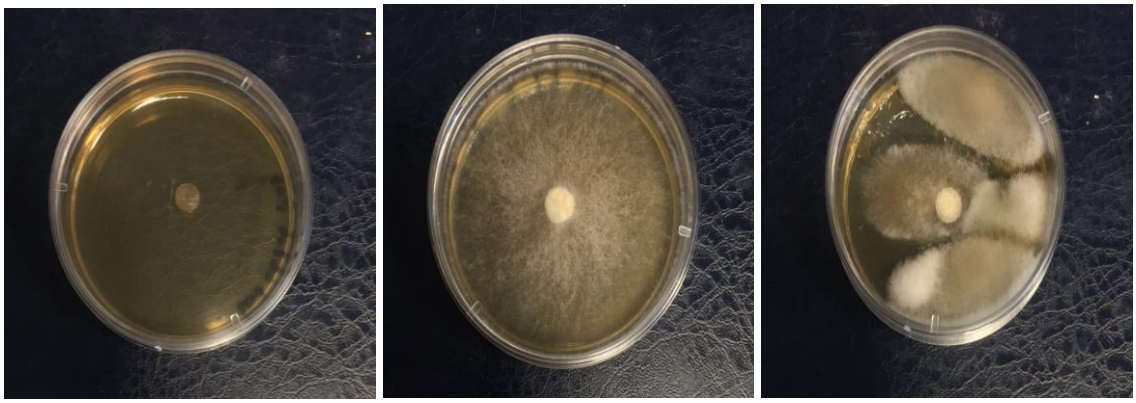
used to solvate the methanol extract of *U. lactuca* as opposed to ethanol. However, this 5% DMSO was further diluted by agar to give a final concentration of just 0.2% aqueous DMSO. From examination of Figure 2.9 it can be seen that this concentration of DMSO had no effect on the growth of *H. annosum* (negative control) since the media supplemented with extract demonstrated a 41.25% inhibition compared to the negative control. Although this activity was not as strong as ethanol, it still illustrated the ability of DMSO as a solvating solvent.



Negative control **Methanol extract of *U. lactuca***

Figure 2.9: The crude methanol extract generated from *U. lactuca* solvated in 200 µL of 0.2% aqueous DMSO tested against *H. annosum* using the poisoned food technique at 5 mg/mL. Negative control: 200 µL of 5% DMSO. Data (n=3).

The potential use of water to solvate the dried extract as opposed to an organic solvent was also investigated, but unfortunately water lacked the ability to sterilise the crude extract and resulted in the plates supplemented with seaweed extract becoming heavily contaminated with other fungal strains as shown in Figure 2.10. The negative control, which only consisted of a plug of *H. annosum* and 200 µL of sterile water, showed strong growth with no contamination, as expected.



Positive control **Negative control** **Methanol extract of *U. lactuca***

Figure 2.10: The crude methanol extract generated from *U. lactuca* solvated in 200 µL sterile water tested against *H. annosum* using the poisoned food technique at 5 mg/mL. Positive control: 50 µg/mL cycloheximide; Negative control: 200 µL of sterile deionised water. Data (n=3).

2.4 Conclusions

Four seaweed species collected from the South-East coast of Ireland were extracted in various solvents including water, methanol, ethanol and acetone and screened against two problematic fungi. Solvent extraction incorporating magnetic stirring was found to generate a higher yield of bioactives compared to automatic shaking and was, therefore, selected as the extraction method for the entire study. It was found that extraction yields differed between solvents, with water producing the highest yield and acetone the lowest. The extraction period also had an influence on yield, with the longer the extraction period (24 h) the greater the yield with an effect on activity also reported for a number of seaweed extracts (Table 2.6).

The crude seaweed extracts were found to exhibit no antifungal activity against the parasitic fungus *A. mellea*. However, the selected antifungal test method (poisoned food technique) lacked the ability to quantitatively assess whether the generated extracts negatively affected the growth of *A. mellea*, mainly due to its growth pattern. Although the test method lacked the ability to quantify reduction, it clearly illustrated the potential growth promoting activity of the water extract generated from the brown seaweed *F. serratus.* This particular activity was suspected to be as result of a hormetic dose response or supply of nutrients from the seaweed itself. It did however highlight a potential novel application of these seaweed extracts as an aid in biocontrol agents, with further investigation required.

This screen also found that the seaweed extracts tested against the phytopathogen, *H. annosum* exhibited two different effects on the mycelia growth including promotion and inhibition with these effects varying between seaweed species, extraction solvent and even extraction period. Similar to *A. mellea*, certain seaweed species increased the growth rate of *H. annosum*, particularly the methanol extract of *P. lanosa*. The methanol extract of *U. lactuca* produced the strongest bioactivity out of all of the generated extracts with a dose response effect observed up to a concentration of 5 mg/mL with bioactive solubility issues encountered thereafter.

Thus, the methanol extract of *U. lactuca* after 24 h of extraction (highest yield and activity unaffected) was chosen for further study in Chapter 3, which investigated different antifungal test methods in order to identify the optimum test method for this particular fungus (basidiomycete). This seaweed extract was also tested *ex vivo* and

efficiency compared to that of a commercially available product currently used to control this pathogen.

This study demonstrated the novel potential of biopesticides derived from seaweed extracts. Additionally, this was the first reported use of seaweed extracts exhibiting antifungal activity against *H. annosum*. Certain species also produced promotional growth on the tested organisms, illustrating a potential application in the area of biocontrols.

CHAPTER 3: *IN VITRO* AND *EX VIVO* ANTIFUNGAL TESTING OPTIMISATION AGAINST *H. ANNOSUM*

3.1 Introduction

Emergence of new plant pathogens is an ever growing problem in the forestry industry mainly as a result of increased travel movement and a lack of regulation in the trade of plant products (379). For these reasons, the international trade of plants has been tightly linked to biological invasions (246). Climate change is also another contributing factor which may put trees and other plants under increased stress, making them more susceptible to infection from native pests and diseases (249). To combat such threats, indiscriminate use of synthetic pesticides have been applied which can have significant negative impacts on the environment, such as their effect on non-target species and soil fertility (302, 380, 381). Therefore, in order to keep pace with these growing concerns, researchers have begun to investigate the use of natural resources as potential novel biopesticides.

A number of different techniques are currently used to assess the antimicrobial capacity of extracts from natural resources, namely disk diffusion, broth dilution and direct bioautography (100); however no single method is suitable for screening all of these different extracts. Thus, careful consideration and investigation must be conducted to ensure the method selected is simple, rapid, sensitive and reproducible with high sample throughput and at low cost.

The first step in this process is the screening of various test methods to see which best suits the specific extract and test pathogen. One problem often faced by researchers is that natural products can have poor solubility in water. Therefore, the disk diffusion assay is one of the most commonly used test methods since the use of water can be avoided. The zones of inhibition around each disk can then be interpreted using the criteria published by the Clinical and Laboratory Standards Institute (382) making it one of the simplest and most practical antimicrobial screening methods (383). Disk diffusion also exhibits many other advantages including; low cost, flexibility and the utilisation of low extract and reagent volumes (101). Pandithurai *et al.* demonstrated the ability of the disk diffusion method to illustrate the antifungal capacity of various solvent extracts of *Spatoglossum asperum* against a number of different fungal pathogens as shown in Figure 3.1 (77). It was found that the chloroform extract of this brown seaweed exhibited the strongest activity against *Aspergillus flavus* with a zone of inhibition of 11.86 ± 0.61 mm.

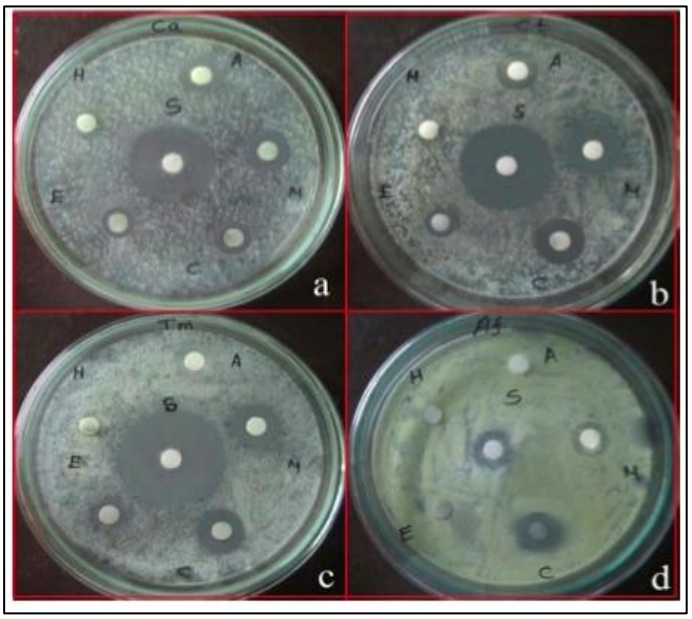


Figure 3.1: Antifungal activity of various solvent extracts of *Spatoglossum asperum* against (a) *Candida albicans*, (b) *Candida tropicalis*, (c) *Trichophyton mentagrophytes* and (d) *Aspergillus flavus* at a concentration of 100 µg/mL via the disk diffusion method (A-Aqueous, M-Methanol, C-Chloroform, E-Ethyl acetate, H-Hexane, S-Standard (flucanozole)) (77).

Peres *et al.* (51) and Wahidi *et al.* (384) also illustrated the effectiveness of this method as a rapid screening technique for the antifungal activity of various seaweed extracts around the world. However, a major downfall to this method is the fact that the polarity of the natural compounds can affect the diffusion of compounds into the culture medium, with less polar compounds diffusing at a slower rate. Therefore, an extract of good antifungal activity but with non-polar compounds might exhibit a small zone of inhibition in a disk diffusion assay (102). Another disadvantage to this method is that the compounds cannot be volatile in nature, as such material would evaporate off during the drying process of the disk (385).

Agar well diffusion is also a well-known technique used to evaluate the antimicrobial capacity of natural product extracts (105, 386, 387). This technique is similar to disk diffusion and like disk diffusion, well diffusion also has limitations arising from diffusion issues. For these reasons, the use of polar solvents such as water is considered optimum. In addition to this, organic solvents can be toxic to the test fungi, however studies applying organic solvents included a negative control ensuring these solvents

did not negatively affect the growth of the fungi (100). Despite this, well diffusion is a simple rapid method of screening that only requires a small amount of sample and, unlike disk diffusion, the drying process is not required, allowing for volatile compounds to be used (10).

The broth dilution assay is considered one of the most basic antifungal susceptibility test methods with two organizations: EUCAST and CLSI having standardised protocols for such testing against moulds, yeast and filamentous fungi (388). These problems have been adopted by researchers in evaluating the antifungal potential of seaweed extracts (81, 336). The broth dilution assay determines the MIC and generally, 50% inhibitory (MIC_{50}) or 90% inhibitory (MIC_{90}) values are reported for natural product extracts (133, 134) and are a good indication of the antimicrobial efficacy of the bioactive compound(s). However, a number of factors including inoculum size, growth medium, incubation time and the preparation method can influence this MIC value and for this reason EUCAST and CLSI have both standardised two similar methods which have defined breakpoints that state whether the test organism is susceptible, intermediate or resistant to the extract (100, 389).

Therefore, the selection of the most appropriate assay is essential for assessing the antifungal activity (due to the importance of correctly determining the antifungal capacity) of the bioactive compound(s) present in the seaweed extracts. For this reason there are a wide variety of antifungal test methods used to determine the antifungal activity of seaweeds including; plate stripping method (390), broth dilution assay (220), disk diffusion assay (335), poisoned food technique (52), direct bioautography technique (51) and well diffusion assay (85). However the antifungal test method chosen can depend on a number of factors, namely the cost and expertise available, whilst not forgetting the suitability of both the extract and fungi towards the test method.

Although the screening of seaweed extracts using a suitable assay gives a strong indication into their potential as antifungal agents, it is very important to evaluate their potential in field trials since results obtained from *in vitro* studies do not always reflect how these bioactives present in the seaweed extracts will behave in the field (348). For this reason, field trials are generally conducted after positive *in vitro* results. Schubert *et al.* evaluated the application of a *Trichoderma* strain as a biological control of wood

decay in trees after this specific strain was identified as highly competitive in an *in vitro* screening against five basidiomycetes and one ascomycete (391). Additionally, the use of biocontrols is restricted due to the shortage of commercial formulations available. As a result they demonstrate poor competiveness towards their synthetic counterparts (392).

The three main objectives of this chapter of work were (a) to compare and optimize a test method for screening antifungal activity in the crude *U. lactuca* extract against *Heterobasidion annosum*, (b) to develop and optimise a standardised method for the testing of the extract on wood samples and, (c) to compare the efficacy of this extract against a commercially available plant protection product (PPP) currently used to control *H. annosum* infection. The suitability of four different test methods were investigated; disk diffusion assay, well diffusion assay, broth dilution assay and agar-plate test. The developed standardised method for the testing of the active extract on wood samples was conducted on disks of *Picea sitchensis* (Bong.) Carrière (Sitka spruce) a coniferous evergreen tree that is susceptible to infection from *H. annosum* (393). Sitka spruce has proven to be one of the most productive conifers in Ireland becoming the mainstay in roundwood processing. It remains the predominant species used in Irish forestry with a total of 335,000 hectares planted (394).

Therefore, this study provides insight into the ability of the extracts to protect a commercially significant tree species when exposed to *H. annosum*. The PPP urea was used to evaluate the competitive efficacy of the antifungal compounds in *U. lactuca* with the potential of reducing the use of this chemical product. This reduction is important as urea is a corrosive substance that damages and reduces the lifetime of the application equipment on harvesters and the high doses applied poses a small risk of increased nitrification of the soil and it is mildly phytotoxic to herbaceous plants (279, 280).

3.2 Experimental Procedure

3.2.1 Production of Crude Ulva lactuca Extracts

The *U. lactuca* collected in July 2016 was extracted in the solvent methanol at the ratio 1:50 *w/v* using the previously optimised extraction method in Chapter 2 section 2.2.2.

3.2.2 Disk diffusion assay

The standard disk diffusion method used in this study was described by Espinel-Ingroff *et al.* with some modification (395). The dried *U. lactuca* methanol extract was re-dissolved in ethanol to give a concentration of 100 mg/mL. The extract (50 µL) was loaded onto a 6 mm blank disk (Oxoid, Basingstoke, UK) at a rate of 10 µL at a time to give a final concentration of 5 mg/disk. Disks loaded with 50 µL of ethanol served as the negative control, and disk loaded with 50 µg/mL of cycloheximide served as the positive control. All disks were allowed to dry and the entire process was carried out under aseptic conditions.

For the antifungal assessment, the fungal isolates of *H. annosum* were cultured on malt extract agar medium and incubated for a seven-day period at 20 °C to allow for sufficient sporulation before harvesting. The spores were then added to a suspension of malt extract broth through the use of a sterile swab and were adjusted to an optical density of 0.08 – 0.1 at 625 nm which is equivalent to the 0.5 McFarland turbidity standard which is comparable to suspension of 1.5×10^8 colony forming units, CFU/mL. (395). A 100 µL aliquot of the adjusted culture was pipetted onto a fresh malt extract agar plate and spread evenly across the plate using a sterile spreader. The disks containing the seaweed extract plus the negative and positive controls were aseptically applied to the inoculated plates, which were then refrigerated for 4 to 5 h in the inverted position. This allowed the diffusion of the bioactive compound(s) whilst the lower temperatures prevented/slowed fungal growth. The pre-inoculated plates were incubated at 20 °C for 5 days. Zones of inhibition were measured to the nearest whole millimeter at the point at which there was 80 – 100% growth inhibition around the disk every 24 h (395). The experiment consisted of three replicates repeated in duplicate.

3.2.3 Well diffusion assay

The standard well diffusion method used in this study was described by Akinpelu *et al.* who evaluated the antifungal properties of *Alchornea laxiflora* leaf extracts with slight modification (396). Similar to the disk diffusion assay, the fungal isolates were prepared on malt extract agar for seven days prior to the experiment to allow for proper sporulation before the spores were harvested. A sterile swab was rolled over the inoculated plate in order to pick up spores and was then mixed in sterile malt extract broth before being adjusted to the 0.5 McFarland turbidity standard spectrophotometrically (396). A 100 µL aliquot of the adjusted fungal suspension was pipetted on to fresh malt extract plates and spread evenly across the plates (396). The inoculated plates were incubated at 20 °C until an even lawn of growth was observed (approx. 5 days). A sterile glass pipette was used to bore holes into the agar medium allowing around 5 mm from the edge of the plate. The dried *U. lactuca* methanol extract was re-dissolved in sterile water (ensure optimum extract diffusion into the agar and non-toxic) to give a concentration of 100 mg/mL. These wells were then filled with 50 µL of *U. lactuca* extracts dissolved in sterile water at a concentration of 5 mg/well. Wells consisting of sterile water served as the negative control while a well consisting of 50 µg/mL of cycloheximide served as the positive control. The plates were allowed to stand on the bench for one hour to ensure sufficient diffusion of the extract solution into the medium (396). The plates were inoculated at 20 °C for 5 days and the zones of inhibition around the wells were measured in mm. The experiment was completed in triplicate and repeated in duplicate.

3.2.4 Broth dilution assay

A standardised microbroth dilution method outlined by CLSI M38-A for filamentous fungi was followed with slight modifications (397). The fungi were cultured for 7 days at 20 °C on malt extract agar to produce conidia. A sterile swab was rolled over the culture and mixed into malt extract broth and was filtered with a Whatman filter model 40 (8 µm) in order to retain hyphae fragments and allow the passage of only *H. annosum* microconidia. The optical density of these suspensions were adjusted spectrophotometrically (520 nm) to a transmittance range of 70 to 72% with inoculum

sizes ranging from 2×10^6 to 4×10^6 CFU/mL (397). Inoculum quantification was achieved by the use of a hematocytometer to count the microconidia followed by the plating of 0.01 mL of suspensions on malt extract agar. The plates were incubated at 20 °C with daily observation until fungal colonies were observed. The colonies were removed and diluted in malt extract broth (1:50 *v/v*) to give a final cell number of between 2×10^4 to 4×10^4 CFU/mL (397).

The extract was re-dissolved in ethanol to give a final concentration of 10 mg/200 µL. A 100 µL aliquot of the extract solution was added into the wells of a sterile microtiter plate (Sarstedt, Hildesheim, Germany). A series of twofold dilutions were performed on the seaweed extracts with malt extract broth. The resulting concentrations ranged from 10 mg per well to 0.3125 mg per well. The wells were inoculated with 100 µL of the diluted inoculum suspension producing final extract concentrations of 5, 2.5, 1.25, 0.625 and 0.3125 mg/200 µL. Inoculated wells loaded with 100 µL of broth served as the negative control and inoculum wells loaded with 100 µL of cycloheximide solution (50 µg/mL) served as the positive control. Sterile control of medium only was also prepared with all wells containing a final volume of 200 µL. The microtiter plate was incubated at 20 °C and the turbidity of each well was measured at 620 nm using BioTek EL×800 Absorbance Microplate Reader (Biotek, VT, USA) after 4, 7 and 10 days of incubation (397). The endpoint determination readings were based on a comparison of the growth in wells containing extract and that of the negative control. The broth dilution assay determines the MIC of the crude extract using Equation 3.1.

Equation 3.1:

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

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

The experiments were completed in triplicate, on three separate weeks.

3.2.5 Agar plate test

The agar plate test is similar to the disk diffusion assay and the poisoned food technique except that one large filter paper was used as described by Alfredsen *et al.* (398). In this test, a sterile Whatman No. 1 filter paper (11 μm pore size, Whatman, Kent, UK) was soaked in the extract solution consisting of 10 mg of *U. lactuca* extract dissolved in 250 μL of ethanol. The solvent was allowed to evaporate before being placed aseptically into a petri dish (60 mm) producing a final concentration of 10 mg/disk. This increased extract concentration was to ensure sufficient diffusion through the agar. A disk soaked in ethanol served as the negative control and a disk soaked in 50 μg/mL of cycloheximide served as the positive control. A thin layer of malt extract agar was poured over the disk and allowed to solidify before a fungal plug of 5 mm in diameter was removed from a 7 to 14 day old culture and was transferred aseptically onto the agar. Figure 3.2 demonstrates the extract loaded filter paper covered by a thin film of agar and inoculated with *H. annosum*. The inoculated plates were incubated at 20 °C and the radial growth was measured (mm) in four perpendicular directions after 7 days of incubation. The antifungal activity of the *U. lactuca* extract was tested in triplicate, with the experiment repeated in duplicate.

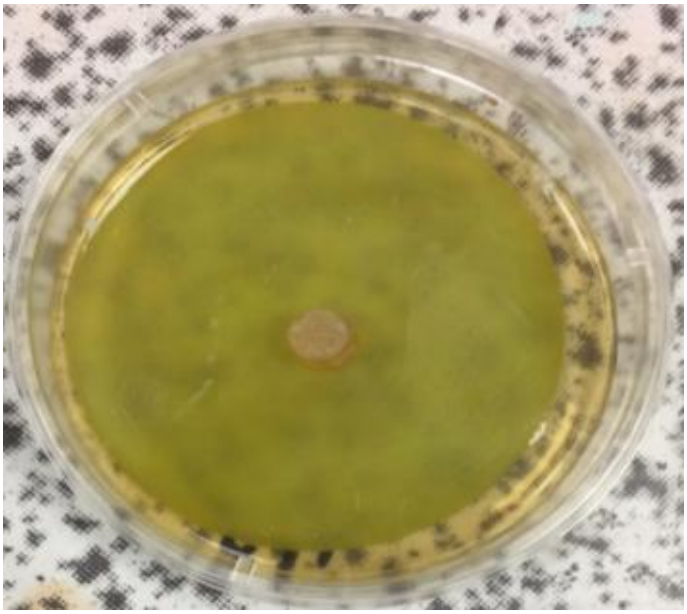


Figure 3.2: The agar plate test used to assess the antifungal capacity of crude methanol extract of *U. lactuca* against *H. annosum* at 10 mg/disk.

3.2.6 Development and optimization of an ex vivo antifungal method on wood samples

The protective effects of the methanol extract of *U. lactuca* against *H. annosum* were studied on disks of *Picea sitchensis* (Bong.) Carrière (Sitka spruce) as described by Aguín *et al*. with slight modifications (399). Sitka spruce was selected because of its known sensitivity to *H. annosum* infection and economic significance. The disks were 2 cm in diameter and 1 cm in thickness and obtained from a young (7-8 years), healthy Sitka spruce tree. This test was carried out within 24 h of collection to ensure the disks resembled the freshly cut stumps on a felling site as these are most susceptible to infection. A number of the disks were sterilised via autoclaving under the waste cycle (30 min at 121 °C) to ensure the disks were free from all types of microbes while others were left un-sterilised (399). The extracts were prepared at a concentration of 5 mg/mL (25 mg of extract dissolved in 5 mL of ethanol) and the disks were soaked in this solution for 3 h (turning the disks half way through) in which the plates were covered in parafilm to prevent any evaporation during this soaking period.

After this time, the ethanol was allowed to evaporate off under aseptic conditions to prevent any contamination from other microbes. Disks soaked in ethanol served as the negative control and disks soaked in an aqueous solution of cycloheximide (50 μg/mL) served as the positive control. The petri dishes containing the dried disks were sandwiched to another petri dish that had been inoculated with *H. annosum* a week prior to the experiment as shown in Figure 3.3. Both the autoclaved and un-autoclaved disks were used with a set of each sandwiched to the inoculated plates for 1 and 2 week periods at 20 °C.

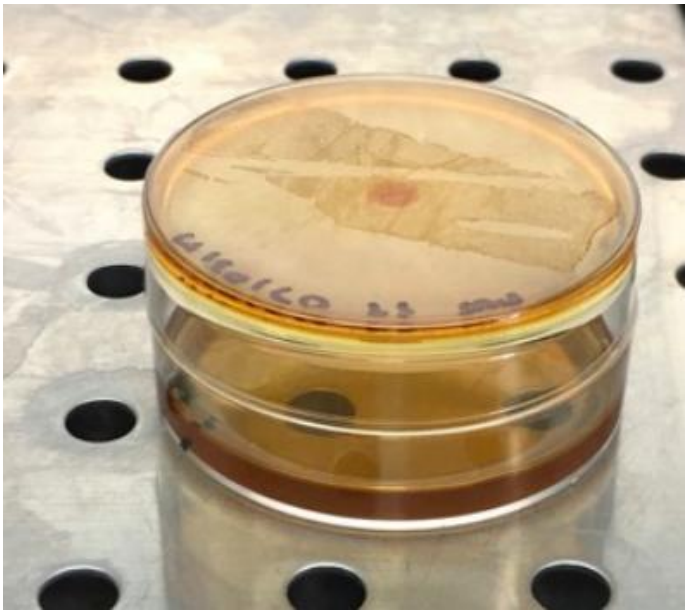


Figure 3.3: Sandwich technique for the *ex vivo* assessment of the protectant activity of *U. lactuca* extracts against *H. annosum*.

The disks were then moved to individual petri dishes (90 mm) containing fresh malt extract agar and incubated at 20 °C for 14 days as demonstrated in Figure 3.4. The plates were checked every 7 days by visual examination with the degree of infection compared to that of the negative control. The *H. annosum* cultures were checked for purity by microscopic examination at 400X using the Species Code (400, 401). The protectant activity of the *U. lactuca* extract was tested in triplicate, with the experiment repeated in duplicate.

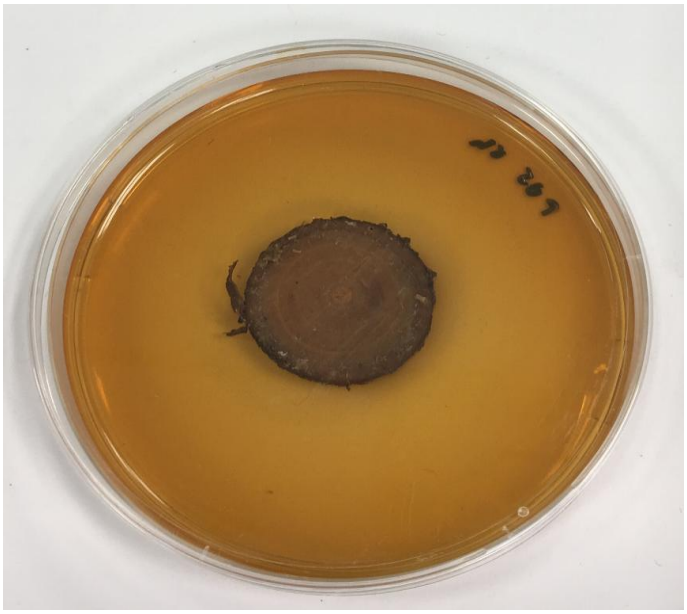


Figure 3.4: Disks of Sitka spruce after exposure to *H. annosum* for a specific length and transferred to malt extract agar to determine the protectant potential of the crude extract of *U. lactuca*.

3.2.7 Comparison to the commercial Plant Protection Product in vitro and ex vivo

The plant protection product urea is currently used as the main method of controlling this problematic root rot disease at its EU approved concentration of 20% *w/v* for manual application and 37% *w/v* for machinery applications (279). For the *in vitro* study, various concentrations of urea were prepared; 0.05%, 0.10%, 0.15%, 0.20%, 0.25%, 0.50%, 0.75%, 1% and 5%, 10% and 20% *w/v* and tested for activity using the poisoned food technique as described in section 2.2.4. The specific concentrations of urea were dissolved in the liquid malt extract agar and allowed to set before a plug of *H. annosum* (5 mm in diameter) removed from a 7 or 14 day old culture was transferred aseptically to the center of the plate with the use of a sterile glass pipette. A plate consisting of only media served as the negative control and a plate consisting of 50 µg/mL cycloheximide served as the positive control. The plates were incubated at 20 °C and the diameter of growth measured (mm) after 7 days. All of the concentrations were repeated in triplicate.

For the *ex vivo* study, the optimised method in section 3.2.6 was applied and the same 11 urea concentrations were used to determine the protective activity on disks of Sitka spruce in order to directly compare to the performance of the crude seaweed extract. Sitka spruce disks that had been sterilised via autoclaving were soaked in urea that was solvated in deionised water and exposed to *H. annosum* over a 14-day period. The results were recorded and purity of the inoculum was checked using microscopic examination. The plant protection product was tested in triplicate, with the experiment repeated in duplicate all on the same day.

3.2.8 Statistical analysis

Data obtained for the disk diffusion assay, well diffusion assay, broth dilution assay and the agar plate test for the *U. lactuca* extracts were performed with repeated measures analysed using one-way ANOVA followed by a post-hoc analysis using Tukey's multiple comparison tests. These analyses were performed by Minitab 17 Statistical Software using a 5% statistical significant level (ρ<0.05). The results are said to be statistically different if ρ<0.05 and are designated with different superscripts with regards to each methods percentage inhibition exhibited against *H. annosum*. In addition, the data obtained from the comparison studies of urea also underwent statistical analysis in order to identify whether the activity of urea was dose dependent. The activity between various urea concentrations was once again said to be statistically different if ρ<0.05. All experiments were performed in at least triplicate and presented as the mean ± SD, unless otherwise stated.

3.3 Results and Discussion

3.3.1 Comparison and optimisation of antifungal test methods

The poisoned food technique was the antifungal test method of choice up to this point of the study in assessing the antifungal capacity of crude seaweed extracts against the problematic root rot disease *Heterobasidion annosum.* This method is commonly used to evaluate the antifungal effect of natural compounds against moulds (402). The details of this methodology is given in Chapter 2 section 2.2.4. However, the poisoned food technique has a number of drawbacks as a test method; (1) high amount of extract are required with 25 mg per plate in order to achieve a final concentration of 5 mg/mL since the extract is further diluted out in the agar, (2) results take a week to be obtained, (3) a high volume of consumables is necessary with each triplicate requiring its own petri dish and this must then be repeated in duplicate and, (4) it is not an accurate methodology to determine an MIC, since it is impossible to quantify the amount of extract that has diffused into the media (100). This opened the door to an investigation into other test methods that could potentially overcome some of these drawbacks experienced by the poisoned food technique and these are discussed below.

3.3.1.1 Disk diffusion assay

The disk diffusion assay is one of the most popular test methods used to determine the antimicrobial activity of plant extracts (387, 403, 404), although it has also been used in many studies investigating the antifungal properties of natural products including seaweeds (51, 334, 335). The disk diffusion assay has been used against fungal plant pathogens. For instance, Chanthini *et al.* who studied the antifungal potential of various seaweed extracts against *Alternaria solani,* a phtyopathogen found that disk diffusion was an efficient method to illustrate this activity, with all extracts of *Chaetomorpha antennina* demonstrating 94% activity against *A. solani* (44). This assay overcomes a particular problem observed in the poisoned food technique by requiring less extract of only 5 mg per disk as opposed to the 25 mg initially required by the poisoned food technique and only one petri dish is necessary per duplicate.

In this assay, ethanol was selected as the solvating solvent since section 2.3.6 demonstrated its superior ability to solvate the required bioactive(s), over that of 5%

DMSO and sterile water. Ethanol was also chosen on the basis of previous studies which used ethanol as the solvating solvent, such as Ertürk *et al.* who dissolved seven seaweed crude extracts in 70% ethanol in order to determine their antifungal capacity against *Aspergillus niger* and *Candida albicans* via the disk diffusion assay (405). *Enteromorpha linza* and *Padina pavonica* showed the highest antifungal activity against *A. niger*, with both illustrating inhibition zones of 17 mm.

A concentration of 5 mg/mL was used in order to be able to directly compare % inhibition to that of the poisoned food technique, as activity was found to be dose-dependent from concentration studies carried out in section 2.3.5 on the active methanol extract of *U. lactuca.* Unfortunately problems started to appear when it came to adjusting and growing the lawn of fungi. The problem arose from the inability to reach the absorbance level required to achieve the McFarland scale and this inevitability lead to the development of a patchy lawn of growth over 14 days of incubation before an even growth was observed. This length of incubation time is not feasible for disk diffusion since after this time the disks containing the extract or the positive control could have degraded resulting in false negative results. For this reason slight modification was made to the assay which involved adding a plug of *H. annosum* 5 mm in diameter to six plates of malt extract agar. Then four disks pre-loaded with cycloheximide (50 μg/mL) were added to a specific individual plate equal distances apart every 24 hours, over a 6 day period excluding one day prior to this for incubation of the plug on its own. Figure 3.5 demonstrates the results obtained for this method development of a slightly modified disk diffusion assay after a week of incubation from the time individual disks were added.

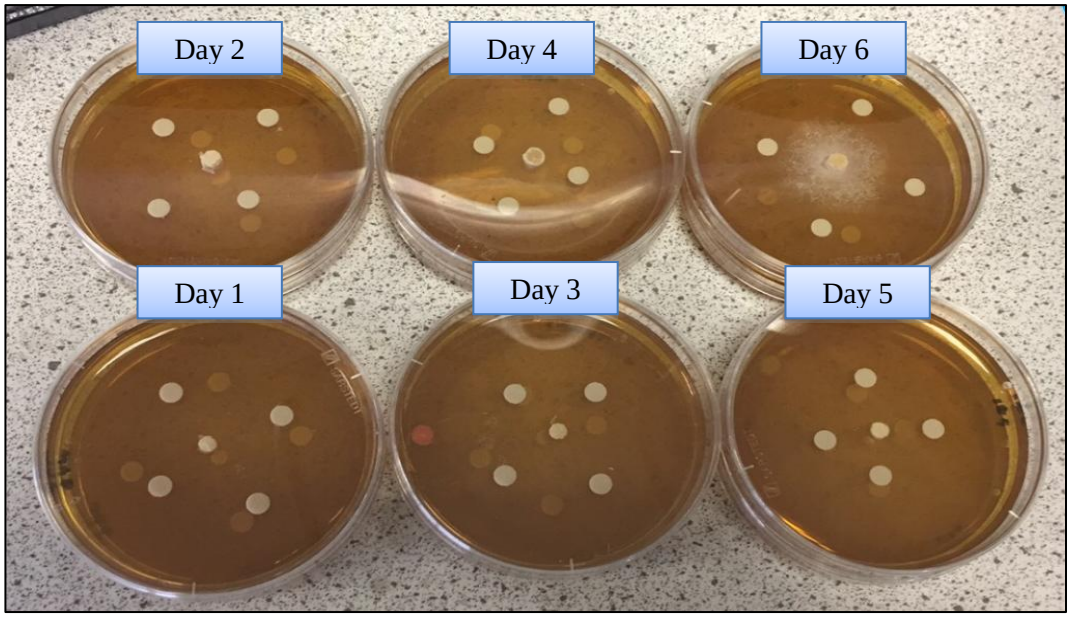


Figure 3.5: Modified disk diffusion assay with disks of the positive control cycloheximide (50 µg/mL) added to individual inoculated plates every 24 h over a 6-day period. Data (n=3).

From examination of the figure above, it can be seen that from day 1 to 4 no growth of *H. annosum* was observed as a result of the cycloheximide disks. But at day 5 (very light) and 6 it can be seen that the fungi had already begun to grow prior to the introduction of the disks but unfortunately the addition of the positive control had no effect on growth with the fungi found to grow over the disks. Therefore, this experiment did not produce a conclusive result on the antifungal activity of *U. lactuca* extracts.

The most likely reason that the disk diffusion assay was unable to assess the antifungal capacity of crude extracts of *U. lactuca* was because the standardised methods published by CLSI and EUCAST have only been developed for yeast disk diffusion testing (document M44-A and for mould disk diffusion testing, document M51-P) (406). Additionally, it has been reported that larger objects including spores or filaments do not follow the rule that established a proportional relationship between dry weight concentration and optical density (407). Inoculum size has also been found to have a significant influence on the MICs for filamentous fungi with other variables including the colour of the spores affecting the optical density value. Therefore, when attempting to test *H. annosum* and adjust it to the McFarland scale, it was incredible difficult. Furthermore, the McFarland standard becomes uncertain when it moves away from

different species of fungi since size and mass differs (408). Also the positive control cycloheximide is not one of the standardised antifungal agents used by EUCAST or CLSI (388). For these reasons, studies that employed the disk diffusion method generally used yeast and/or mould strain pathogens. For example, Demirel *et al.* investigated the antifungal activity of a number of solvent extracts from brown seaweeds against the yeast pathogen *Candida albicans* (409). The disk diffusion method was used and the fungus was adjusted to the McFarland scale and it was found that the hexane extract of *Dictyota dichotoma* exhibited antifungal activity with a zone of inhibition of 6.5 mm. Therefore, the disk diffusion assay would not be suitable for assessing the antifungal activity of seaweed extracts against *H. annosum*.

3.3.1.2 Well diffusion assay

Well diffusion is also a similar methodology to that of disk diffusion meaning it has many of the same advantages including being a rapid, low cost procedure with a high sample throughput capability and ease of interpretation of results, since it has been found to have good correlation to that of the reference CLSI microdilution test (386). However, since the well diffusion assay has a very similar procedure to that of disk diffusion the same problems were encountered when it came to adjusting the fungi to the McFarland scale and once again resulted in the production of an uneven lawn of growth. In order to overcome this modifications to the method were investigated which involved inoculating a plate of malt extract agar and incubating it for a week at 20 °C so as to achieve an even lawn of growth. After the incubation time had elapsed, holes 6 mm in diameter were bored into the plate, three for the extracts, one for the negative control and one for the positive control as shown in Figure 3.6.

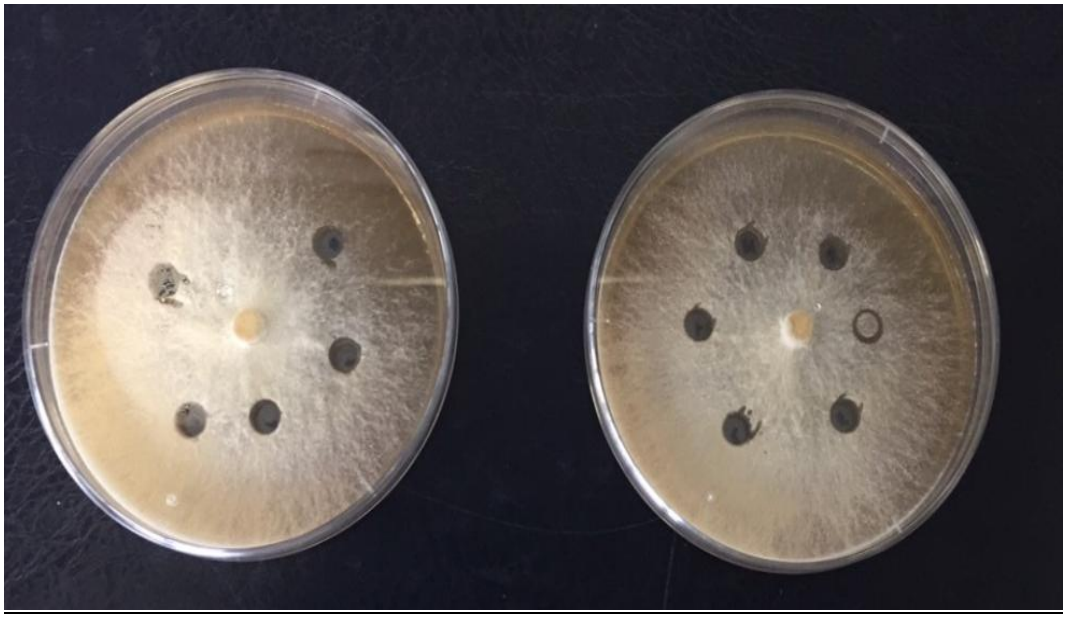


Figure 3.6: Modified well diffusion assay which achieved an even lawn of growth and the 6 mm diameter wells in order to determine the antifungal activity of *U. lactuca* against *H. annosum*. Data (n=3).

The extracts were solvated in ethanol to give a final concentration of 5 mg/mL with 50 μL added to each well, with ethanol serving as the negative control and cycloheximide serving as the positive control. The plates were incubated at 20 °C and checked every 48 h for zones of inhibition over a two week period, Figure 3.7.

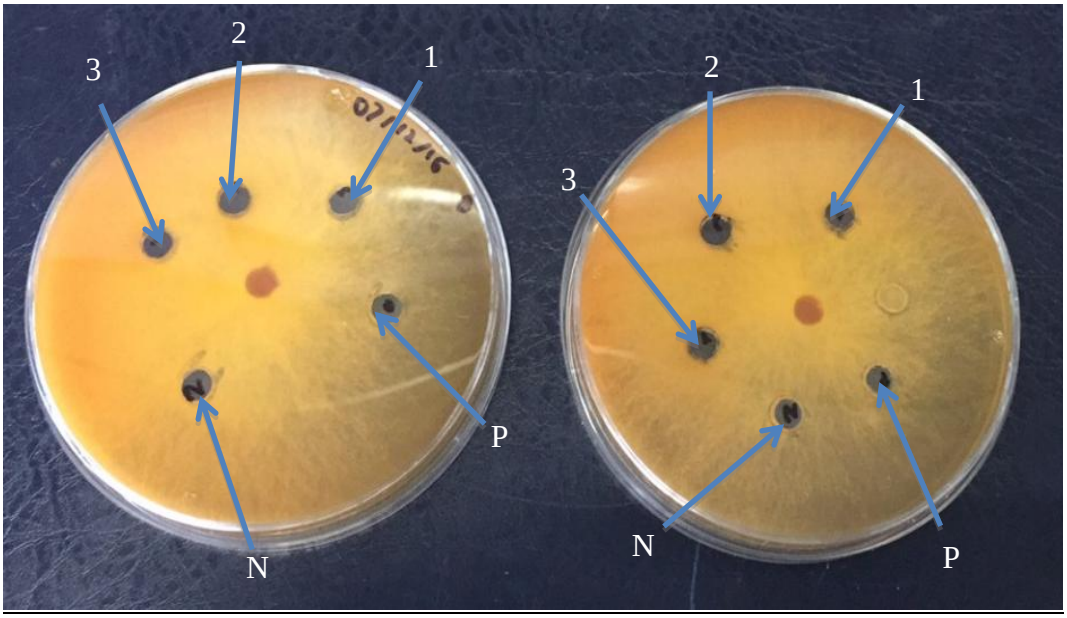


Figure 3.7: Antifungal activity of crude methanol extracts of *U. lactuca* against *H. annosum* via a modified well diffusion assay at 5 mg/mL. Positive control: 50 µg/mL cycloheximide; Negative control: 50 µL of ethanol. (Each extract triplicate is labelled 1, 2 and 3, negative control labelled N and positive control labelled P on each plate). Data (n=3).

From examination of Figure 3.7, it can be seen that the experiment proved unsuccessful, not only because no zone of inhibition was observed around the extract filled wells but also because the method could not be validated since the positive control did not work (no zone of inhibition was found). This once again came back to the fact that the McFarland scale could not be achieved and for this reason well diffusion assays as an antifungal susceptibility tests are generally carried out on yeast or mould strains as they can achieve this McFarland scale. For example, Magaldi *et al.* investigated the effectiveness of a number of antifungal agents such as fluconazole against isolates of *Candida* spp. a common yeast infection through the use of well diffusion (386). Well diffusion was found to be a simple, low cost method that demonstrated its potential to test for antifungal drug susceptibility towards *Candida* spp. in laboratories with few resources. This antifungal test method was also employed in studies that investigated the antifungal activity of seaweed, but once again this was generally against yeast and/or mould strains. For instance, Aruna *et al*. investigated the antifungal properties of six seaweeds against a selection of yeast and mould strains through the use of the well

diffusion assay (85). The assay effectively determined that aqueous extracts of *U. lactuca* had strong activity against the mould *Aspergillus flavus* with an inhibition zone of 56 mm at a concentration of 200 mg. However, in this study all of the extracts including the methanol extracts were re-solvated in sterile water which ensured complete diffusion into the agar medium. Although ethanol is polar in nature, an attempt was made to re-solvate the methanol extracts in water as opposed to ethanol to determine whether that was the reason as to why no inhibition was observed, but this also proved inconclusive and most likely was a consequence of water being an ineffective solvating solvent. Therefore, the well diffusion assay would also not be suitable for assessing the antifungal activity of seaweed extracts against *H. annosum*.

3.3.1.3 Broth dilution assay

Broth dilution assay was the next technique to be investigated and had the added bonus over both disk and well diffusion assays in that it can quantitatively measure the *in vitro* antimicrobial activity against a fungal organism (397). This assay is capable of determining the MIC_{90} and MIC_{50} of an extract, which means the concentration of extract that results in 90% and 50% inhibition respectively of visible growth of the test microorganism. Also this *in vitro* method has been standardised by CLSI and EUCAST for filamentous fungi meaning there are now established quality control parameters and interpretative breakpoints (397). In addition to this, fungal species that aren't under this standardised protocol, such as dermatophytes, have been studied with different modifications or adaptions made to the CLSI methods such as Butty *et al*. (410) and Fernández-Torres *et al*. (411) with success.

Therefore, this method appeared more promising than the previous two which required adjustment to the McFarland scale. An initial "scouting" experiment was conducted which involved inoculating malt extract broth with a known concentration of *H. annosum* and it was found that after 5 days at 110 RPM and at a temperature of 20 °C growth was observed. This, therefore, meant that the broth and growth conditions were suitable for *H. annosum* and led to a second "scouting" experiment which involved the introduction of crude *U. lactuca* extracts solvated in ethanol into the pre-inoculated malt extract broth whilst ethanol alone served as the negative control and cycloheximide (50 μg/mL) served as the positive control. Unfortunately no growth was observed in any

inoculum tube even for the negative control. The lack of growth was confirmed to be due to the presence of ethanol as the media only tube demonstrated strong fungal growth after 5 days of shaking incubation. This was not expected since ethanol was used as the solvating solvent for the poisoned food technique and the negative controls growth was never affected by the solvents presence. In addition to this a number of studies also used organic solvents to solvate their extracts such as Kubanek *et al*. (336) using the solvent acetone and Puglisi *et al*. (412) with methanol. This negative affect on growth may have been due to the initial stages of fungal infection being easily affected by environmental changes such as the presence of an organic solvent or the fact that in the poisoned food technique the ethanol would have been incorporated into the agar compared to broth dilution where the ethanol is present in the liquid broth i.e. in direct contact with the fungus. Water was also tried but once again lacked the ability to dissolve the desired bioactive(s) leading to growth in all tubes containing the extract and additionally, water is unable to sterilise the extracts leading to potential growth from other microbes making this method non-feasible for antifungal assessment of the methanol extracts.

3.3.1.4 Agar plate test

The agar plate test was described by Alfredsen *et al.* who investigated the antifungal activity of bark extracts from European tree species, both decidous and coniferous, against *H. annosum* and *H. parviporum* (398). Due to the similarities between this study and the aim of this body of work, the agar plate test appeared very promising. This method had properties of disk diffusion in which a large filter paper was loaded with extract and allowed to diffuse through the agar with the aim of inhibiting the growth of the test microorganism. Adjustment of the fungi was not required and instead a plug of fungi was added to the center of the agar meaning the issue surrounding an even lawn of growth was avoided. However, since the extract had to diffuse through the agar in order to come in contact with *H. annosum* the volume of agar had to be low with 2 mL of agar chosen.

The ethanol was allowed to evaporate off prior to the introduction of the fungi allowing a higher volume of ethanol to be used, in comparison to the poisoned food technique, which was restricted to 200 μL, as identified in section 2.3.5. Another advantage of this

method is that since the extract is placed on a disk it is not directly diluted by the agar, leading to less extract being required. Therefore, in this study a 10 mg/per disk extract was used to ensure a sufficient amount of extract diffused up through the agar whilst accounting for possible diffusion issues. But even with this increased concentration it required a lot less dried extract compared to the poisoned food technique which required 25 mg of dried extract in order to achieve a 5 mg/disk concentration. Table 3.1 illustrates the inhibition achieved with a 2 mL volume of agar compared to the negative control.

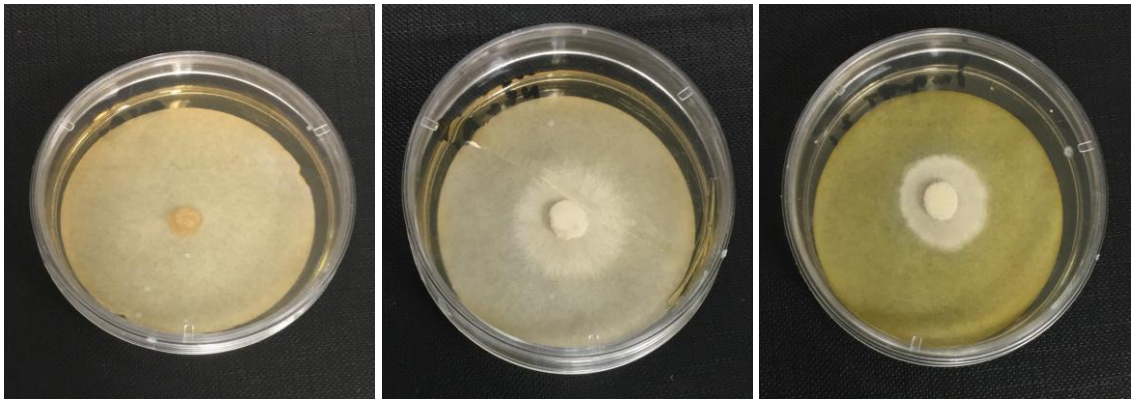
Table 3.1: The effectiveness of the agar plate test at various agar volumes in assessing the antifungal activity of the methanol extract of *U. lactuca* against *H. annosum* at 5 mg/mL compared to the negative control (2 mL = 10 mg, 4 mL = 20 mg and 5 mL = 25 mg of dried extract all dissolved in 250 µL of ethanol).

Volume of malt extract agar	Percentage inhibition (%)	Positive control (mm)	Negative control (mm)
2 mL	18.8 ± 0.75^{a}	0	25
4 mL	27.0 ± 3.82^{b}	0	49
5 mL	14.0 ± 5.30^{b}	0	50

Positive control was cycloheximide solution (50 µg/mL); Negative control was 250 µL of ethanol. Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different (ρ<0.05; One-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test). The percentage inhibition was calculated using Equation 2.2 (100).

From examination of Table 3.1 it can be seen that activity appeared weak with only 18.8 ± 0.75% inhibition compared to the negative control. However, this was not as a result of the poor antifungal ability of the extracts, since the same extracts were tested using the poisoned food technique in section 2.3.4 and demonstrated a 50 ± 9.2% inhibition. Hence, this poor inhibition was as a consequence of the antifungal test method itself with reason to believe this was due to the low volume of agar lacking the nutrients

required for efficient fungal growth. Figure 3.8 illustrates this antifungal activity where it can be clearly seen that both the negative control and inoculated plate exhibited a small diameter of radial growth.



Positive control **Negative control** **Extract soaked filter paper**

Figure 3.8: Antifungal activity of the crude methanol extract of *U. lactuca* against *H. annosum* at 5 mg/disk using the agar plate test with a 2 mL volume of malt extract agar. Positive control: 50 μg/disk cycloheximide; Negative control: 250 μL of ethanol. Data (n=3).

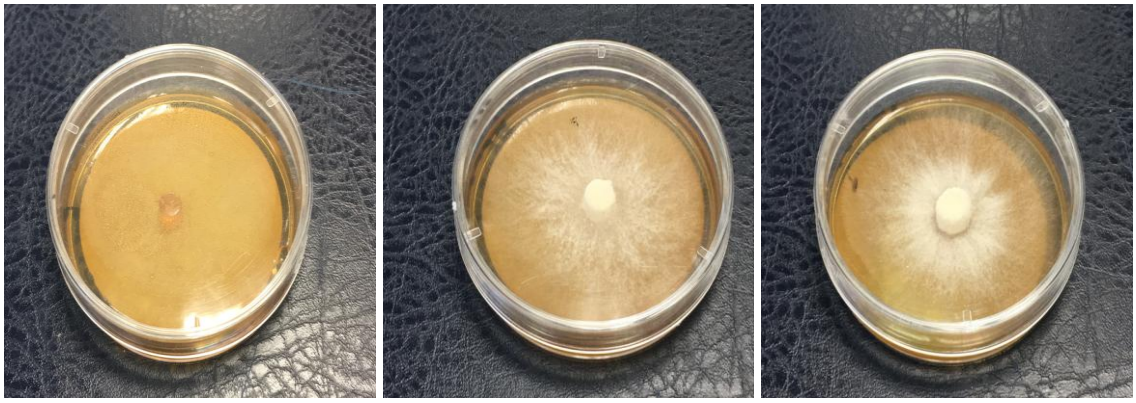
Based on these results an investigation into the effect of agar volume on fungal growth was conducted. It involved various volumes of malt extract agar being poured over blank filter papers which were then inoculated with a plug of *H. annosum*. The radial growth of each plate was recorded after a week of incubation with Table 3.2 outlining the results obtained.

Table 3.2: Investigation into the optimum volume of malt extract agar to allow for sufficient growth of *H. annosum*.

Volume of malt extract agar	Radial growth of *H. annosum* (mm)
2 mL	15.0 ± 1.00^{a}
3 mL	23.0 ± 1.52^{b}
4 mL	37.8 ± 0.58^{c}
5 mL	40.3 ± 4.04^{c}

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

The results from this experiment found that only 4 or 5 mL of media was capable of supplying *H. annosum* with the nutrients necessary for efficient growth. Therefore, the agar plate test was repeated again under the same conditions except this time the volume of agar was increased to 4 and 5 mL of malt extract agar. Table 3.1 summarises the results obtained for each of these volumes. It appeared that the volume of agar was too much for the extract to diffuse through the agar and affect the growth of *H. annosum* with a % inhibition of only 27 ± 3.82% and 14 ± 5.30% for the 4 and 5 mL media, respectively. This can be further seen in Figure 3.9 which illustrates the growth of both the negative control and inoculated plate consisting of a 5 mL volume of malt extract agar.



Positive control **Negative control** **Extract soaked filter paper**

Figure 3.9: Antifungal activity of crude methanol extract of *U. lactuca* against *H. annosum* at 5 mg/disk using the agar plate test with a 5 mL volume of malt extract agar. Positive control: 50 μg/disk cycloheximide. Negative control: 250 μL of ethanol. Data (n=3).

It can be seen that the extract soaked plate demonstrates a very similar degree of radial growth to that of the negative control and hence, confirms the inability of the extract to diffuse through the now larger volume of media and inhibit the growth of *H. annosum*. Although the agar plate test appeared promising at first due to the similarities with the Alfredsen *et al.* (398) study, diffusion problems associated with the crude extracts proved to be fundamental and resulted in the inability of this test method to effectively assess the antifungal activity of *U. lactuca* extracts. However, the agar plate test is a novel test method with no other literature describing such a method other than Alfredsen *et al.* (398), making it a very novel technique with potential as an antifungal susceptibility test particularly as it does not require fungal adjustment.

Overall the four antifungal test methods investigated in this study proved to be incapable of evaluating the antifungal capacity of the crude *U. lactuca* extract to the same degree as the poisoned food technique due to issues around colony adjustment and agar diffusion. Therefore, explaining why the poisoned food technique is the method of choice in so many studies assessing the antifungal effects of natural products including seaweeds against common fungal pathogens (124, 413, 414). For example, Ambika *et al*. evaluated the antifungal activity of *Sargassum myricocystum* and *Gracilaria edulis* against the mycelial growth of the fungal pathogen *Colletotrichum falcatum* with

aqueous and ethanol extracts at a concentration of 5% and 10% (126). The poisoned food technique was the test method used and revealed that the ethanol extract of *S. myricocystum* at 10% had significant activity against *C. falcatum* with a mycelial growth of 52 mm compared to the negative control which recorded a growth of 90 mm. Studies have investigated and compared different antifungal test methods (161, 415). For example, Hadacek *et al.* investigated various dilution and diffusion bioassays in determining the antifungal activity of two antifungal natural products against three selected microfungi (416). Microdilution was found to exhibit the greatest potential of all the tested bioassays since it allows for high sample throughput, direct detection of morphological anomalies, detection of concentration response effects by regression analysis and determination of MIC values. However, the choice of methods are generally restricted by the properties of the bioactives, fungal species, cost or simply from a lack of expertise or equipment.

3.3.2 Protectant activity of the crude extract on wood samples

In this section of work the ability of the crude extract to protect disks of Sitka spruce from *H. annosum* infection was evaluated. This method was based on work by Aguín *et al*. who investigated the protective activity of a number of fungicides against the root rot disease *Armillaria mellea* using fragments of hazel (*Corylus avellana* L.) (399). This method had to be initially developed to specifically suit *H. annosum*. Therefore, the first objective was to determine whether the disks had to undergo sterilisation via autoclaving as in the work by Aguín *et al*. or could be left as they were. It was feared that sterilisation would prevent/reduce the ability of this wound pathogen to infect the disks as its basidispores/conidiophores would be too small and low in nutrients to penetrate a hardened layer that may be formed during the autoclaving process (417). However on the other hand, by not conducting a method of sterilisation would allow for natural infection by *H. annosum* but also from other microbes that were already present on the disks or picked up during transportation. The second part of this development plan was to determine the optimum exposure time for the disks in order to achieve infection specifically on untreated disks. Therefore, a set of disks both autoclaved and un-autoclaved was subjected to one or two week exposure to *H. annosum*.

It was established that sterilisation of the disks was essential as shown in Figure 3.10. High levels of contamination arose from other microbes in the absence of sterilisation, specifically for the positive control which was only soaked in an aqueous solution of cycloheximide. The level of contamination in the negative control and the extract soaked disks were less severe, most likely caused by the ethanol surface sterilising the disks.

Positive control **Negative control** **Extract soaked disk**

Figure 3.10: Protectant activity of the crude methanol extract of *U. lactuca* against *H. annosum* at 5 mg/mL after one week of exposure to un-autoclaved disks of Sitka spruce. Positive control: 50 μg/mL cycloheximide; Negative control: 5 mL of ethanol. Data (n=3).

The autoclaved disks, which demonstrated no form of contamination still allowed for colonisation by *H. annosum* (Figure 3.11). Additionally, one-week of exposure to *H. annosum* via the sandwich technique was found to be sufficient for the disks to become infected. The infection observed on the negative control confirmed that the sterilisation process had not altered the chemical and physical features of the disk, thus preventing *H. annosum* infection. Furthermore, microscopic examination of the culture morphology confirmed it was *H. annosum* with a consistent anamorph stage and no contamination observed (400, 401). Therefore, an effective validated method had been developed capable of assessing the protectant activity of *U. lactuca* on wood samples against *H. annosum ex vivo*.

Positive control **Negative control** **Extract soaked disk**

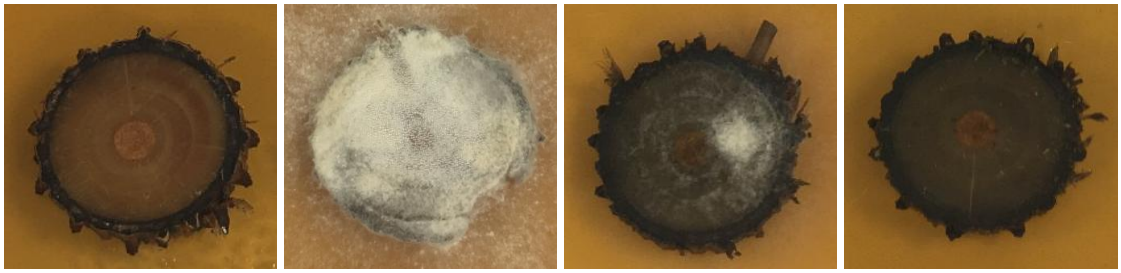
Figure 3.11: Protectant activity of the crude methanol extract of *U. lactuca* against *H. annosum* at 5 mg/mL after one week of exposure to autoclaved disks of Sitka spruce at 20 °C for two weeks. Positive control: 50 µg/mL cycloheximide; Negative control: 5 mL of ethanol. Data (n=3).

Unfortunately Figure 3.11 also showed that the 5 mg/mL extract concentration demonstrated no protectant activity after 3 h of soaking. However, since the solvating solvent was allowed to evaporate off prior to exposure to *H. annosum*, the amount of ethanol was not limited and for this reason the extract concentration was increased to 20 mg/mL for longer soaking times of 18 h, 24 h, 48 h and 72 h. Figure 3.12 illustrates the results obtained for the 18 h soaked disks after 1 week of incubation.

Positive control **Negative control** **Extract soaked disk**

Figure 3.12: Protectant activity of the crude methanol extract of *U. lactuca* against *H. annosum* at 20 mg/mL after 18 h of soaking and one week of exposure and incubation at 20 °C for one week. Positive control: 50 μg/mL cycloheximide. Negative control: 5 mL of ethanol. Data (n=3).

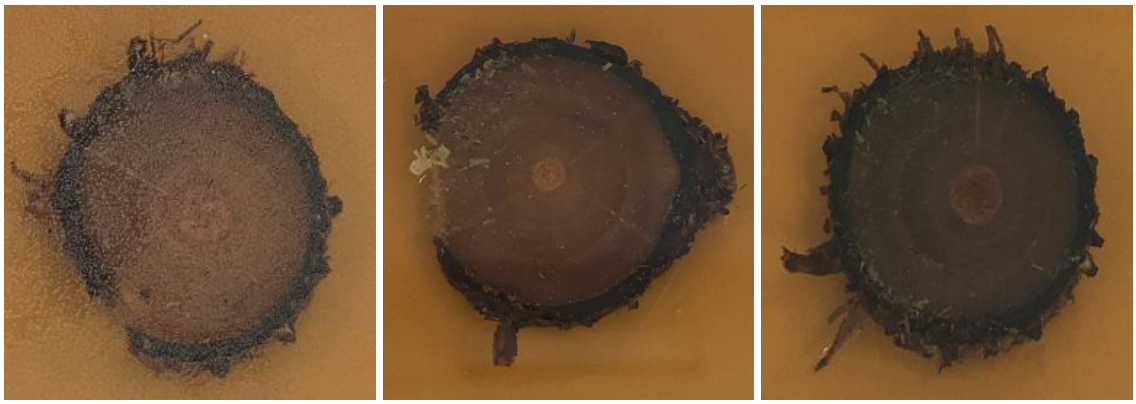
From examination of Figure 3.12, it can be seen that after a week of incubation, the crude extracts at 20 mg/mL appeared to exhibit strong protectant activity with the disks appearing free from infection compared to the negative control which illustrated the initial pattern of *H. annosum* infection. Figure 3.13 shows the results obtained a week later. From examination of these images it can be seen that the negative control's infection had increased as expected with only a small degree of infection observed on some of the extract soaked disks with others even remaining infection free. The 24 h soaked disks also illustrated similar bioprotectant capabilities.



Positive control **Negative control** **Extract soaked disk (Small degree of infection)** **Extract soaked disk (No infection)**

Figure 3.13: Protectant activity of crude methanol extracts of *U. lactuca* against *H. annosum* at 20 mg/mL after 18 h of soaking and one week of exposure and incubation at 20 °C for two weeks. Positive control: 50 µg/mL cycloheximide; Negative control: 5 mL of ethanol. Data (n=3).

However, the 48 h and 72 h soaked disks remarkably illustrated no infection, including the negative control, as shown in Figure 3.14.



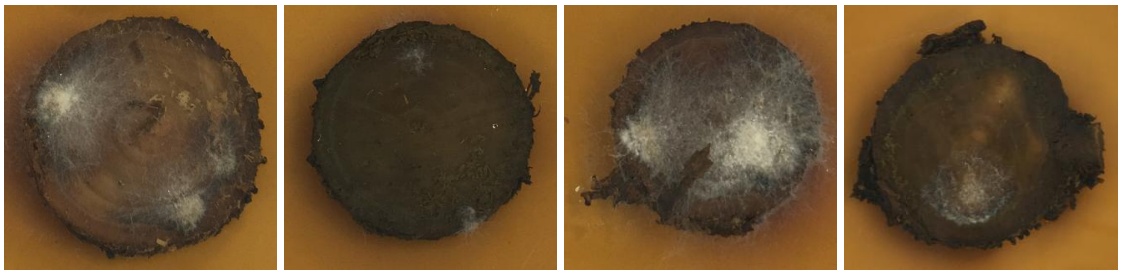
Positive control **Negative control** **Extract soaked disk**

Figure 3.14: Protectant activity of the crude methanol extract of *U. lactuca* against *H. annosum* at 20 mg/mL after 48 h of soaking and one week of exposure and incubation at 20 °C for two weeks. Positive control: 50 µg/mL cycloheximide; Negative control: 5 mL of ethanol. Data (n=3).

This unusual result is suspected to be as a result of the disks forming a hardened layer over the extended soaking period and due to the fact that *H. annosum* as a wound pathogen can only infect an exposed tree as its basidispores/conidiophores are too small and low in nutrients to penetrate the bark layer of the tree (417). For this reason, freshly cut trees are the most vulnerable and as a result of this the PPP urea is sprayed immediately over tree stumps (418). Over time these stumps form a hardened layer via the sap present in the stump, which is also capable of preventing infection and it is believed that this layer formed over the 48 h and 72 h disks resulting in no infection being observed. It must also be noted that throughout these experiments disks that were only autoclaved and exposed to *H. annosum* were also assessed to ensure that any contact with solvent either organic or aqueous didn't affect this infection process. These disks for the 48 h and 72 h experiment also demonstrated no infection and further confirmed this formation of a hardened layer.

Overall these experiments facilitated the development of an effective method which found that a concentration of 20 mg/mL and soaking period between 18 h and 24 h had a strong protectant activity. The key to this success was believed to be as a result of the increased extract concentration which was four times stronger than that of the original (5 mg/mL). These high concentrations have also been used in other studies such as Aruna *et al.* who investigated the antifungal activity of six seaweeds against *Aspergillus* spp. (85). The extract concentrations used by Aruna *et al.* were 50, 100 and 200 mg, with the well diffusion assay effectively determining that the aqueous extracts of *U. lactuca* had strong activity against *Aspergillus flavus* with an inhibition zone of 56 mm at a concentration of 200 mg (85).

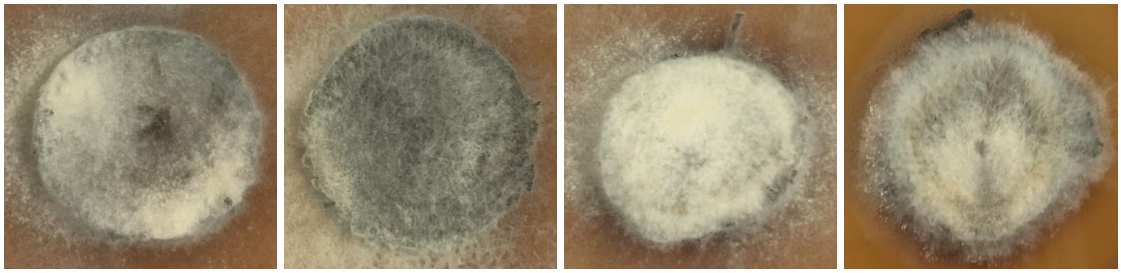
The soaking time was also found to be a contributing factor with 48 h and 72 h leading to no infection at all. However, since the freshly cut stumps must be sprayed immediately after felling in the forestry industry, as this is when the stump is most vulnerable to infection, the shorter soaking times would achieve such a requirement. In order to determine the full potential of the extracts, much shorter times were investigated including 0 h (no soaking period – disk introduced into the extract solution and removed immediately), 1 h, 3 h and 6 h at 20 mg/mL. It was determined that all of the shorter soaking periods demonstrated the same degree of protectant activity against *H. annosum* after 1 week of incubation as shown in Figure 3.15.



0 h Negative **0 h Extract** **6 h Negative** **6 h Extract**

Figure 3.15: The protectant activity of crude methanol extracts of *U. lactuca* against *H. annosum* at 20 mg/mL after 0 h and 6 h of soaking and incubation at 20 °C for one week. Negative control: 5 mL of ethanol. Data (n=3).

Unfortunately, this degree of protection diminished after another week of incubation as demonstrated in Figure 3.16 with both the 0 h and 6 h extract soaked disks illustrating the same degree of infection as their corresponding negative controls.



0 h Negative **0 h Extract** **6 h Negative** **6 h Extract**

Figure 3.16: The protectant activity of crude methanol extracts of *U. lactuca* against *H. annosum* at 20 mg/mL after 0 h and 6 h of soaking and incubation at 20 °C for two weeks. Negative control: 5 mL of ethanol. Data (n=3).

This, therefore, meant that shortening the soaking times in turn reduced the protectant activity of *U. lactuca* extracts with activity only being observed for up to one week after exposure and after which activity fell dramatically. Thus, 18 h or 24 h soaking periods at 20 mg/mL with crude extracts exhibited the optimum protectant activity against *H.*

annosum. This method could be transferred to a forestry setting with soaked/sprayed disks placed in a surrounding felling site for a set timeline before returning to the lab for analysis in order assess the degree of protectant activity specifically against *H. annosum* infection through morphology evaluation.

3.3.3 Comparison of the crude extracts effectiveness to a commercial product

The most commonly used chemical agent against *H. annosum* infection through airborne basidiospores/conidiophores is the licensed PPP urea. As mentioned previously, the EU approved concentration of urea is 20% *w/v* for manual application and 37% *w/v* for harvester application (279). Therefore in order to compare the competitive efficiency of the extracts to this commercial product, various concentrations of urea were prepared; 0.05% to 20% *w/v* (to align with manual application) and tested for activity using the poisoned food technique. Unfortunately, the media did not solidify above a concentration of 5% *w/v* and this was due to the acidic nature of urea resulting in a lowering of the pH. In this acid pH range it causes the hydrolysis (breakdown) of the agarose polysaccharides, the units that bind together to form the agar. Therefore, the effect of urea on the growth of *H. annosum* was investigated within the new range of 0.05% to 5% *w/v* as demonstrated in Table 3.3.

Table 3.3: Concentration study of urea at 0.05% - 5% *w/v* using the poisoned food technique against *H. annosum*.

Concentration of urea % (*w/v*)	Concentration of urea (mg/mL)	Percentage inhibition (%)
0.05	0.5	17.0 ± 1.32^{a}
0.10	1	42.0 ± 2.65^{b}
0.15	1.5	74.1 ± 0.58^{c}
0.20	2	92.3 ± 0.76^{d}
0.25	2.5	100.0 ± 0^{e}
0.50	5	100.0 ± 0^{e}
0.75	7.5	100.0 ± 0^{e}
1.00	10	100.0 ± 0^{e}
5.00	50	100.0 ± 0^{e}

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

Table 3.3 also includes the concentrations in mg/mL to allow for direct comparison to that of the concentrations used in the screen study (Chapter 2). Although a maximum concentration of only 5% *w/v* could be tested, it still showed the extreme effectiveness of urea in preventing the growth of *H. annosum in vitro*. For instance, 100% inhibition was achieved using 2.5 mg/mL which is half the concentration used in the *U. lactuca* extracts experiments which achieved 50% inhibition. The difference in activity between urea and the *U. lactuca* extracts could lie in the fact that these extracts are crude with studies showing that purification of crude extracts typically has a positive effect on activity (419, 420). Since most of the non-active compounds would be removed including antagonistic compounds.

Although strong activity by urea was observed at these low concentrations, the currently approved much higher concentrations were selected after concentration studies completed in field trials. Nicolotti *et al.* investigated the effectiveness of various concentrations of urea against *Heterobasidion* airborne infections on Norway spruce

stumps in four forests in the Western Alps (421). It was reported that the capability of urea greatly depended on its concentration with little control observed by the 10% concentration, with higher concentrations such as 30% required for significant decrease in *Heterobasidion* infections. These high concentrations are needed to maintain the high pH values for the duration of time in which the stumps are susceptible, which is estimated to be approximately one month (422, 423).

Although the *U. lactuca* extract did not demonstrate the same level of activity as the urea, it did compete favourably for a crude product. But to allow for more realistic comparison, the urea was also tested on disks of Stika spruce. The 10% *w/v* and 20% *w/v* urea soaked disks exhibited strong protectant activity against *H. annosum* with no visual signs of mycelia growth; infection was found to be dose dependent (Figure 3.17). The disks were once again checked for purity using microscopic examination of the culture morphology to confirm it was *H. annosum* and free from other forms of possible contaminates including wood fungi (400, 401).

20% *w/v* **10% *w/v*** **1% *w/v*** **Negative control**

Figure 3.17: Effect of urea at various concentrations in controlling *H. annosum* infection on Sitka spruce disks after 18 h of soaking and incubation at 20 °C for two weeks. Negative control: 5 mL of sterile deionised water. Data (n=3).

Comparing Figure 3.13 and Figure 3.17, the methanol extract of *U. lactuca* exhibited very similar activity to the 10% (100 mg/mL) and 20% *w/v* (200 mg/mL) concentrations of urea, demonstrating its potential to be used as a biopesticide and also its comparability with the currently available product used to control this plant pathogen on Sitka spruce. However, the possible mode of action of urea against *Heterobasidion* spp. on conifers was elucidated by Johansson *et al.* who concluded that enzymes present in the wood itself hydrolysed the urea into ammonia causing a rise in pH above the

thershold for *H. annosum* to germinate and mycelia to survive (424). Therefore, since these disks had been previously autoclaved, such enzymes would have been denatured preventing hydrolysis. Figure 3.17 demonstrated a heavily infected negative control compared to the urea soaked disks indicating some degree of activity potentially as a result of the toxicity from urea itself or urea derviative compounds including ammonia and ammonium ions. In order to determine a direct comparsion of the *U. lactuca* extracts to that of this commerical PPP it would require field trials in a natural forest setting on freshly cut stumps of Sitka spruce which still contained constitutive enzymes. Nonetheless, this was a novel result in itself since a literature review found no such studies with only a study by Sultana *et al.* who compared the comparative efficacy of a red seaweed *Solieria robusta* to the chemical fertiliser urea on the growth of soybean plants (329). The seaweed was found to have a slightly better effect on plant growth than urea by achieving taller plants, better root length and increased numbers of flowers per plant.

3.4 Conclusions

The poisoned food technique has a number of disadvantages as a test method including high amount of extract required, results take a week to be obtained and it is not accurate enough to determine an MIC, since it is impossible to quantify the amount of extract that has diffused into the media (100). An investigation into other antifungal test methods including the disk diffusion assay, well diffusion assay, broth dilution assay and agar plate test was conducted in order to determine whether a more efficient method could be developed for assessing the antifungal activity of the crude extracts against *H. annosum*. Unfortunately, none of the four methods investigated proved effective, with both disk diffusion and well diffusion struggling to achieve the "McFarland scale". The broth dilution assay was unsuccessful as a result of *H. annosum* being unable to grow in the presence of ethanol, the solvating solvent. The most promising technique was the agar plate test which has similar properties to that of the disk diffusion assay and the poisoned food technique, but the volume of the agar over the disk necessary for *H. annosum* to grow proved problematic with too low a volume of agar (2 mL) leading to poor growth of the fungi and percentage inhibition of just 18.8 ± 0.75% compared to the negative control. Although a larger volume of agar allowed for stronger growth of the fungi, activity fell as it reduced the ability of the extracts to diffuse through the agar to reach the plug of fungi and resulted in a % inhibition of 27 ± 3.82% and 14 ± 5.30% compared to the negative control for 4 mL and 5 mL volume of agar respectively. Therefore the optimum antifungal test method for *H. annosum* remains that of the poisoned food technique.

The second part of this study involved the development of a method to assess the protectant activity of the crude extracts against *H. annosum* on disks of Sitka spruce. A method was successfully developed that allowed this qualitative assessment and involved the use of autoclaved disks soaked in extract for a specific period of time before being dried and sandwiched to a pre-inoculated plate of *H. annosum* for one week at 20 °C. A crude extract concentration of 5 mg/mL was investigated with a 3 h soaking time, but it proved unsuccessful with strong growth observed on all extract soaked disks. However, an increased concentration of 20 mg/mL with a appropriately longer soaking time of 18 h and 24 h resulted in reduced infection with some disks demonstrating no growth at all even after two weeks of incubation.

A comparison study was conducted against the commercial PPP urea both *in vitro* using the poisoned food technique and *ex vivo* on disks of Sitka spruce. The extracts competed favourably with this commercial product considering these were crude samples, but unfortunately elucidation of urea's mode of action against *Heterobasidion* spp. on conifers confirmed that a direct comparison to this commercial product would require field trials.

Nevertheless, this study highlights a potential novel application of seaweeds as biopesticides which can be used to protect this commercially valuable tree species. This could also lead to a reduction in the use of urea with resultant environmental benefits. This potential application can be further verified through field trials on a Sitka spruce felling site by the methanol extracts of *U. lactuca* alone or in combination with the PPP urea.

As this study focused on a crude extract, purification and characterisation of the active compounds present in *U. lactuca* through Thin layer chromatography (TLC), semi-preparative-HPLC, GC-MS, NMR etc. will provide greater insight into its potential as a natural antifungal based product. An investigation also needs to be conducted into the mode of action of the active extracts against this fungal species using the sorbitol protection assay, ergosterol effect assay, potassium efflux assay and leakage of substances absorbing at 260 nm (425).

CHAPTER 4: ANTIBACTERIAL POTENTIAL OF SEAWEEDS AGAINST PLANT PATHOGENS

*All data in this chapter (except for the phytotoxicity data) have been published in the *Journal of Bacteriology and Mycology* (O' Keeffe, E., Hughes, H., McLoughlin, P., Tan, S. P. and McCarthy, N. (2019) Antibacterial activity of seaweed extracts against plant pathogenic bacteria. **6**(3): 1105).

4.1 Introduction

Bacterial plant pathogens can cause widespread devastation in the agricultural sector. This is a critically important sector to many economies including Ireland where in 2016, the agri-food sector generated €13.6 billion (426). Losses in crop yields can in turn affect food supply which is constantly under demand as a result of the growing human population (427). To combat this ever-growing issue, intensive applications of synthetic pesticides is used, but this current control strategy is losing its efficacy due to the development of resistance through biofilm formation which has been shown to be an attribute involved in bacterial resistance to copper bactericides (427) as reported for the phytopathogen *Xylella fastidiosa* (428). Pesticide-degrading microorganisms employ several mechanisms of resistance including membrane transport systems, efflux pump systems, production of enzymes and encoded catabolic genes for degradation with some microorganisms even inheriting this pesticide-degrading functional trait (429).

Additionally, the negative health effects associated with the use of these pesticides (427) has led to a well-recognized need for the development of eco-friendly control measures with the use of natural products receiving high interest, particularly marine algae due to their potential as a source of novel bioactive compounds that could aid in protecting plants against pathogenic microbes (219). Many seaweed species have been reported to exhibit a wide variety of biological activities including anti-inflammatory (40), antioxidant (34), antifouling (430) and antiviral (431). They also overcome other problems associated with synthetic pesticides such as exhibiting biodegradable properties, making them a potentially low environmental impact product (432) with a long history of safe use (433-435). In addition to this, pesticides derived from biological origin maybe less toxic and generally only affect the target pest and closely related species (436).

The antibacterial activity of seaweeds has been known for years with one of the first studies conducted by Vacca *et al.* who found that the brown seaweed, *Ascophyllum nodosum* exhibited a wide range of activity against both Gram positive and Gram negative bacteria such as *Bacillus subtilis* and *Escherichia coli* (437). These promising results have led to many more studies on the antibacterial activity of seaweeds (42, 56, 59, 438, 439). For example, Jaswir *et al*. demonstrated the antibacterial capacity of the four brown seaweeds *Sargassum plagyophillum*, *Sargassum flavellum*, *Padina australis*

and *Sargassum binderi* against *Staphylococcus aureus, Bacillus subtilis, E. coli* and *Pseudomonas aeruginosa* with the methanol extract of *S. plagyophillum* exhibiting the best yield (4.72%) and antimicrobial activity with inhibition zone of 12 mm reported against *B. subtilis* (440). Although a lot of literature is available on the antibacterial capacity of seaweeds against human pathogens, their bioactive potential against bacterial plant pathogens is a relatively new concept. Kulik was one of the first researchers who investigated the possible use of cyanobacteria and algae against plant pathogenic bacteria and fungi (222). Since then studies have been completed on a wide range of bacterial pathogens including *Xanthomonas* sp. (70), *Clavibacter michiganensis* subsp. *sepedonicus* (223) and *Pseudomonas syringae* (72). These studies showed very promising results since the majority of these pathogens are Gram-negative which are harder to kill because of their protective outer membrane (224). For instance, Paulert *et al*. studied the antibacterial activity of the green seaweed, *Ulva fasciata* against two Gram-negative plant pathogens *Xanthomonas campestris* and *Erwinia carotovora* (70). The methanol extracts exhibited activity against both pathogens with the highest activity against *E. carotovora* and a MIC of 1 mg/mL (70). These studies therefore highlight the potential of seaweeds as a novel source of biopesticides against bacterial pathogens.

The main objective of this chapter was to screen five species of seaweeds including *Fucus serratus*, *Fucus vesiculosus, Ascophyllum nodosum, Polysiphonia lanosa* and *Ulva lactuca* that were collected from the Irish coast for antibacterial activity against nine quarantine plant pathogens. The bacterial plant pathogens were supplied by the DAFM with Table 1.7 in Chapter 1 compiling the information regarding each pathogen's hosts, signs of infection and current control methods. The main problem with these pathogens is the lack of effective treatment methods resulting in critical impacts on a wide range of crops and trees worldwide. This in turn results in significant economic losses with bacterial diseases of plants estimated to cause losses of over $1 billion worldwide to the food production chain annually (441).

This need formed the basis of this screen study as an initial study in Chapter 2 found that the methanol extract of *Ulva lactuca* exhibited strong antifungal activity against the root rot pathogen *H. annosum*. As with antifungal activity, there are similar factors that influence the antibacterial capacity of seaweeds including; reproductive state, geographical location and seasonality. Arunkumar *et al*. demonstrated higher activity

against *Xanthomonas axonopodis* pv. *citri* from seaweeds collected in post-monsoon seasons (February) compared to seaweeds collected in summer, (May) pre-monsoon (August) and monsoon season (November) (436). Therefore, antibacterial activity can be maximized by manipulation of the time of seaweed harvesting. Other than environmental factors, the selection of extracting solvents has a major influence on the activity observed and, therefore, requires important consideration. The four seaweed species used in this investigation were used in Chapter 2 with the addition of *F. vesiculosus* and were also selected on the basis of being indigenous to the South-East coast of Ireland and had demonstrated antibacterial activity in previous studies (384, 439, 442-445). The solvents water, methanol, ethanol and acetone were also used in Chapter 2 and were applied again here as they ranged in polarity and so aided in determining the seaweed with the best antibacterial activity. In addition to this, these solvents have shown their effectiveness in extracting antibacterial compounds in previous studies (438, 446, 447).

For instance Borbón *et al.* investigated the antimicrobial potential of ethanol and acetone extracts from 13 marine algae with the acetone extracts of *Sargassum polyceratium* illustrating strong activity against the plant pathogen *E. carotovora* (448). The ethanol extracts of a number of seaweeds were active against *S. aureus*. However, in general methanol appears to be the best extraction solvent with Kumar *et al*. completing an antimicrobial screen on 12 different seaweeds using a number of different solvents including petroleum ether, methanol, ethyl acetate, chloroform, water and acetone against the phytopathogenic bacterium *P. syringae* (72). The methanol extracts exhibited the strongest activity, particularly for the brown seaweed *S. wightii* which demonstrated a greater zone of inhibition of 21.00 ± 0.67 mm compared to the positive control (100 µg tetracycline) of 12.67 ± 0.33 mm (72). Esserti *et al.* found that aqueous extracts of *Cystoseira myriophylloides* and *Fucus spiralis* significantly reduced crown gall disease caused by the plant bacterial pathogen *Agrobacterium tumefaciens* (46).

In this study a relatively low extract concentration (1:50 *w/v*) was generated for all extracts as in Chapter 2 to ensure all the bioactives were extracted and were then tested against the three Gram-positive and six Gram-negative pathogens via the disk diffusion assay. Once the extracts with the best antibacterial activity were identified, the next step was to determine their antimicrobial efficacy. This can be achieved in a number of ways

including the determination of the MIC value for that particular extract. MIC is defined as the lowest concentration of the antimicrobial compound that will inhibit the visible growth of the bacteria after overnight incubation (449). However, this concentration may not actually kill the bacteria and once the compound is removed the bacteria will start to grow again. Therefore, a simple test following MIC can be carried out known as the MBC which determines the lowest concentration of the antimicrobial compound that will prevent (≥99%) the growth of the bacteria when sub-cultured on fresh media (449). MIC and MBC values are important with regards to administration of the correct dosage of the antimicrobial agent that can fully inhibit/kill the bacteria and ensure the correct concentration of extract is used to achieve control over a certain plant pathogen.

This study also aimed to determine the antibiofilm potential of the crude extracts since a major issue with the effectiveness of antibacterial agents is the development of exopolysaccharide biofilms by bacteria (450). Bacteria produce biofilms for a multitude of reasons and these biofilm-growing bacteria can cause chronic infection due to increased resistance to antibacterial agents and disinfectant chemicals. This is particularly prevalent in the healthcare industry where researchers have estimated that 60 – 80% of microbial infections in the body are caused by biofilm-forming bacteria compared to planktonic (free moving) bacteria (146). Bacterial plant pathogens also have the ability to form biofilms, with all nine bacterial pathogens tested in this body of work exhibiting this property. Therefore, for a biopesticide to be effective against bacterial pathogens, it must exhibit antibiofilm activity. This assessment was completed via two assays; biofilm prevention and biofilm disruption based on colony counts.

It is also critical to establish the safety of the seaweed extracts to the plant itself and also non-target species. This is typically achieved through a phytotoxicity study. Assessment of relative phytotoxicity involves a number of studies on both the whole/part of the plant (*in vivo*) and at cell level (*in vitro*) using a variety of concentrations of the test compound to ensure precise evaluation. A method to examine the phytotoxic effect at the cellular level is through the use of a suspension cell line that is treated with the compound and the rate of cell viability is compared to that of a control cell line (176). In this study, the phytotoxicity of the methanol extract of *P. lanosa* was determined using the *in vivo* root hair assay on the model plant species, *Arabidopsis thaliana* (L.) Heynh. *A. thaliana* is the premier model for plant biology for a number of reasons including the availability of its entire genome sequence allowing for the quantitative analysis of

proteomics (proteins) which can change in abundance, form, location or activity in response to alterations in environmental conditions (451, 452). In addition to this, *A. thaliana* exhibits a rapid life cycle with germination to mature seed in roughly 6 weeks with abundant seed production through self-pollination. It can also be easily cultivated in a minimum space making this plant very suitable for phytotoxicity studies, particularly in a laboratory setting (453).

4.2 Experimental Procedure

4.2.1 Seaweed collection and production of crude extracts

The four seaweed species, *Fucus serratus*, *Ascophyllum nodosum*, *Polysiphonia lanosa* and *Ulva lactuca* used in Chapter 2 were also applied in this screen study. Another *Fucus* species known as *Fucus vesiculosus* was also included. The seaweed species were harvested from Fethard-On-Sea, Co. Wexford, Ireland in June 2017. The seaweeds were processed and powdered to 850 μm as described in section 2.2.1. The seaweeds were then extracted in four solvents of increasing polarity; acetone (99.8%), ethanol (96%), methanol (99.8%) and deionised water, except for *F. vesiculosus* which was only extracted in water. The production of the crude dried extracts was achieved via rotary evaporation for the organic extracts and freeze-drying was utilised for the aqueous extracts as outlined in section 2.2.2. The % yield for each extraction was calculated using Equation 2.1. All extractions were completed in triplicate.

4.2.2 Bacteria stocking and growth conditions

The quarantine bacterial species *Xanthomonas arboricola* pv. *Pruni* ATCC 19316, *Xanthomonas hyacinthi* (Wakker) Vauterin *et al*. ATCC 19314, *Clavibacter michiganensis* subsp. *nebraskensis* (Vidaver and Mandel) Davis *et al*. ATCC 27794, *Clavibacter michiganensis* subsp. *michiganensis* Davis *et al*. ATCC 7433, *Clavibacter michiganensis* subsp. *tessellarius* (Carlson and Vidaver) Davis *et al*. ATCC 33566, *Xanthomonas fragariae* Kennedy and King emend. Van den Mooter and Swings ATCC 33239, *Xanthomonas campestris* (Pammel) Dowson pathovar *uppalii* ATCC 11641, *Ralstonia* sp., and *Erwinia amylovora* (Burrill) Winslow *et al*. were supplied by the DAFM. The cultures were stocked in sterile broth containing 25% glycerol and stored at -20 °C. A master stock of each bacterium was stored at -80 °C. Table 4.1 presents details on each strains particular sub-group and growing conditions.

Health and Safety: It must be noted that since these bacterial strains are regarded as international quarantine pathogens all work was conducted in the pathogen lab at WIT within a Class II laminar flow cabinet. Benches and surfaces were sterilised with 70% ethanol before and after laboratory work to avoid cross contamination. All consumables

including plates, spreaders, pipette tips, etc and the cultures themselves used were autoclaved under the waste cycle (30 min at 121 °C) and disposed of in the general waste bin.

Table 4.1: Bacterial strains and their respective incubation temperatures and growth media.

Bacteria	Gram positive/negative	Incubation temp (°C)	Growth media	Media information
X. arboricola	Gram negative	26 °C	Nutrient agar	Lennox: Biolab Zrt., Budapest, Hungary (Product code: CXBLNUA20500)
X. hyacinthi	Gram negative	26 °C	Nutrient agar	Lennox: Biolab Zrt., Budapest, Hungary (Product code: CXBLNUA20500)
E. amylovora	Gram negative	26 °C	Nutrient agar	Lennox: Biolab Zrt., Budapest, Hungary (Product code: CXBLNUA20500)
X. campestris	Gram negative	26 °C	Nutrient agar	Lennox: Biolab Zrt., Budapest, Hungary (Product code: CXBLNUA20500)
X. fragariae	Gram negative	27 °C	Nutrient agar + 1% glucose	Lennox: Biolab Zrt., Budapest, Hungary (Product code: CXBLNUA20500)
C. michiganensis subsp. *nebraskensis*	Gram positive	26 °C	Brain heart infusion agar	Lennox: Biolab Zrt., Budapest, Hungary (Product code: CXBLNUA20500)
C. michiganensis subsp. *michiganensis*	Gram positive	26 °C	Brain heart infusion agar	Lennox: Biolab Zrt., Budapest, Hungary (Product code: CXBLBHA20500)
C. michiganensis subsp. *tessellarius*	Gram positive	26 °C	Brain heart infusion agar	Lennox: Biolab Zrt., Budapest, Hungary (Product code: CXBLBHA20500)
Ralstonia sp.	Gram negative	30 °C	Trypticase soy agar	Thermo: Oxoid, Basingstoke, UK (Product code: CM0131)

4.2.3 Antibacterial activity of the crude extracts assessed by the disk diffusion assay

The disk diffusion assay was the test method used in this study and is the standard protocol recommended by the Clinical and Laboratory Standard Institute (382, 454). The dried extracts were re-dissolved in their respective solvent to give a concentration of 100 mg/mL. Each individual extract (50 µL) was loaded onto a 6 mm blank disk (Oxoid, Basingstoke, UK) at a rate of 10 µL at a time to give a final concentration of 5 mg/disk. Disks loaded with 50 µL of the respective solvent served as the negative control, and disks containing 10 µg/disk of chloramphenicol (Oxoid, Basingstoke, UK) served as the positive control for all bacterial pathogens except for the *Clavibacter* species where 10 µg/disk of streptomycin (Oxoid, Basingstoke, UK) was used instead. As agar that has been supplemented with chloramphenicol has been used previously to maintain *Clavibacter* species (455-457). All disks were allowed to dry and the entire process was carried out under aseptic conditions.

For the antibacterial assessment, 5 µl of each strain was used to inoculate 5 mL of brain heart infusion broth under static conditions at 26 °C with the exception of *Ralstonia* sp., which was incubated at 30 °C and *X. fragariae* which was incubated at 27 °C. After incubation, 1 mL of the cultures of each bacteria was centrifuged at 13,000 RPM for 2 min. The supernatant was removed and the cell pellet was washed with 1 mL of maximum recovery diluent (MRD), vortexed and centrifuged once again. This step was repeated a total of three times to remove metabolic waste from the bacterial culture. The bacterial turbidity was adjusted to 0.5 on the McFarland turbidity standard as measured by absorbance (0.08 to 0.1 at 625 nm) in a spectrophotometer, corresponding to approximately 1.5×10^8 CFU/ml. Each bacterial culture was used within 15 min of being adjusted. A sterile swab was placed into this bacterial suspension and was removed by pressing the swab on the side of the tube before inoculating the plate. This technique was repeated for all bacterial strains to ensure consistency of the bacterial volume introduced onto the media plates. The bacterial cultures were spread using the swab onto the surface of their particular media (Table 4.1) with the plates rotated 60° after each application. The disks containing the seaweed extracts plus the negative and positive controls were aseptically applied to the inoculated plates, which were then refrigerated for 4 to 5 h in the inverted position. This allowed the diffusion of the

bioactive compound(s), whilst the lower temperatures prevented/slowed bacterial growth. The plates were incubated for 24 h at 26 °C except for *Ralstonia* sp., which was incubated at 30 °C and *X. fragariae* which was incubated at 27 °C. The diameter of the zones of inhibition were measured as a clear zone of no bacterial growth around the disk. The antibacterial activity of the seaweed extracts were tested in triplicate and repeated in duplicate.

4.2.4 Determination of positive controls for Clavibacter species

For the initial screen, the commercial antibiotic disk streptomycin (10 µg) was used as the positive control for the three *Clavibacter* species. However, small zones of inhibition for this positive control (≤ 18 mm) were observed, leading to the use of 10 µg/disks of chloramphenicol. The disk diffusion assay (section 4.2.3) was repeated on *C. michiganensis* subsp. *nebraskensis* with both 10 µg/disks of chloramphenicol and streptomycin tested in order to select the optimum positive control i.e. produced the largest zone of inhibition. The optimization study was conducted in triplicate.

4.2.5 Comparison of filtering and autoclaving as a method of sterilisation of the crude extracts

In order to carry out certain tests such as MIC, MBC or cell culture analysis, the crude extracts must be initially sterilised. A comparative study to examine the efficacy of filtering to that of autoclaving as a sterilisation method was performed on the methanol extract of *P. lanosa*. For the filter sterilisation method, the crude extracts were re-dissolved in sterile deionised water to give a final concentration of 100 mg/mL before being filtered through a 0.2 µm filter (Filtropur S plus 0.2, Hildesheim, Germany). For autoclaving sterilisation, the crude extracts were autoclaved under the media sterilisation cycle (steam heating for 15 min at 121 ˚C) before being re-solvated in sterile deionised water to give a final concentration of 100 mg/mL. The antibacterial activity of both set of extracts were determined using the disk diffusion assay as outlined in section 4.2.3 at a concentration of 5 mg/disk against the most susceptible organisms (*X. arboricola*, *C. michiganensis* subsp. *nebraskensis* and *X. fragariae*). All the experiments were completed in triplicate and repeated in duplicate.

4.2.6 Determination of the minimum inhibitory concentration of the methanol extract of P. lanosa

The standard microbroth dilution assay was used to determine the MIC value for the methanol extract of *P. lanosa* as recommended by the CLSI (458) and Srikong *et al*. (66). The test was performed in sterile 96 well microtiter plates (Sarstedt, Hildesheim, Germany). The MIC was determined on the bacterial pathogen *X. fragariae* against which the methanol extracts of *P. lanosa* exhibited strong activity, even after sterilisation. Inoculum (1%) of this microorganism was prepared in brain heart infusion broth and involved inoculating 5 mL of broth with 5 µL of the *X. fragariae* stock. The culture was incubated for 24 h at 27 ˚C under static conditions. The culture was then washed as described in section 4.2.3 and the absorbance adjusted according to the 0.5 on the McFarland turbidity standard. The culture was further diluted by 1:100 using MRD to produce a final concentration of $10^5 – 10^6$ CFU/mL.

The extracts were initially sterilised via autoclaving and re-dissolved in the respective growth media (nutrient broth supplemented with 1% glucose) to give a final concentration of 10 mg/200 µL. For the MIC procedure, 200 µL of the seaweed extracts were added into the microtiter plate. A series of twofold dilutions were performed on the seaweed extracts with the broth serving as the diluent. The resulting concentrations ranged from 5 mg per well to 0.156 mg per well. The wells were inoculated with 100 µL of the adjusted bacteria and the extract control wells, which ensured complete sterility of the seaweed extracts, had the addition of 100 µL of their respective broth as opposed to the bacterial culture. The bacterial control wells consisted of 100 µL of adjusted bacteria and 100 µL of broth, excluding the seaweed extracts. The negative control wells had 200 µL of broth and the positive control well contained 100 µL of a 5 µg/mL chloramphenicol solution and 100 µL of adjusted bacteria. The contents in each were mixed thoroughly. The plates were incubated for 18 – 20 h at 27 ˚C under static conditions. After the incubation period, the turbidity of each well was measured at 620 nm using BioTek EL×800 Absorbance Microplate Reader (Biotek, VT, USA). The absorbance values for each of the wells were recorded with the % reduction calculated for each extract concentration using Equation 3.1. The method was performed in triplicate and repeated on three separate occasions. A reduction of more than 80% of bacterial growth was considered valid (132).

4.2.7 Determination of the minimum bactericidal concentration of the methanol extract of P. lanosa

The MBC was determined on the methanol extract because although an antimicrobial agent inhibits the growth of bacteria, this does not mean the bacteria will not grow once the agent is removed. Such activity is known as bacteriostatic. The MBC method used in this study was adapted from CLSI (459) and Indira *et al*. (460). A 100 µL aliquot from each well of the MIC plate was spread-plated onto their respective media plate. The lowest extract concentration that represented the MBC was where bacterial inhibition of ≥ 99% was observed after overnight incubation at 27˚C without the antimicrobial agent present i.e. no colonies present on the media plates. In the majority of cases, the MBC value was greater than the MIC value.

4.2.8 Antibiofilm properties of the seaweed extract

4.2.8.1 Biofilm prevention assay

The biofilm disruption assay is conceptually similar to the microbroth dilution assay described in the CLSI document M07-A9 with several modifications (382). Dried methanol extracts of *P. lanosa* were autoclaved and dissolved in sterile broth (nutrient broth supplemented with 1% glucose) to a starting concentration of 50 mg/mL. A 100 µL aliquot of the extract solution was added in triplicate to a sterile 96-well microtiter plate with a lid. Serial twofold dilutions were then carried out on the extracts with sterile broth. A 100 µL aliquot of sterile broth was added to two rows to serve as the negative and media only controls. A 100 µL aliquot of 0.5 mg/mL chloramphenicol solution was loaded into the remaining row to serve as the positive control.

A 1% inoculation of *X. fragariae* was prepared in BHI and incubated overnight at 27 °C under static conditions. The subsequent cells were washed three times in MRD and adjusted to the 0.5 on the McFarland turbidity standard as described in section 4.2.3. The adjusted bacteria were diluted 1:100 in nutrient broth supplemented with 1% glucose and 100 µL was loaded into the extract containing wells, to give a final of concentration 5 – 0.3125 mg /200 µL. The negative and positive control wells also had 100 µL added to them to give a final volume of 200 µL.

Following to overnight incubation at 27 °C under static conditions, the supernatants were transferred to a new 96-well microtiter plate. The original wells were washed in triplicate in sterile phosphate buffered saline (PBS) carefully without affecting the biofilm formed at the bottom of the plate in order to remove any planktonic cells. This was followed by the addition of 110 µL of MRD into the wells to allow sufficient volume of bacterial suspension for the twofold dilutions and neat plate. The bacteria/biofilms in the wells were scraped into the MRD solution with the use of a 20 – 200 µL pipette tip. This suspension solution was uniformly aspirated and removed to a separate 96-well microtiter plate. Serial tenfold dilutions were performed on the extract and negative control wells and "were plated" neat – 10^{-7} for the purpose of plate counts. A 100 µL aliquot of each dilution was pipetted onto fresh media plates and spread evenly across the plate immediately to avoid diffusion of the solution through the agar leading to the growth of colonies in clumps making it impossible to count. Positive and media only controls were plated neat (without any dilutions). Plate counts for the supernatants were performed in the same manner. Following overnight incubation the number of colonies present on each plate were counted and recorded. Biofilm prevention was calculated as a percentage against the negative control using Equation 4.1. The assay was repeated in triplicate and further repeated on three separate occasions.

Equation 4.1: $$\% \text{ prevention} = 100 - \left(\frac{sample\ cfu/ml}{negative\ control\ cfu/ml} \times 100\right)$$

4.2.8.2 Biofilm disruption assay

The biofilm disruption assay is also similar to the microbroth dilution assay described in the CLSI document M07-A9 with several modifications (382). A 1% inoculation of *X. fragariae* was prepared in BHI and incubated overnight at 27 °C. The subsequent cells were washed in triplicate in MRD and adjusted to the 0.5 on the McFarland turbidity standard as described in the section 4.2.3. A 1:100 dilution of the adjusted bacteria was prepared in nutrient broth supplemented with 1% glucose and 100 µL of this bacterial stock was then loaded into a 96-well microtiter plate. Three rows remained empty and were loaded with 100 µL of broth to serve as the media only controls. The microtiter

plate was incubated for 48 h at 27 °C under static conditions to allow the formation of a biofilm.

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

Subsequent to the incubation period, the supernatant containing media, samples and planktonic cells were removed and the wells were washed in triplicate in PBS and then loaded with 110 µl of MRD. The bacteria/biofilm containing wells were then carefully scraped with the use of a 20 – 200 µL sterile pipette tip and the resulting suspensions were uniformly aspirated. Serial tenfold dilutions were carried out on the bacteria containing extracts and the negative control in a separate microtiter plate. These were then plated from neat – 10^7 to achieve plate counts on nutrient agar supplemented with 1% glucose in the same manner as biofilm prevention (section 4.2.8.1). The positive control and media only controls were plated neat. Biofilm disruption was calculated as a percentage against the negative controls using Equation 4.2 in order to determine the minimum biofilm eradication concentration (MBEC50 and MBEC90), which is the minimum concentration of seaweed extract capable of inhibiting the mature biofilm by 50 and 90% (461). The method was performed in triplicate and repeated on three separate occasions.

Equation 4.2: $$\% \text{ disruption} = 100 - \left(\frac{sample\ cfu/ml}{negative\ control\ cfu/ml} \times 100\right)$$

4.2.9 Phytotoxicity of the P. lanosa extract

The phytotoxic effect of the methanol extract of *P. lanosa* was evaluated by an *in vivo* root hair assay as described by Kacprzyk *et al.* (462). The plant species used in this assay was the dicotyledonous model plant *Arabidopsis thaliana* and is commonly used to investigate the effect various treatments have on plant viability (462, 463). Seedlings of *A. thaliana* were stored in eppendorf tubes at room temperature. The seedlings were grown on Murashige and Skoog (MS) half-strength basal salt mixture (Sigma, St. Louis, USA) with 1% *w/v* sucrose (Sigma, St. Louis, USA). The MS solution pH was adjusted to 5.8 with 1 M potassium hydroxide (KOH) and 1.5% *w/v* plant agar (Duchefa, Haarlem, The Netherlands) was added before being autoclaved (15 min at 121 °C). The growth medium was poured into 90 mm sterile petri dishes and allowed to set. The seedlings were sterilised with 1 mL of 20% *v/v* of commercial bleach to give a final concentration of sodium hypochlorite of approx. 1% *v/v*. The seeds were sterilised for 10 min under constant mixing.

The sterilising solution was removed by pipetting and the seeds were thoroughly washed with 1 mL of sterile deionised water until all the bleach had been removed. The seeds were sown on the solid MS growth medium using a 200 μL pipette tip at least 0.5 cm apart. The petri dishes were sealed with parafilm and stored for 1 day in the fridge (4 °C) to allow for vernalization of the seeds. The petri dishes were then incubated at 22 °C under constant light in a vertical position to allow the seeds to germinate in a horizontal manner for 5 days.

After the 5 days, the *A. thaliana* seedlings were ready for treatment with the *P. lanosa* extracts. The extracts were initially sterilised via autoclaving and were dissolved in sterile deionised water to obtain extract concentrations that ranged from 5 to 0.156 mg/mL. The wells of a 24 well plate were filled with 1 mL of extract solution. A 1 mL aliquot of sterile deionised water served as the negative control and 65 mM salicylic acid served as positive control. Using forceps, the *A. thaliana* seedlings were transferred root first into the individual wells. The multiwell plate was incubated at 22 °C under constant light for 16 h with the work up to this point carried out in an aspectic laminar flow cabinet. Following incubation, the *A. thaliana* seedlings were transferred to microscope slide and were stained directly with a small volume of 0.001% *w/v* fluorescein diacetate (FDA) stain which was prepared in deionised water. The seedlings

were immediately examined under white and fluorescent light using Olympus BX51 Fluorescence Microscope (Mason Technology, Dublin, Ireland). Only viable roots were able to cleave FDA to form fluorescein which, when excited by a wavelength of 485 nm, fluoresces green. Therefore, these root hairs were scored as viable (exhibited green fluorescence) whereas roots negative for FDA staining were examined further and scored as either programmed cell death (exhibited no fluorescence, cytoplasm condensation and retraction of protoplast away from the cell wall) or necrotic (exhibited no fluorescence or programmed cell death morphology). Results were recorded using mechanical counters with at least 100 root hairs scored starting from the root tip. The percentage for each category was calculated as a percentage of the total number of root hairs scored. The method was performed in triplicate and repeated on three separate occasions. Images (Figure 4.9) were taken using an Olympus DP71 and captured using the Olympus Cell F imaging software for life science microscopy. Data is expressed in a bar chart as percentage of viable, PCD and necrosis for each extract concentration (Figure 4.8).

4.2.10 Statistical analysis

Data obtained for the extraction yields for the five seaweed species, comparison of sterilisation methods, biofilm assay and phytotoxicity results were performed with repeated measures using one-way ANOVA followed by a post-hoc analysis using Tukey's multiple comparison tests. These analyses were performed by Minitab 18 Statistical Software using a 5% statistical significant level (ρ<0.05). The results were said to be statistically different if ρ<0.05 and were designated with different superscripts. All experiments were performed in at least triplicate and are presented as a mean, ± SD, unless stated otherwise.

4.3 Results and Discussion

4.3.1 Extraction yields for crude seaweed extracts using different solvents

In this chapter of work, the five seaweed species *Fucus serratus, Ascophyllum nodosum, Polysiphonia lanosa, Ulva lactuca* and *Fucus vesiculosus* collected in June 2017 were extracted over a 2 h period under the optimised solvent extraction method as designed in Chapter 2, section 2.3.2. Table 4.2 illustrates the average % yields obtained for the five seaweeds and as expected, different solvents yielded different amounts of crude extracts, with water achieving the highest yields. This was as a result of seaweeds consisting of a large quantity of polar constituents including carbohydrates, which can account for almost 20% of the dry weight followed by proteins and lipids (349, 353). This meant that methanol produced the second highest yields followed by ethanol and acetone based on their relative polarities. This trend was also observed in Chapter 2 section 2.3.3 and hence, further work on the ethanol and acetone extractions of *P. lanosa* and *U. lactuca* was not continued due to their known low yields. *F. vesiculosus* was extracted only in water as previous research at Waterford Institute of Technology (WIT) found that this extract collected at the same geographical location exhibited strong antibacterial activity against human pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA) (348).

Table 4.2: Extraction yields for the five seaweed species collected in June 2017 and extracted using solvent extraction in solvents of varying polarity for 2 h.

Solvent	Average % yield				
	F. serratus	***A. nodosum***	***P. lanosa***	***U. lactuca***	***F. vesiculosus***
Water	31.6 ± 0.86^{a}	27.3 ± 0.05^{b}	16.6 ± 1.61^{c}	19.6 ± 0.53^{d}	25.4 ± 0.47^{b}
Methanol	13.5 ± 2.11^{e}	28.1 ± 1.45^{f}	4.1 ± 0.74^{g}	2.7 ± 0.52^{g}	N/A
Ethanol	11.4 ± 1.17^{h}	11.3 ± 1.18^{h}	-	-	N/A
Acetone	6.3 ± 0.88^{i}	11.2 ± 1.22^{j}	-	-	N/A

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

There were significant differences in the % yields of the crude extracts generated in Chapter 2 (Table 2.5) compared to extract yields obtained here in Table 4.2. The *F. serratus*, *A. nodosum* and *P. lanosa* extracts were collected in two different seasons; September 2015 (autumn) and June 2017 (summer) giving rise to seasonal variation. For example, the % yield of *F. serratus* generated from ethanol was much lower in September 2015 compared to those harvested 2 years later in June 2017. This trend was also observed for the other brown seaweed *A. nodosum* where the % yield for the methanol extracts was double that for the species harvested in June. Overall the extracts harvested in June generally produced higher yields. These seasonal fluctuations could potentially be related to the growth of seaweeds as different compounds are produced at different stages of growth. However, this growth cycle differs between seaweeds and is also effected by environmental factors with Luning (464) reporting the growing season for most seaweeds starting in winter with a reduction in growth observed in the summer months. Rani *et al.* also reported this growth cycle effect on the extraction yield obtained for the compound fucoidan, which was extracted from brown seaweeds collected along the Gulf of Manner, India (465). The highest yields were acquired

during the maturation stage of the seaweeds, which occurred in September for *Turbinaria ornata* and *Sargassum wightii* and January for *Padina tetrastromatica*. Mak *et al*. also found that the growth cycle of *Undaria pinnatifida* influenced the fucoidan content with the yield from sporophyll increasing significantly from July (25.4-26.3%) to September (57.3-69.9%) which is the sporophyll maturation period of *U. pinnatifida* (466). Both of the *U. lactuca* samples were collected in summer and as a result the yields obtained for the water and methanol extracts were found not to be statistically significant with a P value of 0.085 for the water extracts and 0.069 for the methanol extracts (ρ>0.05).

4.3.2 Antibacterial activity of the crude seaweed extracts

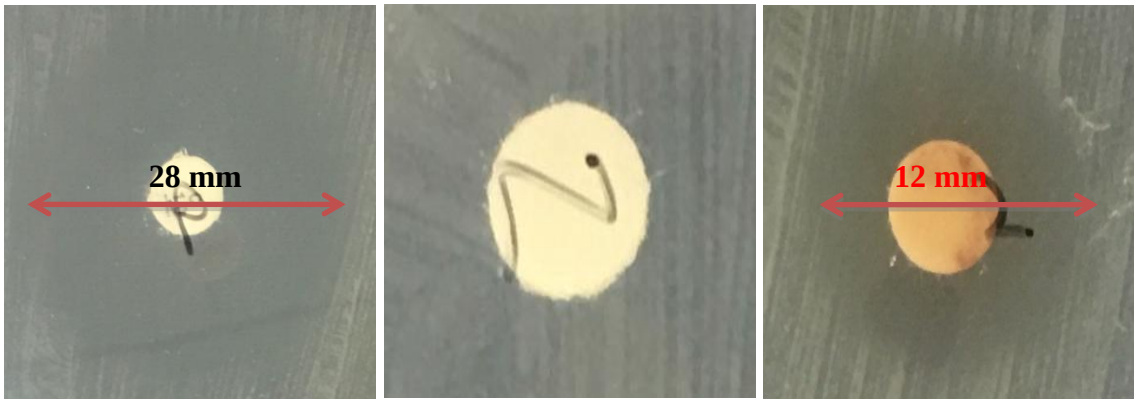
The antibacterial activity of thirteen extracts was investigated against nine quarantine plant pathogens supplied by the DAFM. The extracts had been generated from four different solvents which varied in polarity and allowed the determination of the optimum solvent with regards to its ability to extract the antibacterial compounds from the specific seaweed species. This initial screen found that the red seaweed *P. lanosa* exhibited the broadest range of activity, as it displayed inhibition against eight of the nine bacteria tested (Table 4.3). Hornsey *et al.* also reported this antibacterial potential of *P. lanosa* when they conducted an antimicrobial screen on eleven species of marine algae against *S. aureus* (330). *P. lanosa* exhibited the strongest activity with antimicrobial production occurring uniformly throughout the year. Hellio *et al.* investigated the use of seaweed extracts against 35 marine isolates with dichloromethane fractions of *P. lanosa* producing inhibition against seven sensitive strains of bacteria at 24 μg/mL (444). Shanab also reported greater antibacterial activity in red seaweed species compared to the brown seaweed *S. dentifolium* (467).

Table 4.3: Antibacterial activity of crude P. *lanosa* extracts using the disk diffusion assay at 5 mg/disk against nine bacterial plant pathogens.

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

[a]Positive control was a chloramphenicol antibiotic disk (10 µg/disk) with streptomycin antibiotic disk (10 µg/disk) used for the *Clavibacter* species; [b]Negative control was 50 µL of the respective solvents; Inhibition zones are reported as clear zones (including 6 mm diameter of blank disks); - indicates no activity; + indicates zone of inhibition of 6 – 8 mm; ++ indicates zone of inhibition of 8.1 – 10 mm; +++ indicates zone of inhibition of 10.1 – 13 mm; ++++ indicates zone of inhibition of 13.1 – 16 mm; +++++ indicates zone of inhibition of >16 mm. Data (n=3).

The water extract of *P. lanosa* exhibited activity against a number of pathogens and was the only extract that produced activity against the Gram-negative pathogen *E. amylovora* (Figure 4.1). This is a very promising result since water is a green solvent and overcomes many of the problems associated with the use of organic solvents. Esserti *et al.* also found that the aqueous extracts of *C. myriophylloides* and *F. spirallis* were effective in controlling the Gram-negative plant pathogen *A. tumefacien* (46). Christobel *et al.* also showed that the aqueous extracts of seven seaweed species exhibited antibacterial activity against a range of clinical and fish pathogens with a greater inhibitory activity against Gram-negative pathogens (468).



Positive control **Negative control** ***P. lanosa* extract**

Figure 4.1: The antibacterial activity of the crude water extract of *P. lanosa* against *E. amylocora* using the disk diffusion assay at 5 mg/disk. Positive control: 10 µg/disk chloramphenicol; Negative control: 50 µL of water. Data (n=3).

The methanol extract of *P. lanosa* demonstrated the strongest activity with a zone of inhibition of 15.8 ± 0.41 mm against the Gram-negative pathogen *X. arboricola* (Figure 4.2), the causal agent of bacterial spot disease of stone fruit. This pathogen can have a significant economic impact, with estimated crop losses over €10,000 per hectare in epidemic years on commercial plum orchards (469). Methanol has been demonstrated to be the optimum solvent in extracting the antibacterial compounds with Kumar *et al.* reporting that methanol extracts exhibited the broadest range of activity in controlling the phytopathogen *P. syringae* in the medicinal plant *Gymnema sylvestre* (72). Kim *et al.* also demonstrated a broad spectrum of activity from the methanolic extracts of 33

seaweed species against a range of Gram-positive and Gram-negative bacteria with crude extracts of *Esiena bicyclis* and *Sargassum* sp. exhibiting strong activity against MRSA (71).

Positive control **Negative control** ***P. lanosa* extract**

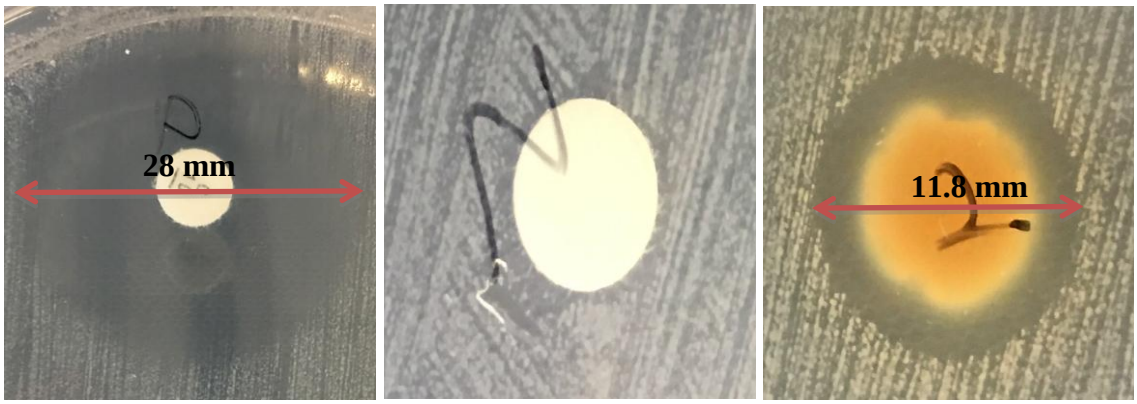
Figure 4.2: The antibacterial activity of the crude methanol extract of *P. lanosa* against *X. arboricola* using the disk diffusion assay at 5 mg/disk. Positive control: 10 µg/disk chloramphenicol; Negative control: 50 µL of methanol. Data (n=3).

However the other methanol extracts generated from *F. serratus*, A. *nodosum* and *U. lactuca* exhibited no activity against the tested bacteria. This is possibly as a result of different seaweed species producing different bioactive compounds. The crude extracts generated from the green seaweed species *U. lactuca* produced no activity against all nine phytopathogens. This was not expected since this green seaweed had been collected at the same geographical location by Tan *et al.* who reported that the methanol extract exhibited low inhibitory activity against both *S. aureus* and MRSA (122). However this may be as a result of seasonal variation since Tan *et al*. harvested the *U. lactuca* in both spring and autumn whereas the *U. lactuca* in this study was harvested in summer. Tan *et al.* also reported this effect of seasonality on activity since the methanol extracts collected in September 2009 (autumn) were only active against MRSA, whereas the extracts collected in March 2010 (spring) were found to be active against *S. aureus* (122).

Differences in activity among different locations have also been reported. For instance, methanol extracts of *U. lactuca* harvested in New York did not produce activity against

E. coli at 1:10 *w/v* concentration (470), whereas "the same extracts" harvested in Morocco at a lower concentration (1:40 *w/v*) exhibited activity against *E. coli* (471). Hence, different compounds might be produced in seaweeds at different geographical locations as a result of environmental variation.

The water extract of *F. vesiculosus* demonstrated activity against five of the nine tested bacteria (Table 4.4). It exhibited the broadest spectrum of activity out of the three Phaeophyceae species screened, with the highest activity against the Gram-negative pathogen *X. fragariae*, responsible for several scorch diseases (Figure 4.3).



Positive control **Negative control** ***F. vesiculosus* extract**

Figure 4.3: The antibacterial activity of the crude aqueous extract of *F. vesiculosus* against *X. fragariae* using the disk diffusion assay at 5 mg/disk. Positive control: 10 µg/disk chloramphenicol; Negative control: 50 µL of water. Data (n=3).

Lustigman *et al.* reported activity from the methanol and chloroform extracts of *F. vesiculosus* collected in New York against *S. epidermidis* with the water extract exhibiting no activity (470). This difference in activity between the water extracts of *F. vesiculosus* here and in the Lustigman *et al.* study may have been as a result of geographical location, since Tan *et al.* who also harvested *F. vesiculosus* at Baginbun beach, Wexford, Ireland found that the water extracts exhibited moderate activity against MRSA (122). Additionally, previous research at WIT, which also harvested *F. vesiculosus* at the same location observed very strong inhibitory activity by water extracts against a range of clinical bacterial strains (348).

Table 4.4: Antibacterial activity of crude *F. vesiculosus* water extracts using the disk diffusion assay at 5 mg/disk against nine bacterial plant pathogens.

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

[a]Positive control was a chloramphenicol antibiotic disk (10 µg/disk) with streptomycin antibiotic disk (10 µg/disk) used for the *Clavibacter* species; [b]Negative control was 50 µL of the respective solvents; Inhibition zones are reported as clear zones (including 6 mm diameter of blank disks); - indicates no activity; + indicates zone of inhibition of 6 – 8 mm; ++ indicates zone of inhibition of 8.1 – 10 mm; +++ indicates zone of inhibition of 10.1 – 13 mm; ++++ indicates zone of inhibition of 13.1 – 16 mm; +++++ indicates zone of inhibition of >16 mm. Data (n=3).

The aqueous extracts of *A. nodosum* were active against four out of the nine bacteria with the methanol extract the only other extract to exhibit activity specifically against *X. fragariae* (Table 4.5). This was unexpected since Vacca *et al*. reported strong antimicrobial activity from alcohol extracts of *A. nodosum* against a range of Gram-negative and positive bacteria (437). Tan *et al.* also observed a broader range of activity from the methanol, ethanol and acetone extracts of *A. nodosum* compared to the water extracts. But this again may have been as a result of seasonal variation and different bacterial strains tested (122). These results differed from the results obtained from the red seaweed *P. lanosa* (epiphytic) which grows on *A. nodosum* (basiphyte) and, therefore, suggests that both species exhibit a different antibacterial profile. This is explained by the fact that a symbiotic relationship exists between the two species in the form of carbon exchange only (472).

The brown seaweed, *F. serratus* is widely used in the cosmetic industry and grows throughout the year along the South-East coast. Unfortunately, *F. serratus* exhibited poor antibacterial activity as seen in Table 4.6 with both the water and methanol extract producing low inhibitory activity against *X. frragariae* as seen in Figure 4.4 for the water extract.

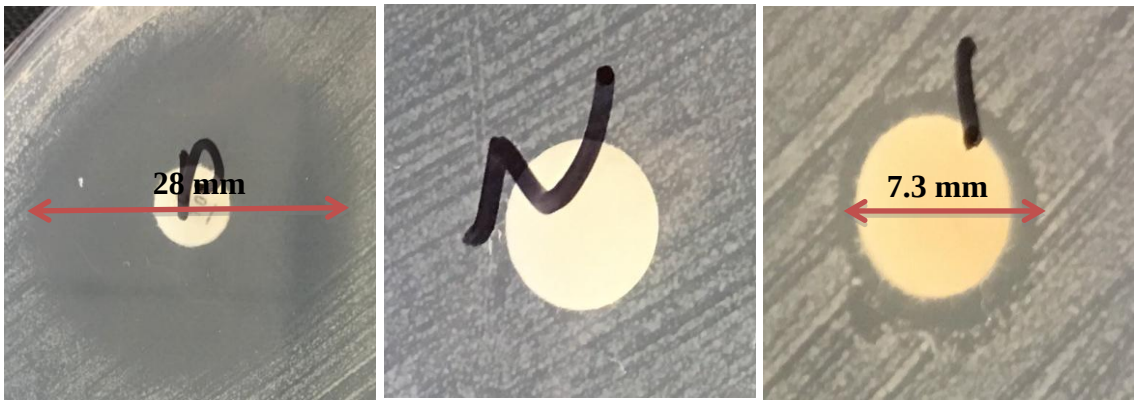


Figure 4.4: The antibacterial activity of the crude water extract of *F. serratus* against *X. fragariae* using the disk diffusion assay at 5 mg/disk. Positive control: 10 µg/disk chloramphenicol; Negative control: 50 µL of water. Data (n=3).

Table 4.5: Antibacterial activity of crude *A. nodosum* extracts using the disk diffusion assay at 5 mg/disk against nine bacterial plant pathogens.

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

[a]Positive control was a chloramphenicol antibiotic disk (10 µg/disk) with streptomycin antibiotic disk (10 µg/disk) used for the *Clavibacter* species; [b]Negative control was 50 µL of the respective solvents; Inhibition zones are reported as clear zones (including 6 mm diameter of blank disks); - indicates no activity; + indicates zone of inhibition of 6 – 8 mm; ++ indicates zone of inhibition of 8.1 – 10 mm; +++ indicates zone of inhibition of 10.1 – 13 mm; ++++ indicates zone of inhibition of 13.1 – 16 mm; +++++ indicates zone of inhibition of >16 mm. Data (n=3).

Table 4.6: Antibacterial activity of crude *F. serratus* extracts using the disk diffusion assay at 5 mg/disk against nine bacterial plant pathogens.

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

[a]Positive control was a chloramphenicol antibiotic disk (10 µg/disk) with streptomycin antibiotic disk (10 µg/disk) used for the *Clavibacter* species; [b]Negative control was 50 µL of the respective solvents; Inhibition zones are reported as clear zones (including 6 mm diameter of blank disks); - indicates no activity; + indicates zone of inhibition of 6 – 8 mm; ++ indicates zone of inhibition of 8.1 – 10 mm; +++ indicates zone of inhibition of 10.1 – 13 mm; ++++ indicates zone of inhibition of 13.1 – 16 mm; +++++ indicates zone of inhibition of >16 mm. Data (n=3).

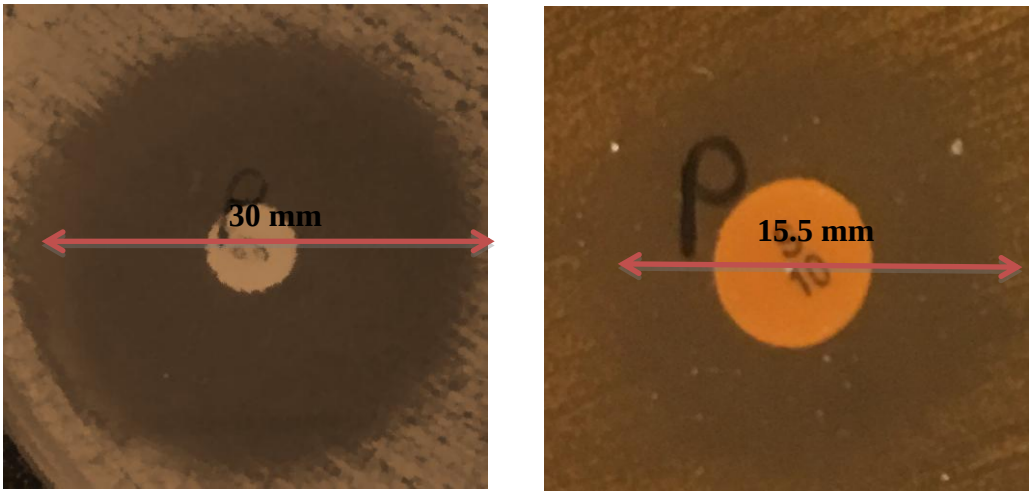
Interestingly, the ethanol and acetone extracts for all four seaweed species exhibited no activity against the tested bacteria. However, Bonbón *et al.* found that the ethanol and acetone extracts of a number of seaweeds produced strong activity against *S. aureus* (448). Moderate activity was only observed by the acetone extracts against the plant pathogen *E. carotovora*, whilst the ethanol extracts exhibited no activity against this pathogen. This is most likely as a result of extraction of antimicrobial compounds from seaweeds being solvent dependent and also activity being specific to the organism i.e. Gram-positive or Gram-negative (473).

This initial screen study found that the crude extracts exhibited a wide spectrum of activity against both Gram-negative and Gram-positive bacteria. The most notably activity was from the methanol and water extracts of *P. lanosa* with zones of inhibition against *X. arboricola* reported to be 15.83 ± 0.41 and 12.33 ± 0.82 mm, respectively. The methanol extracts also exhibited strong activity against the Gram-positive species *C. michiganensis* subsp. *nebraskensis* and *C. michiganensis* subsp. *tesselarius* with zones of inhibition of 14.17 ± 1.60 and 11.17 ± 0.41 mm, respectively. This was an interesting result; as mentioned previously it is harder to kill Gram-negative bacteria compared to Gram-positive due to the complexity of their cell wall. This is a very positive result since a number of studies have reported no activity by seaweed extracts against Gram-negative bacteria (440, 474). Rao *et al.* studied the antibacterial activity of seaweed extracts collected from the Saurashtra coast, India and found that none of the extracts were active against the tested Gram-negative bacteria including *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhosa* Para A, *Pseudomonas aeruginosa* and *Shigella sonnei* (475). The methanol extracts of *P. lanosa* demonstrated the strongest activity and was therefore selected for further study with regards MIC, MBC, antibiofilm activity and phytotoxicity analysis.

4.3.3 Investigation into the optimum positive control for Clavibacter species

Clavibacter michiganensis is an aerobic Gram-positive bacterium, which is responsible for sustainable economic losses through the damage of tomatoes and potatoes (476). In this screen study, three *Clavibacter* sub-species were screened i.e., *Clavibacter michiganensis* subsp. *nebraskensis, Clavibacter michiganensis* subsp. *michiganensis, Clavibacter michiganensis* subsp. *tessellarius*. Although all of the *Clavibacter* species were maintained on BHI agar, in a number of studies the agar has been supplemented with chloramphenicol (455-457). Chalupowicz *et al.* investigated the virulence factors of C. *michiganensis* subsp. *michiganensis* on the systemic and local infection of tomatoes. This involved growing the *Clavibacter* on medium that was supplemented with 10 μg/mL of chloramphenicol (476). For this reason, streptomycin was used as the positive control (10 μg/disk) in the disk diffusion assay for the three *Clavibacter* species. Streptomycin was chosen on the basis that it is a registered pesticide by the Environmental Protection Agency (EPA) and is used to control bacterial and fungal infections on certain fruits, vegetables, seeds and ornamental crops (477). Milijašević *et al.* reported that streptomycin demonstrated the best ability in controlling *Clavibacter michiganensis* subsp. *michiganensis* in tomato seedlings (478).

In this screen study, streptomycin (10 μg/disk) gave moderate zones of inhibition (≤18 mm) against the *Clavibacter* species compared to the larger zones of inhibition (27-30 mm) obtained for chloramphenicol (10 μg/disk) against the majority of the other bacterial pathogens. Therefore, an investigation was carried out where chloramphenicol was used as the positive control against these three *Clavibacter sp.*, with Figure 4.5 illustrating the much larger clear zone of inhibition obtained by the chloramphenicol disk compared to the streptomycin disk against *C. michiganensis* subsp. *nebraskensis.* Thus, from this point on chloramphenicol was used as the positive control against *Clavibacter* sp. for antibacterial assays.



Chloramphenicol (10 µg/disk) **Streptomycin (10 µg/disk)**

Figure 4.5: Comparison of the antibiotic chloramphenicol and streptomycin against *C. michiganensis* subsp. *nebraskensis* using the disk diffusion assay at 10 µg/disk. Data (n=3).

4.3.4 Comparison of sterilisation methods for the crude P. lanosa extracts

Since the extraction process was not conducted under sterile conditions, it was essential that the extracts were sterile for certain assessments, particularly for broth dilution methods which include MIC and MBC studies. This is as a result of the broth dilution assay measuring antimicrobial activity on the reduction of growth of the indicator bacteria and hence, non-sterile extracts could cause contamination leading to unreliable results. Therefore, an investigation was carried out to determine the best sterilisation method for the specific extract between filtration and heat sterilisation.

In general, the chosen method for sterilisation of a liquid in laboratories is filter sterilisation, which uses force to separate rather than to kill. In this method, the re-solvated extract was passed through a sterile nylon filter, pore size 0.2 µm, with this particular pore size preventing the passage of larger particles including bacteria and other microbes. Hence, this results in a sterile extract without the use of heat, making it suitable for heat-sensitive compounds. Arullappan *et al.* filter sterilised crude extracts of the ornamental plant *Hibiscus rosa sinensis* through 0.2 µm filter paper in

order to evaluate the extracts antimicrobial potential (479). Amorim *et al.* also used this method of sterilisation using a 0.22 µm filter membrane to determine the effect of crude sulfated polysaccharides extracted from the red seaweed *Gracilaria ornata* on the development of eubacteria in mineral liquid medium (180).

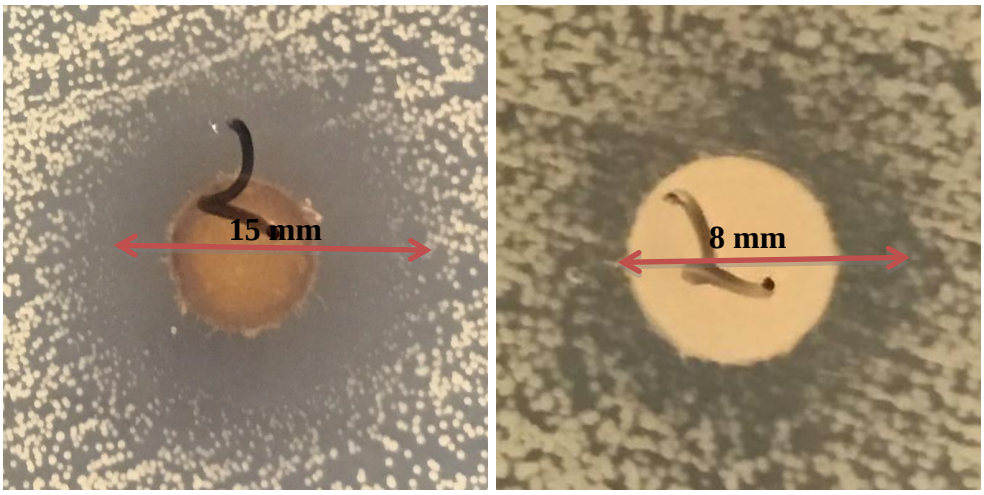
In this study, the methanol extracts of *P. lanosa* were investigated since it exhibited the broadest spectrum of activity (Table 4.3). The methanol extracts were also re-solvated in 100% sterile deionised water as the solvent would be in direct contact with the bacteria for MIC and MBC studies making methanol unsuitable due to its potential toxicity. The methanol extracts were syringe filtered but required 400 µL of extract solution in order to obtain 150 µL of sterile crude extract as a result of some of the extract embedding onto the filter. The extract was then tested for activity via the disk diffusion assay at 5 mg/disk against the three most susceptible bacteria identified in the initial screen including *X. arboricola*, *C. michiganensis* subsp. *nebraskensis* and *X. fragariae* (Table 4.7).

Table 4.7: Antibacterial activity of the filtered methanol extract of *P. lanosa* against three bacterial pathogens in comparison to un-filtered extracts at 5 mg/disk.

***P. lanosa* extract**	**Antibacterial activity (mm)**		
	X. arboricola	***C. michiganensis* subsp. *nebraskensis***	***X. fragariae***
Un-filtered extract (100% MeOH)	15.8 ± 0.41^{a}	14.2 ± 1.60^{c}	14.8 ± 2.10^{e}
Filtered extract (100% water)	6.8 ± 0.98^{b}	8.3 ± 1.75^{d}	8.0 ± 1.47^{f}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript for the specific bacterial species are statistically different in terms of activity (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

From examination of the results, it can be seen that activity remained after filtration, however, it was much lower compared to that of the un-filtered extracts. Figure 4.6 illustrates the difference in activity observed between the filtered and unfiltered extracts against *X. arboricola* where the un-filtered extracts exhibited a large clear zone whereas the filtered extracts exhibited a much smaller hazier zone. This could possibly be as a result of the antimicrobial compounds sticking/absorbing onto the filter due to a polarity issue since these extracts had been re-solvated in water as opposed to their respective solvents, meaning slightly non-polar compounds may not be resolvated. Although activity was reduced, it did show that filtration had the ability to achieve a sterile extract since no contamination was observed around the disks loaded with the extract (Figure 4.6).



Un-filtered methanol extracts **Filtered methanol extracts**

Figure 4.6: Comparison of the antibacterial activity exhibited by the methanol extracts of *P. lanosa* both un-filtered and filtered against *X. arboricola*. Activity was assessed by the disk diffusion assay at 5 mg/disk. Data (n=3).

Autoclaving of the extracts was also investigated and had the added advantage over syringe-filtration of being cheaper and more convenient, although it had the risk of deactivating heat sensitive antimicrobial compounds or oxidation of the bioactives in the crude extracts. Dried *P. lanosa* crude extract was autoclaved with the methanol extract becoming a dark purple liquid (water vapor from the autoclave) with solid particles possibly as a result of the oxidation of the sugars/polysaccharides present in

the extracts. The extract was re-solvated and the solid particles were removed by centrifugation. The extract was once again tested to see whether activity was retained against the same three susceptible bacterial species. In addition to this, the methanol extracts were re-solvated in 5% methanol to determine whether this supplement of organic solvent would overcome any potential polarity issues. Table 4.8 demonstrates the results obtained for the autoclaved methanol extracts of *P. lanosa*.

Table 4.8: Antibacterial activity of the autoclaved methanol extracts of *P. lanosa* that were re-solvated in sterile water/5% methanol using the disk diffusion assay against three bacterial pathogens in comparison to un-autoclaved extracts at 5 mg/disk

***P. lanosa* extract**	**Antibacterial activity (mm)**		
	X. arboricola	***C. michiganensis* subsp. *nebraskensis***	***X. fragariae***
Un-autoclaved extract	15.8 ± 0.41^{a}	14.2 ± 1.60^{c}	14.8 ± 1.47^{e}
Autoclaved extract (100% water)	0 ± 0^{b}	8.3 ± 0.52^{d}	13.8 ± 1.48^{e}
Autoclaved extract (5% methanol)	0 ± 0^{b}	8.3 ± 0.52^{d}	13.7 ± 1.21^{e}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript for the specific bacterial species are statistically different in terms of activity (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

Similar to the filtered methanol extracts, activity against *X. arboricola* was lost meaning these specific bioactives were heat sensitive, whereas the activity against *C. michiganensis* subsp. *nebraskensis* was reduced compared to the un-autoclaved extracts, but this may have been as a result of some bioactive(s) being heat sensitive. This adds to the probability that different bioactives are active against different bacteria possibly as result of their different modes of action. The addition of 5% methanol had no effect in improving activity with both the 100% water and 5% methanol exhibiting statistically the same activity against *C. michiganensis* subsp. *nebraskensis*. However, both the 100% water and 5% methanol autoclaved extracts exhibited the same degree of activity against *X. fragariae* compared to the un-autoclaved extracts. This meant that the specific bioactives against *X. fragariae* were not the same compounds that exhibit activity against *X. arboricola* and *C. michiganensis* subsp. *nebraskensis*. This result also demonstrated that the bioactives against *X. fragariae* were thermostable and also that water had the ability to solvate the required bioactives avoiding possible polarity issues leading to reduced activity.

Therefore, in this study heat sterilisation produced better ranges of inhibition compared to filter sterilised extracts. This was also observed by Muniandy *et al.* who investigated the effects of heat and filter sterilisation on the antibacterial efficiency of *Coleus aromaticus* (480). It was found that heat sterilisation (15 min at 121 °C) was more efficient in maintaining activity of the extract than filter sterilisation. For instance, the zone of inhibition achieved for the ethanolic extract of *C. aromaticus* was 27 ± 1.4 mm compared to the heat sterilised ethanolic extract of *C. aromaticus* at 20 ± 0.7 mm and filter sterilised ethanolic extract of *C. aromaticus* at 12 ± 1.4 mm (480). Hashemi *et al.* also found that autoclaving caused less damage to the antibacterial activities of the aqueous extracts of herbal plants compared to syringe filtration (481). For this reason heat sterilisation was the method of choice for the remainder of this study with the extracts re-solvated in 100% sterile deionised water in order to avoid the use of organic solvents.

4.3.5 Determination of minimum inhibitory concentration and minimum bactericidal concentration of the methanol extracts of P. lanosa

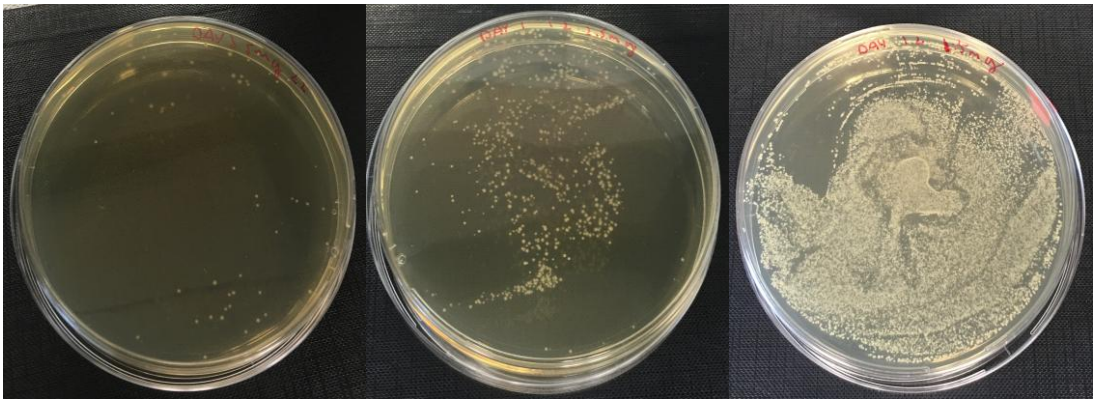
The MIC and MBC was conducted using the previously optimised sterilisation method in which the methanol extracts of *P. lanosa* were autoclaved and re-solvated in sterile deionised water. The MIC and MBC was assessed against the Gram-negative bacteria *X. fragariae* with this bacteria selected on the basis of section 4.3.4, where it was found that both the un-autoclaved and autoclaved extracts exhibited statistically the same activity. The MIC assay was conducted in the concentration range of 5 – 0.156 mg per well. The inhibitory effect of *P. lanosa* extract against *X. fragariae* started at 1.25 mg per well (200 µL) in a dose response manner, with the higher the extract concentration the higher the kill rate. Therefore, the MIC value of the methanol extracts of *P. lanosa* was 6.25 ± 1.96 mg/mL or 6.25 ± 1.96 mg/1000 µL (greater than 80%). These results were in accordance with those of Sasidharan *et al.* who reported MIC values of 6.25 and 3.125 mg/mL against *Pseudomonas aeruginosa* and B*acillus subtilis* respectively, for the crude red seaweed *Gracilaria changii* (482). The concentrations required by this extract are similar to the *P. lanosa* extract, demonstrating the potent antimicrobial compounds contained in *P. lanosa*. Similarly Natheer *et al.* found that the methanol extract from the brown seaweed, *Turbinaria conoides* exhibited the highest activity, with a MIC value of 12 mg/mL against the Gram-negative bacteria *E. coli* (483).

There are other novel studies that have demonstrated more potent antimicrobial compounds isolated from other seaweeds including Solomon *et al.* who purified two compounds known as A_1 and C_1 which were found to be related to terpenoid secondary metabolites isolated from the seaweed *Dictyota acutiloba* and exhibited MIC values of 0.69 and 0.7 µg/mL respectively against MRSA, while the positive control methicillin (commercial antibiotic) showed an MIC value of 50 µg/mL (484). Therefore, purification of crude extracts typically has a positive effect on activity with most of the non-active compounds been removed including antagonistic compounds. This increase in activity was also reported by Ha *et al.* who investigated the antibacterial activity of 44 seaweed species against the vaginal bacterium *Gardnerella vaginalis* (419). The MIC value for the crude ethanolic extract of *Ulva*

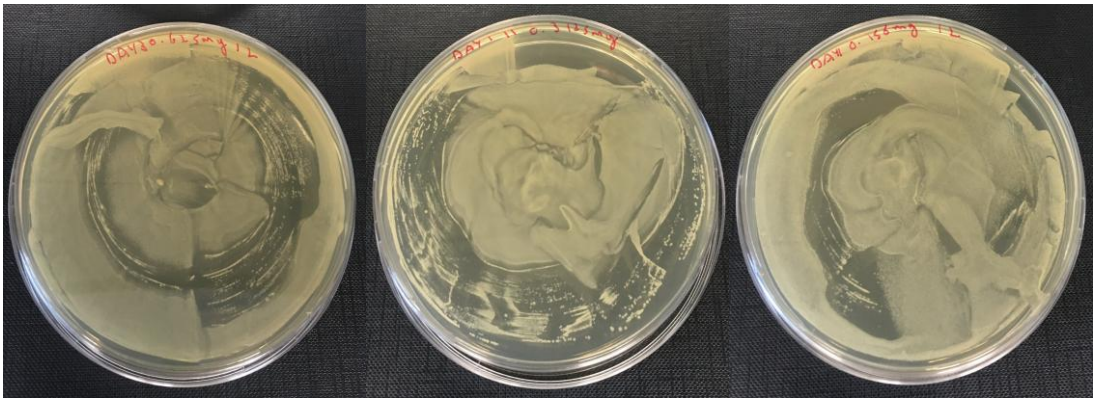
pertusa was found to be 312 μg/mL, whereas the nitrogenous compounds fractioned from this crude extract was twice as potent with a MIC of 156 μg/mL (419).

The MBC value of the methanol extracts against *X. fragariae* was found to be above the tested concentration range, with extract concentration above 5 mg per well or 25 mg/mL required. Unfortunately, solubility issues prevented the concentration of the methanol extract being increased. However, it is common to have a higher MBC compared to MIC, since a higher concentration of the antimicrobial compound is required to completely eliminate the bacteria (485). Eom *et al.* also found that their hexane, dichloromethane and butanol fractions generated from the brown seaweed *Eisania bicyclis* required concentrations above their MIC to achieve an MBC value against MRSA (486). Furthermore, these MIC and MBC concentrations can vary and depend on a number of factors including the specific antimicrobial compounds and the type of test bacteria used (485). For instance, isolates of a particular species will have variable MIC's; sensitive strains producing low MICs, compared to the more resistant strains which will exhibit relatively higher MICs.

Although in this study the methanol extracts of *P. lanosa* were not found to be bactericidal over the tested concentration range, there was a dose response effect (Figure 4.7) due to the fact that the higher extract concentration would result in a higher kill rate.



25 mg/mL **12.5 mg/mL** **6.25 mg/mL**



3.125 mg/mL **1.56 mg/mL** **0.780 mg/mL**

Figure 4.7: Dose response effect of the autoclaved methanol extracts of *P. lanosa* in determination of the MBC against *X. fragariae* using the microdilution assay. Data (n=9).

4.3.6 Investigation into P. lanosa antibiofilm potential and mode of action

As mentioned previously, microbial biofilms are causing great concern in the management of bacterial and fungal infections due to their high resistance to antibacterial treatments. This resistance is a result of the planktonic bacterium irreversibly adhering to biotic or abiotic surfaces and the formation of a three-dimensional extracellular matrix with regulated motility (487). There are a number of studies which have noted the antibiofilm potential of seaweed extracts against human biofilm-forming bacteria (156-158) and marine biofouling (488, 489). The vast majority of studies have been conducted on bacteria from marine environments

(490-492), including bacteria symbionts extracted from the surface of seaweeds (493). Since bacteria produce secondary metabolites similar to seaweeds in response to external pressure including competition for nutrients or space (492) as demonstrated by coral-associated bacteria against *Pseudomonas aerginosa* biofilm formation (494). A basic mechanism by phylogenetically different bacteria is the production of extracellular molecules that degrade adhesive components in the biofilm mechanism (495, 496).

Bacterial attachment and biofilm formation are regarded as critical steps in the establishment of biofilms. The crude *P. lanosa* extracts were initially assessed for their ability to inhibit biofilm formation of *X. fragariae* using the colony counting method. It was found that the crude extracts prevented biofilm formation in a dose response manner with 6.25 mg/mL preventing over 80% biofilm formation (Table 4.9). Jun *et al.* also reported antibiofilm prevention from marine algae against dental plaque, particularly the compound fucoidan isolated from *Fucus vesiculosus* with a concentration above 125 μg/mL completely suppressing biofilm formation and planktonic cell growth (497). This concentration was 50 times less compared to the concentration used in this study and further demonstrates the high potency of purified compounds compared to crude extracts. Below the 6.25 mg/mL concentration, a promotion in biofilm formation was noted.

This phenomena was also reported by Omwenga *et al.* who found that the methanol extracts of *Elaeodendron buchananii* and 50% ethanol *Acacia gerrardii* extracts did not exhibit any antibiofilm activity and on the contrary promoted biofilm formation (498). This was concluded to be as a result of the tested extracts not possessing any compounds capable of targeting and inhibiting biofilm formation, such as phytochemical compounds including N-[4-(phenylamino) phenyl]-benzamide which has been reported to exhibit such activity (499). This was not the case in this study, as after a certain extract concentrations antibiofilm activity was observed (Table 4.9). This phenomenon was also described in section 2.3.4 for certain seaweed extracts against two fungal species. It was suspected to be either the effect of nutrients present in the extracts promoting the growth of the bacteria or a dose-response relationship categorised by opposing effects of low and high extract doses, known as a hormetic response (500). This biphasic dose-response relationship was reported by other recently published studies in which high concentrations of

antibiotics eradicated bacteria, whilst at low concentrations, biofilm formation was encouraged (501, 502). Salta *et al.* reported such a phenomenon from *Chondrus crispus* extracts and two isolated compounds from terrestrial sources, (+)-usnic acid and juglone against marine biofilm forming bacteria at low extract concentrations (503). The extract concentration to inhibit biofilm growth was also found to be dose dependent with 6.25 mg/mL the lowest concentration to achieve this in the supernatant. This is technically the MIC against *X. fragariae* and from comparison of the MIC obtained in section 4.3.5 using a colorimetric assay it can be seen that the same MIC values were recorded for both assays, demonstrating the accuracies of both methods.

Table 4.9: Antibiofilm activity of crude methanol extracts of *P. lanosa* against *X. fragariae*. Extract concentration tested: 1.563 – 25 mg/mL.

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

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

For biofilm disruption assessment, the bacteria were incubated over a 48 h period to allow sufficient time for biofilm formation. The same concentrations of extract (1.563 – 25 mg/mL) were applied in this assay, with antibiofilm disruption activity also found to be concentration dependent with 100% disruption observed above the concentration of 6.25 mg/mL and falling to 78.2 ± 9.27% for 3.125 mg/mL. Therefore, the MBEC50 and MBEC90 of *P. lanosa* were found to be 3.125 mg/mL and 6.25 mg/mL, respectively. These results demonstrated the antibiofilm activity of the crude *P. lanosa* extracts against biofilms. This was a very promising result since mature biofilm communities are complex and once established are very difficult to eradicate (504). For instance, Jun *et al.* reported strong biofilm prevention from the compound fucoidan isolated from *F. vesiculosus* at 125 μg/mL (497). But it was found that the fucoidan compound was unable to disrupt and eliminate the completed biofilm. A promotion in bacterial growth was also observed for the lowest tested concentration of 1.563 mg/mL with such a promotion also suspected to be as a result of nutrient supplement or a hormesis response (372).

Even though the antimicrobial nature of seaweed extracts has been widely reported, their specific underlying mode of action is still relatively unknown. It is well known that the antibiofilm activity of seaweed extracts is tightly linked with the content of secondary metabolites including phenols and flavonoids which have been reported in seaweed, including methanol extracts (505, 506). The antimicrobial activity of phenolic compounds is believed to lie in its ability to effect the permeability in bacterial cell membranes causing the widening of the pores resulting in the loss of intercellular macromolecules including nucleotides and proteins (507). The presence of flavonoids such as apigenin and kaempferol in natural products have been associated with antibiofilm activity by modulating bacteria cell to cell communication through the suppression of the autoinducer-2 (508). Halogenated furanones isolated from the red seaweed, *Delisea pulchra* has demonstrated antibiofilm properties due to their effectiveness as quorum sensing inhibitors in both Gram negative and positive bacteria (509). In order to gain more insight into the antibiofilm compounds present in the methanol extracts of *P. lanosa*, and determine its specific mode of action phytochemical analysis is required on the methanolic extract.

This study indicated the potent antibiofilm compounds present in the methanol extracts of *P. lanosa* against the plant pathogen, *X. fragariae*. This pathogen can enter the plant through penetration of the stomata into the interior air spaces of the mesophyll, where biofilms are produced consisting of a large volume of xanthans (510). Infections in this manner results in plasmolysis and deformation of the plant cells making this international quarantine pathogen a considerable concern to strawberry nurseries and growers (511). Strawberries are an economically and socially significant crop worldwide. In 2014, the United States produced three billion pounds of strawberries estimated at a value of $2.9 billion (512). *X. fragariae* has been reported to cause losses in yield from 8% up to 80% in North America (513).

Additionally, no current effective control measure is available and therefore, new alternatives are required to ensure the future success in the management of this disease on strawberries, further demonstrating the importance of this antibacterial activity by the *P. lanosa* extracts.

The effectiveness of the *P. lanosa* extract was not totally surprising since seaweed extracts were previously applied in strawberry management connected to both their biostimulant effects and antimicrobial properties (45). Meszka *et al.* reported that the application of laminarin, a compound that has been extracted and purified from the brown seaweed, *Laminaria digitata*, was effective in controlling *Botrytis cinerea*, *Podosphaera aphanis* and *Mycoshaerella fragariae* infections in strawberries under field conditions (514). Although studies have demonstrated the strong antifungal potential of seaweed extracts against common fungal pathogens effecting strawberries (130, 515), no such study has been found on the use of *P. lanosa* extracts against *X. fragariae* adding to the novelty of this result.

4.3.7 Determination of the phytotoxicity of P. lanosa extracts

Since these seaweed extracts are intended for use in the biopesticide industry, the small flowering plant *Arabidopsis thaliana* was used to identify potential phytotoxic properties exhibited by these extracts. The root hair assay was capable of rapidly quantifying the effect that the crude extracts had on *A. thaliana's* root hairs by simultaneously determining the percentage of viable (live) and dead root hairs through distinct morphology differences (462). These dead root hairs were further

divided based on their mode of death: programmed cell death (PCD) is a highly controlled and organized process of cell destruction occurring in every part of the plant, throughout its life cycle (462). PCD is crucial in plant development but has also been shown to be part of the plants response to environmental stresses such as the presence of a phytotoxin (463). On the other hand, necrosis is a rapid uncontrolled death generally caused by severe stress or injury (462).

The *P. lanosa* methanol extracts demonstrated all three effects on the *A. thaliana's* root hairs in a dose response manner (Table 4.10). The 5 and 2.5 mg/mL concentrations were found to be toxic with 0% viability and elevated levels of necrosis demonstrating the high degree of stress that these concentrations caused to the plant compared to the negative control which exhibited over 88 ± 10.97% viability and any cell death recorded was most likely the consequence of physical damage caused during the experimental process.

Table 4.10: Effect of crude *P lanosa* extracts on the root hair viability of *A. thaliana* over the concentration range 0.156 - 5 mg/mL (PCD – programmed cell death; TCD – total cell hair death).

Concentration (mg/mL)	% Viability	% PCD	% Necrosis	% TCD
0.156	69.2 ± 12.85[a]	30.4 ± 12.83[ab]	0.2 ± 0.71[a]	30.8 ± 12.85[a]
0.313	70.9 ± 11.93[a]	27.0 ± 10.75[ab]	2.1 ± 1.98[a]	29.1 ± 11.93[a]
0.625	64.3 ± 11.57[a]	32.4 ± 12.32[a]	3.3 ± 3.14[a]	35.7 ± 11.57[a]
1.25	36.5 ± 27.39[b]	38.7 ± 12.68[a]	24.8 ± 22.84[b]	63.5 ± 27.39[b]
2.5	0[c]	25.5 ± 19.12[ab]	74.5 ± 19.12[c]	100 ± 0[c]
5	0[c]	11.2 ± 7.58[b]	88.8 ±7.58	100 ± 0[c]
Positive control[a]	0 ± 0	82 ± 6.43	25 ± 4.16	100 ± 0
Negative control[b]	88 ± 10.97	15 ± 6.43	3 ± 1.15	6 ± 9.33

[a]Positive control was 60 mM salicylic acid; [b]Negative control was 1 mL of sterile deionised water. Data (n=9) are presented as the mean ± SD. Data that do not share a common superscript are statistically different in terms of % viability, % PCD, % necrosis or % TCD between each extract concentration (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

However, as the concentration of extract was reduced, root hair viability gradually increased and the majority of death was as result of PCD with this change starting at 1.25 mg/mL where viable root hairs were observed and TCD dropped from 100% to 63.5 ± 27.39%. A high standard deviation was obtained for the 1.25 mg/mL treatment, as this concentration was not quiet enough to kill all the root hairs. The *P. lanosa* extract affected the viability of *A. thaliana* root hairs (i.e. 30% root hair viability) for all the concentrations tested as seen in Figure 4.8. Unfortunately, it meant that the *P. lanosa* extracts could affect plant species at and below its MIC (6.5 mg/mL against *X. fragariae*; Table 4.10) and MBC (<25 mg/mL against *X.*

fragariae; Table 4.10), with 100% inhibition at 5 mg/mL. The crude extracts, therefore, contained a number of compounds that were toxic to *A. thaliana in vivo* and required isolation and purification of the active bioactive(s) to allow for a more accurate phytotoxicity study. For instance, Tan reported that the crude *P. lanosa* extracts were cytotoxic to L-929 mouse fibroblast cells below its MIC and MBC compared to a semi-pure *U. lactuca* extract which was found to be non-toxic to the L-929 fibroblast cells at concentrations up to 63 µg/mL (348). This was most likely as a result of the antagonistic compounds being removed from the *U. lactuca* extract during the separation process.

The observed toxicity points to the potential use of such extracts in anti-cancer therapies as explored in a number of studies (516, 517). *In vitro* cytotoxic study using bromophenols present in P. *lanosa* demonstrated a toxic effect on human colon carcinoma lines (DLD-1 and HCT-116) (518). More relevantly, the phytotoxicity properties demonstrated by the crude *P. lanosa* could be utilised in a natural herbicide for problematic plant species such as rhododendron.

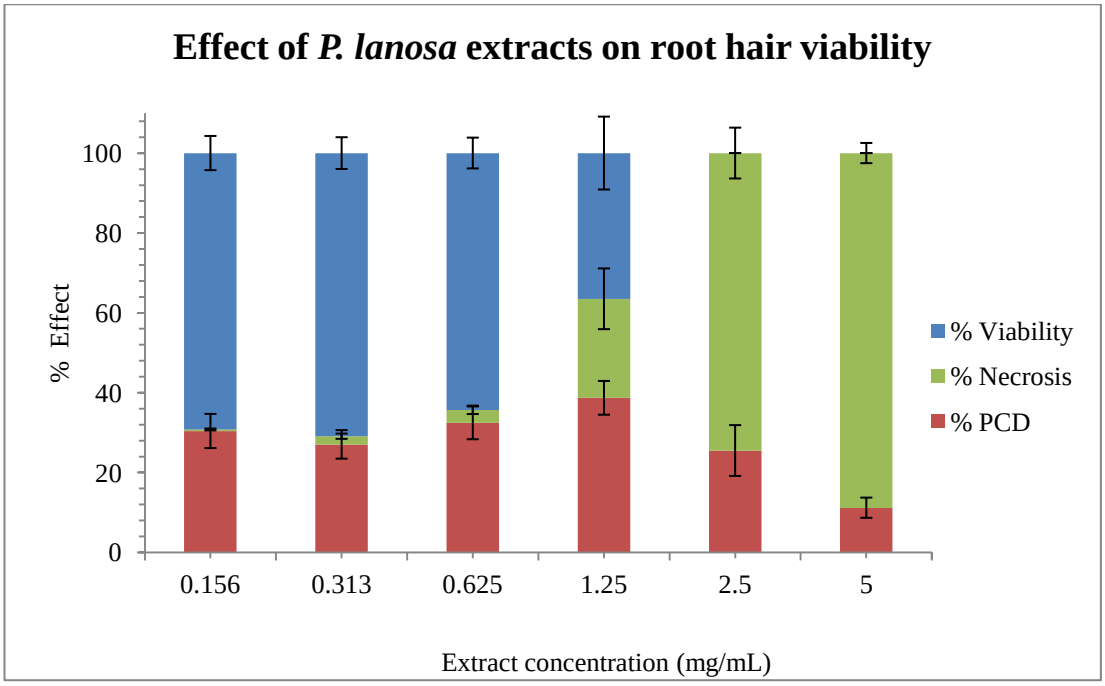


Figure 4.8: Effect of *P lanosa* on the root hair viability of *A. thaliana* over the concentration range 0.156 - 5 mg/mL. Data (n=9) are presented as the mean ± standard error (SE).

Regardless of the results, this root hair assay proved to be an effective method in assessing the phytotoxic potential of seaweed extracts based on morphological changes of dying root hairs as shown in Figure 4.9. No study was identified which had conducted phytotoxicity studies with seaweed extracts using the root hair assay method. This method is generally applied to determine cell death in response to various treatments including elevated temperatures and exposure to toxins (462, 463, 519). The majority of studies evaluating the potential toxicity of natural products assess its effects on germination and development of a specific plant species (520, 521). For example, Imatomi *et al.* investigated the phytotoxic effects of the aqueous leaf extracts of Myrtaceae species on both the germination and development of three weeds (522). It was found that the majority of extracts resulted in a significant delay in germination and inhibited the growth of all seedlings.

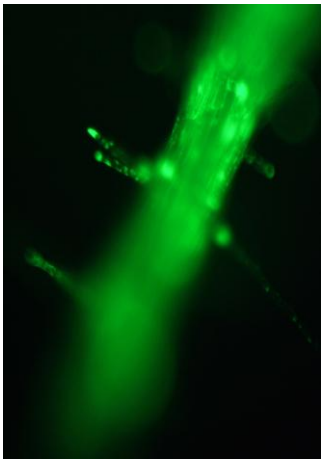
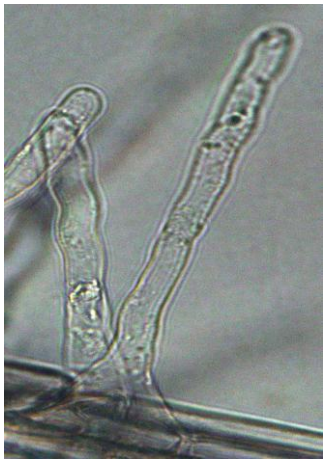
(a)  (b)  (c) 

Figure 4.9: Effect of different concentrations of crude *P. lanosa* extracts on the root hairs of *A. thaliana* stained with FDA and examined under a fluorescent microscope at 485 nm: (a) living root hair at 0.156 mg/mL exhibiting green fluoresce (normal), (b) root hair undergone apoptosis-like PCD at 2.5 mg/mL as did not fluoresce and demonstrated cytoplasm condensation and retraction of the protoplast from the cell wall and (c) root hair undergone necrosis at 2.5 mg/mL as did not fluoresce nor exhibit apoptosis-like PCD morphology.

4.4 Conclusions

In conclusion, the extracts from four seaweed species demonstrated antimicrobial activity against at least one of the tested bacteria. *U. lactuca* was the only seaweed that did not demonstrate any antibacterial activity. This confirms that seaweeds contain antibacterial compounds even in low concentrated extracts (1:50 *v/w*) as used in this study, compared to other studies where much more concentrated extracts were applied including 1:25 *v/w* or 1:5 *v/w* indicating the high potency of seaweed extracts in inhibiting bacterial growth at low concentrations. This study looked at the potential antibacterial capacity of seaweed extracts against plant bacterial pathogens as opposed to human pathogens, illustrating its degree of novelty.

P. lanosa exhibited the broadest range of activity compared to the other four species with activity found against eight of the nine tested bacteria with the methanol extract exhibiting the maximum activity. Therefore, this extract was selected for an investigation into the optimum sterilisation method including syringe filtration and heat sterilisation. Heat sterilisation was found to be the best method and demonstrated the thermal-stability of the antimicrobial compounds present in the methanol extracts against the phytopathogen, *X. fragariae*. This investigation also demonstrated the fact that different antimicrobial compounds are present in a seaweed extract and are active against different pathogens. For example, the antimicrobial compounds in the methanol extract of *P. lanosa* active against *X. arboricola* were found to be thermally unstable.

The MIC of the autoclaved sterilised methanolic extracts was determined to be 6.25 mg/mL against *X. fragariae*, demonstrating the efficacy of the bioactive(s) present in *P. lanosa*. The MBC was above the tested concentration range (<25 mg/mL), which can be expected since a higher concentration of the antimicrobial compound is typically required to completely eliminate the bacteria.

The formation of biofilms is an important adaption affecting the survival and persistence of bacteria in various habitats. Unfortunately, this makes biofilms a major problem in the healthcare sector, with bacterial biofilms requiring up to 1,000 times more antibiotics to kill cells within a biofilm compared to their planktonic counterparts (523). Biofilm formation also occurs in bacterial plant pathogens, which protects the bacteria from desiccation, UV radiation, other environmental stresses as

well as the plants immune system and antimicrobial treatments. The antibiofilm potential of the methanol extract of *P. lanosa* was assessed against this problematic pathogen. The same concentration of crude extract required for MIC inhibited biofilm formation by over 80% with MBEC50 and MBEC90 values of 3.125 mg/mL and 6.25 mg/mL. This suggests that the methanol extract of *P. lanosa* might be a potential antibiofilm agent capable of inhibiting biofilm formation and/or disruption of an already established biofilm. The crude *P. lanosa* extracts were found to be phytotoxic to *A. thaliana* in a dose response manner with isolation and purification of the active bioactive(s) required to allow for a more accurate phytotoxicity study. However, this result highlighted the potential use of crude *P. lanosa* as a natural herbicide for weed species or other problematic plants.

This is the first study on the antibacterial and antibiofilm activity of *P. lanosa* extracts against *X. fragariae*. Additionally, this is the first report of using the root hair assay to determine the phytotoxicity of a seaweed extract on a model plant adding to the novelty of this study.

The methanol extract of *P. lanosa* was selected for further study in Chapter 5 to investigate the effect different extraction techniques had on yield and antibacterial activity. A separation and purification process of this seaweed extract was also conducted in Chapter 6 with the aim of identifying the compounds responsible for this antibacterial activity.

CHAPTER 5: INVESTIGATION INTO THE EXTRACTION OF ANTIBACTERIAL COMPOUND(S) FROM *P. LANOSA*

*The dose response data in this chapter have been published in the *Journal of Bacteriology and Mycology* (O' Keeffe, E., Hughes, H., McLoughlin, P., Tan, S. P. and McCarthy, N. (2019) Antibacterial activity of seaweed extracts against plant pathogenic bacteria. **6**(3): 1105).

5.1 Introduction

Seaweeds are considered a significant source of bioactive compounds that have been found to exhibit a broad spectrum of biological activities such as antimicrobial activity (335); management of body weight and obesity (24); anticancer (43); anti-inflammatory (40) and pesticidal activity (230). However, a number of steps are needed before these bioactives are obtained with a clean up of the seaweed samples carried out first to remove sand, epiphytes and other forms of debris, followed by the drying of the fresh seaweed samples to prevent microbial and enzymatic degradation during the storage period (524). The third step is extraction which is needed to separate the desired compounds from the seaweed. Conventional extraction methods are commonly used and include solvent extraction and Soxhlet extraction; each having its own advantages and disadvantages.

Solvent extraction is the simplest method and is commonly used for the extraction of bioactives from seaweeds (333, 525) and was, therefore, the method of choice for chapters 2 to 4. This technique involves the blended seaweed sitting in the extraction solvent for a specific set of time, with or without homogenization at a certain temperature until the solvent is saturated in bioactive(s) (526). However, extraction over extended periods and/or at high temperatures can result in the degradation of unstable compounds and this method is also prone to high solvent usage (79). Soxhlet extraction is also another popular conventional technique (53, 64). It follows the principle of refluxing and siphoning to allow the bioactives to be continuously extracted with fresh solvent. It is considered more efficient than solvent extraction and generally requires less time and solvent (79). For instance, Zakaria *et al*. reported that their Soxhlet extracts exhibited more profound radical scavenging activity compared to the extracts generated from solvent partitioning (liquid-liquid extraction) (86). The main limitation with this technique is that it is not suitable for the extraction of thermo-labile compounds as the sample is constantly heated (526).

Sonication is commonly used for the cleaning of materials, extraction and other chemical processes (527). Sonication employs ultrasound ranging from 20 kHz to 2000 kHz (528), which produces the mechanical effect of acoustic cavitation that accelerates surface contact between the solvent, sample and permeability of cell walls. Both the physical and chemical properties of the sample are altered and

disruption of the plant cell wall results in the release of compounds, whilst the mass transport of the extracting solvent into the plant cells is also enhanced and is, therefore, known as UAE (529). UAE is a simple, relatively low cost, efficient alternative to conventional techniques suitable for both small and large phytochemical extractions (89). It exhibits the benefits of a reduced extraction time and solvent consumption. Crespo *et al.* investigated the potential of extracting hydrocarbons from various seaweed samples using UAE and MAE (530). It was found that UAE was the more suitable technique achieving higher recovery yields compared to MAE with hexane as the extraction solvent. However, the formation of free radicals at irradiation above 20 kHz may have a negative effect on the active phytochemicals (527).

Selection of the extraction solvent also plays a major part regardless of the technique chosen, as its polarity must achieve maximum recovery of the target compounds (10). Therefore, if the target compounds are hydrophilic in nature, polar solvents such as methanol, water, ethanol and acetone are utilised, while for lipophilic compounds, hexane, chloroform or other non-polar compounds are employed. However, if the polarity of the bioactive(s) is unknown, a number of different solvents that vary in polarity must be used. Extraction conditions will also affect the extraction efficiency including temperature, with higher temperatures generally resulting in higher yields due to increased solubility and diffusion (79). Hwang *et al.* reported such an affect with extraction conducted at 100°C achieving the highest yield for *Porphyra tenera* (531). If the temperature is too high, it can cause the evaporation of the extracting solvent and the decomposition of thermolabile components leading to a loss in activity.

The particle size of the seaweed extracts can also affect extraction efficiency with the smaller (finer) the particle size, the greater the surface area leading to improved penetration of solvents and diffusion of solutes. Saleh *et al.* observed this trend with the particle size having a significant effect on the methanol extraction yield of *Silybum marianum*, where an extraction yield of 8.5% was obtained for normally ground seeds compared to the 10% yield achieved for the nano sized particles (532). However, if the particle is too small it can result in excessive absorption of solute in the solid matrix and subsequent difficulty in filtration (79). The feed:solvent ratio has also been shown to affect extraction efficiency with higher ratios generally

resulting in greater extraction yields (79). The extraction duration is a major factor in extraction methods with an increase in extraction efficiency observed with longer times. Within a certain time range equilibrium will occur leading to no further improvement in yields and more importantly, excessive time in the extracting solvent could potentially result in the breakdown of unstable bioactives (78).

Therefore, it is essential that these parameters be investigated to ensure optimum extraction conditions are being applied to achieve the highest possible yield, whilst reducing the use of solvents and costs. There are other newer techniques available such as SFE and MAE that offer some advantages over these conventional techniques including lower solvent consumption, shorter extraction time and higher selectivity. They have become popular extraction techniques for natural products (533-535) including seaweeds (93-95). However a major drawback with these techniques is the initial start up cost of the equipment which can be very high and hence, not always justifiable for small companies.

Therefore, before selecting a technique one must consider the cost, time and most importantly the associated properties of the compounds of interest. Such considerations influenced the main objectives of this study which was to investigate the effect of time and feed:solvent ratio on the extraction efficacy of two conventional extraction methods: solvent extraction and Soxhlet extraction and one non-conventional technique UAE with regards to yield and antibacterial activity. The red seaweed, *P. lanosa*, was used in this study as it exhibited both the strongest and broadest spectrum of activity against the tested plant pathogens in Chapter 4, particularly the methanol extract. Methanol was therefore applied as the extracting solvent for all techniques. Whilst the extraction time and feed:solvent ratio was varied, all other parameters remained constant. The newly generated extracts were tested for differences in activity against the indicator bacteria, *Xanthomonas fragariae* Kennedy and King emend. (ATCC 33239) using the disk diffusion assay.

5.2 Experimental Procedure

5.2.1 Optimisation of extract concentration

This study examined the effect of various concentrations of the methanol extract of *P. lanosa* against *X. fragariae* and hence, determined the optimal extract concentration. The concentrations investigated included: 1, 2, 3, 4, 5, 7.5 and 10 mg/mL using the standard disk diffusion assay recommended by the CLSI (454). The disk diffusion method was described in section 4.2.3 and involved re-dissolving the dried extracts in methanol to give a concentration of 100 mg/mL. The extracts were loaded onto 6 mm blank disks at a rate of 10 μL, with each load equivalent to 1 mg/disk. Hence, 10 μL = 1 mg/disk, 20 μL = 2 mg/disk, 30 μL = 3 mg/disk, 40 μL = 4 mg/disk, 50 μL = 5 mg/disk, 75 μL = 7.5 mg/disk and 100 μL = 10 mg/disk. Disks loaded with an equivalent quantity of methanol served as the negative control and disks containing 10 μg/disk of chloramphenicol (Oxoid, Basingstoke, UK) served as the positive control. All disks were allowed to dry to remove the solvent and the entire process was carried out under aseptic conditions. The extracts were tested against *X. fragariae* using the 0.5 on the McFarland turbidity standard. The plates were incubated for 24 h at 27 °C. The diameter of the zones of inhibition were measured as a clear zone of no bacterial growth around the disk. The antibacterial activity of the seaweed extract was tested in triplicate and repeated in duplicate.

5.2.2 Effect of time and feed:solvent ratio for solvent extraction yields

For this investigation, the experimental conditions are outlined in Table 5.1 with both feed:solvent ratio and time the two varying factors. The seaweed powders were extracted in methanol (99.8%) at the three different feed ratios over the set time periods. Approximately 3 g of the seaweed powder was extracted in 75 mL (1:25 *w/v*), 150 mL (1:50 *w/v*) and 300 mL (1:100 *w/v*) of methanol. The solutions were stirred with a magnetic stirrer at room temperature. Once the specific time had elapsed, the solid seaweed particles were removed from the solvent containing the bioactives by filtering under a vacuum with Whatman No. 1 filter paper (11 μm pore size, Whatman, Kent, UK). The solvent was rotary evaporated under vacuum (Biddy heated water bath, Heidolph Laborota 4000 motor unit condenser), at temperatures

no higher than 30 °C, to yield a crude dried extract. The extract was re-dissolved in a minimum amount of methanol so they could be transferred to pre-weighed amber bottles. Methanol was removed from the bottles via the nitrogen concentrator and the dried crude extract was stored at -20 °C until further analysis. Extractions were completed in triplicate for all the different experimental conditions.

Table 5.1: Solvent extraction conditions for methanol extracts of *P. lanosa*.

Parameter	**Experimental condition**
Solvent	Methanol
Temperature (°C)	Room temperature
Particle size (μm)	850 μm powdered seaweed
Feed:solvent ratio (*w/v*)	1:25, 1:50 and 1:100
Time (h)	1 h, 2 h, 6 h, 12 h and 24 h

5.2.3 Effect of time and feed:solvent ratio for Soxhlet extraction yields

As with the previous extraction technique, feed:solvent ratio and extraction time were varied for Soxhlet extraction, as shown in Table 5.2, to determine the effect that these parameters have on extraction yield and antibacterial activity of the resultant extracts. Approximately 6 g of the seaweed powder was placed in a thimble and extracted in 150 mL (1:25 *w/v*) of methanol, 3 g of the seaweed powder was extracted in 150 mL (1:50 *w/v*) of methanol and 1.5 g of the seaweed powder was extracted in 150 mL (1:100 *w/v*) of methanol. The methanol was removed using rotary evaporation as per the solvent extraction procedure (section 5.2.2) followed by concentration of the samples to yield dried extracts which were stored at -20 °C until further analysis. Extractions were completed in triplicate for all the different experimental conditions.

Table 5.2: Soxhlet extraction conditions for methanol extracts of *P. lanosa*.

Parameter	Experimental condition
Solvent	Methanol
Temperature (°C)	64.7°C – boiling point of methanol
Particle size (μm)	850 μm powdered seaweed
Feed:solvent ratio (w/v)	1:25, 1:50 and 1:100
Time (h)	2 h, 4 h, 6 h, 8 h and 10 h

5.2.4 Effect of time and feed:solvent ratio for ultrasound-assisted extraction yields

Table 5.3 demonstrates the parameters investigated for the UAE of antibacterial compounds from *P. lansoa* with the feed:solvent ratio and time varying. Approximately 3 g of the seaweed powder was extracted in 75 mL (1:25 *w/v*), 150 mL (1:50 *w/v*) and 300 mL (1:100 *w/v*) of the extracting solvent methanol. The samples were extracted in an ultrasonic bath (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) for the specific time at room temperature. To prevent the rise in temperature during sonication, ice water was circulated through the treatment chamber. After extraction, the same procedure was applied as in solvent extraction to remove the solvent via rotary evaporation and concentrating down the samples to yield dried extracts (section 5.2.2). The extracts were stored at -20 °C until required for further analysis. Extractions were completed in triplicate for all the different experimental conditions.

Table 5.3: UAE conditions for methanol extracts of *P. lanosa*.

Parameter	Experimental condition
Solvent	Methanol
Temperature (°C)	Room temperature
Particle size (μm)	850 μm powdered seaweed
Frequency (kHz)	40 kHz (set)
Feed:solvent ratio (*w/v*)	1:25, 1:50 and 1:100
Time (h)	1 h, 2 h, 3 h, 4 h and 5 h

5.2.5 Comparison of the antibacterial activity of P. lanosa extracts generated from solvent extraction, Soxhlet extraction and UAE

A comparative study to examine the efficacy of the three extraction techniques in extracting antibacterial compounds for *P. lanosa* with the solvent methanol was performed using the disk diffusion method. The antibacterial activities of these crude extracts were tested against the indicator bacteria, *X. fragariae* as described in section 4.2.3 at a concentration of 5 mg/disk. The extracts were assessed in triplicate, repeated in duplicate and were reported as the mean ± SD.

5.2.6 Statistical analysis

The concentration study data was analysed with repeated measures using one-way ANOVA followed by a post-hoc analysis using Tukey's multiple comparison tests. These analyses were performed by Minitab 17 Statistical Software using a 5% statistical significant level (ρ<0.05). The results were said to be statistically different if ρ<0.05 and were designated with different superscripts. Factorial design was completed on each extraction technique with the two factors feed:solvent ratio and extraction time, with three levels for feed:solvent ratio and five levels for extraction

time. The extraction yields and antibacterial activity data was also analysed using one-way ANOVA followed by a post-hoc analysis using Tukey's multiple comparison tests to determine the effect of time and feed ratio for each technique. Determination into whether a 2-way interaction between both parameters existed was performed by the use of the factorial design feature present in Minitab 17 Statistical Software. The extractions were performed in triplicate and the antibacterial assays were performed in triplicate and repeated in duplicate. All results were presented as the mean ± SD, unless otherwise stated.

5.3 Results and Discussion

5.3.1 Dose response effect of the antibacterial activity of the crude P. lanosa extracts

The concentration study found that the seaweed extracts exhibited a dose response effect against the phytopathogen, *X. fragariae* with an increase in concentration resulting in greater activity as shown in Table 5.4. The 1 and 2 mg/disk concentrations produced no activity, while the 10 mg/disk concentration produced the highest activity with an inhibition zone of 18.33 ± 1.2 mm.

Table 5.4: Concentration study of the antibacterial activity of crude P. *lanosa* extracts using the disk diffusion assay at 1-10 mg/disk against *X. fragariae*.

Extract	**Antibacterial activity (mm)**						
	1 mg/disk	**2 mg/disk**	**3 mg/disk**	**4 mg/disk**	**5 mg/disk**	**7.5 mg/disk**	**10 mg/disk**
P. lanosa	0^{a}	0^{a}	8.3 ± 1.20^{b}	10.2 ± 0.40^{bc}	14.3 ± 1.00^{c}	15.7 ± 0.82^{c}	18.3 ± 1.20^{d}
Positive control[a]	28.5	28.5	29	29	29.5	28	28.5
Negative control[b]	0	0	0	0	0	0	0

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

Kolanjinathan *et al*. also reported this dose response effect when the ethanol extracts of *Gracilaria edulis, Calorpha peltada* and *Hydroclothres* sp. were screened against six bacterial pathogens (446). The crude extracts of *C. peltada* tested against *Bacillus cereus* exhibited a zone of inhibition of 27 ± 0.2 mm at 1% concentration, while at 0.6% inhibition reduced to 1.1 ± 0.2 mm and no activity was observed at 0.3%. Rattaya *et al.* also found this concentration effect for the ethanol and methanol extracts of *Turbinaria ornata* and *Sargassum polycystum* (536). The concentrations investigated included: 0, 100, 200, 300, 400 and 500 mg/L, with only the 500 mg/L extract concentration demonstrating activity against *Staphylcoccus aureus*.

This result also determined the optimum concentration to be used for the investigation into the effect of extraction time and feed:solvent ratio on the antibacterial activity of *P. lanosa* extracts using various extraction techniques. The concentration applied must not over-saturate the disk which could result in not all of the bioactives diffusing into the agar. This in turn would lead to no difference in inhibition zones, regardless of the fact that more bioactives were applied. As a result, an increase in activity would not be identified. From Figure 5.1 it can be seen that even up to 10 mg/disk, the disk is not over saturated, with differences in zones of inhibition observed. The extract concentration selected for the remainder of this study was 5 mg/disk as this required less crude extract and time spent in preparing the disk compared to if the 10 mg/disk was applied. Additionally, the 5 mg/disk concentration still produced a clear zone of inhibition to ensure accurate interpretation of results.

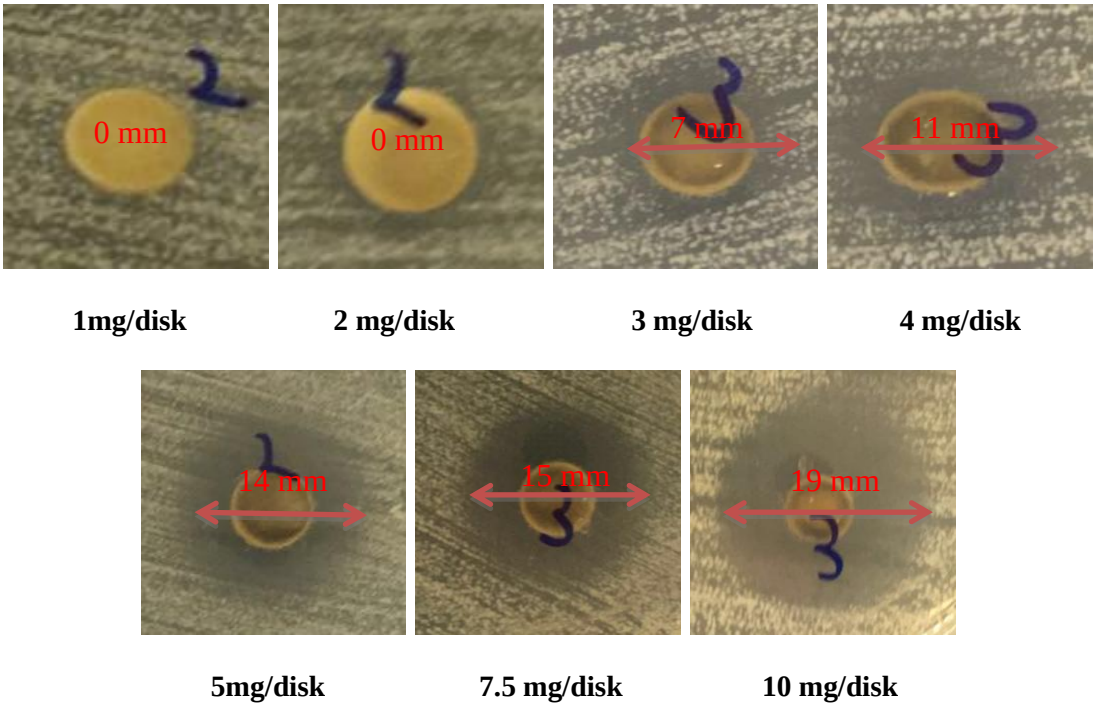


Figure 5.1: Dose response effect of crude methanol extracts of *P. lanosa* at 1, 2, 3, 4, 5, 7.5 and 10 mg/disk against *X. fragariae*. Data (n=3).

5.3.2 Determination of the effect of time and feed:solvent ratio on the extraction efficiency

5.3.2.1 Solvent extraction

Solvent extraction was the first technique to be investigated with regards to the effect of time and feed:solvent ratio on extraction yields. The extraction times selected were 1 h (357), 2 h (357, 431), 6 h (42), 12 h (537) and 24 h (65), commonly used times in solvent extraction. Although some studies have conducted extractions for longer periods such as 48 h (403), 72 h (538) and 7 days (436) for the extraction of antimicrobial compounds, such a prolonged exposure to the extracting solvent can lead to the degradation of labile compounds resulting in reduced activity (96). The effect of feed:solvent ratio was also investigated with three ratio's 1:25, 1:50 and 1:100 *w/v* used in this study, although other ratios have being applied including 1:10 - 1:70 *w/v* (539).

Table 5.5 demonstrates the mean results obtained for the % yields of *P. lanosa* extracts at the specific times and feed ratios. It was found that as extraction time

increased so did the percentage yields. Since the longer the extraction periods the greater the likelihood that more compounds would be extracted into the liquid phase. Dayuti *et al.* also reported this when investigating the effect of solvent and extraction time on antibacterial activity of the red seaweed, *Gracilaria verrucosa* against *E. coli* and *Salmonella typhimurium*. The highest yield and inhibition activity among all the solvent extracts was achieved with an extraction time of 72 h as opposed to 24 h and 48 h extracts (525). This effect of time was also observed in Chapter 2 section 2.3.3 where the % yields for 24 h extraction were generally higher than the 2 h extractions.

Table 5.5: Extraction yields of the methanol extracts of *P. lanosa* at different times and feed:solvent ratios for solvent extraction.

Time (h)	**Average % yield**		
	1:25 *w/v*	**1:50 *w/v***	**1:100 *w/v***
1 h	2.9 ± 0.003^{ax}	3.4 ± 0.002^{ax}	3.1 ± 0.001^{ax}
2 h	3.3 ± 0.002^{ax}	3.6 ± 0.001^{ax}	2.9 ± 0.007^{ax}
6 h	4.7 ± 0.010^{abx}	5.9 ± 0.014^{ax}	5.6 ± 0.003^{abx}
12 h	6.8 ± 0.004^{bx}	10.4 ± 0.016^{bxy}	8.2 ± 0.017^{bcy}
24 h	9.1 ± 0.014^{cx}	11.8 ± 0.012^{bx}	10.6 ± 0.018^{cx}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different in terms of yield for the various times are denoted by a, b, or c and for the three feed:solvent ratios by x, y or z at the specific extraction time (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

However, statistical analysis using Tukey's multiple comparison tests carried out on the results found that the yields remained constant after 12 and 24 h for the 1:50 and 1:100 *w/v* feed:solvent ratios. This was due to equilibrium of the solute being reached inside and outside of the solid material, meaning increasing time had no further affect on yield (79). This is known as Flick's law and is driven by the

difference in the concentration between the seaweed matrix and the extracting solvent (540). Mokrani *et al.* also observed this phenomenon in which the extraction yield of phenolic content and antioxidant capacity from peach fruit remained unchanged after 180 min (361). Tukey's multiple comparison test was also carried out on the results regarding the three feed:solvent ratios and no effect was discerned on % yields for each extraction time, except for the 12 h extraction which showed a statistical difference between the 1:25 and 1:100 *w/v*. This is not uncommon, since the greater the solvent to solid ratio, the higher the extraction yield (79).

A main effects plot was constructed on minitab by plotting the mean response for each factor of the two categorical variables, time and feed:solvent ratio (Figure 5.2). A line connects the points for each variable and by examining this line one can determine whether a main effect is present for that categorical variable (541). For instance, a horizontal line (parallel to the x-axis) means there is no main effect present and hence, the response mean is the same across all of the tested levels. A non-horizontal line means a main effect does exist and the steeper the slope the higher the magnitude of the effect. Figure 5.2 demonstrates that extraction time was a significant main effect on the % yield of *P. lanosa* achieved, with a steep slope observed and ρ-value below the significant level. It appears that the 24 h extraction is associated with the highest yield and one-way ANOVA analysis on all yields regardless of feed:solvent ratio indicated that this main effect was statistically significant ($\rho<0.05$). Figure 5.2 also demonstrates the main effect plot for the feed:solvent ratio and found that it also exhibited a main effect ($\rho>0.05$), but not to the same degree as extraction time since the slope is of less magnitude. However, the plot showed that 1:50 *w/v* produced the highest % yield, but when one-way ANOVA analysis on all yields regardless of extraction time was carried out, this indicated that this main effect was not statistically significant ($\rho=0.376$, $\rho>0.05$). The difference was most likely as a result of natural variability present in the seaweed samples.

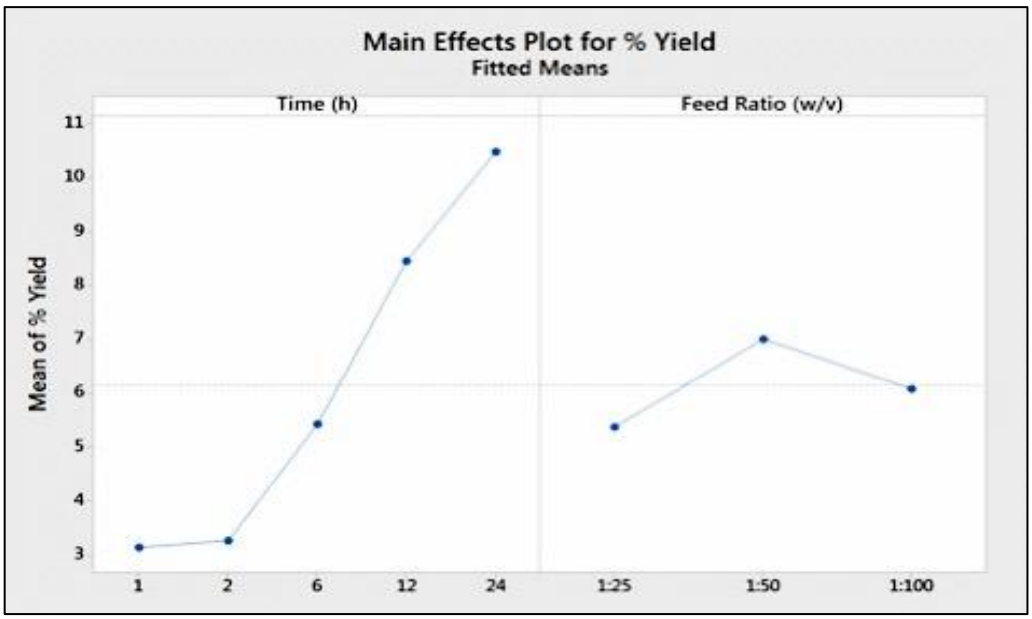


Figure 5.2: Main effects plot for % yield of *P. lanosa* using solvent extraction for the categorical variables time (h) and feed:solvent ratio (*w/v*).

An interaction plot was then constructed to determine whether the effect of one variable e.g. time depends on the level of the other variable e.g. feed:solvent ratio. If this is the case then a 2-way interaction exists meaning the independent variables interact or combine to affect the response (542). The interaction plot obtained for these results (Figure 5.3) exhibited relatively parallel lines and, therefore, (the ρ-value was greater than 0.05 (ρ=0.175)) there was no interaction between time and feed:solvent ratio when all other parameters remained constant.

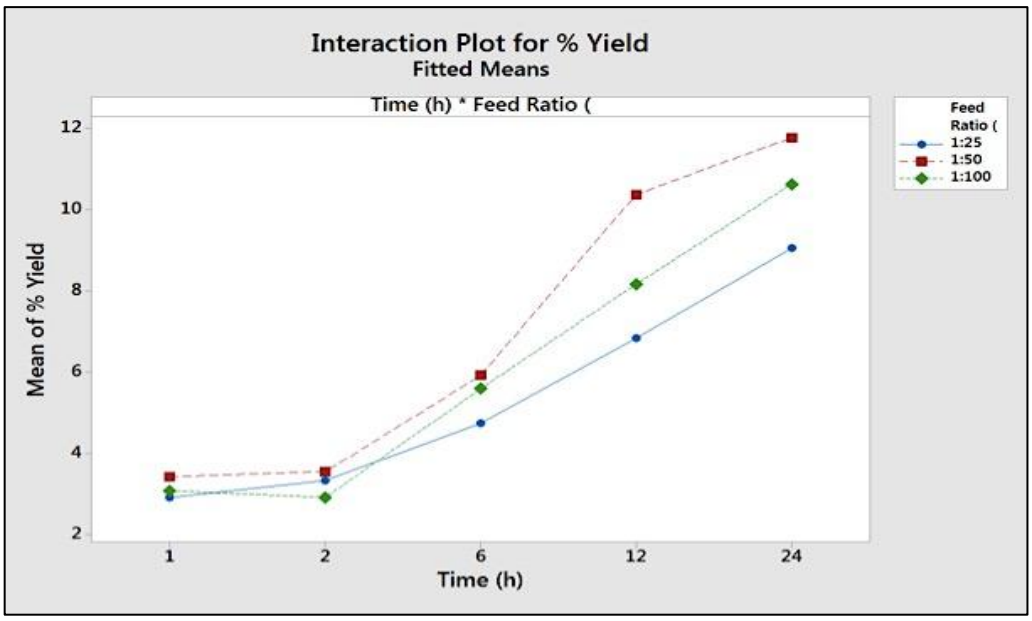


Figure 5.3: Interaction plot of the variables time and feed:solvent ratio on the % yield of *P. lanosa* extracts using solvent extraction.

5.3.2.2 Soxhlet extraction

Soxhlet extraction is another classic extraction technique and is based on the power of the extracting solvent coupled with the application of heat in order to obtain the desired bioactives (543). The times selected for the Soxhlet extraction were similar to that of solvent extraction with the longest time being 10 h due to the fact that the thermolability of these bioactives is unknown and, therefore, extended exposure to methanol at high temperatures could result in degradation of these target compounds and also increase the running costs associated with Soxhlet extraction (544, 545). Soxhlet extraction on seaweeds has been conducted at a variety of extraction times including 4 h (335), 8 h (334) and 24 h (409). Table 5.6 presents the results obtained for the five extraction times investigated with the longer times achieving higher yields. Extraction equilibrium was observed after 6 h with extractions beyond 6 h for 1:25 and 1:50 *w/v* causing no further increase in yield. The 2 h and 10 h extraction yield for the 100 *w/v* were statistically the same and this high extraction efficiency was most likely as a result of the kinetic energy generated by the application of heat causing the rapid diffusion of the bioactives into the liquid phase. Mampouya *et al.* also reported that a 2 h 30 min extraction resulted in 90% of the oil in the safou pulp being extracted with longer extraction times found to be not worthwhile (546). The

feed:solvent ratio exhibited no effect on % yields except for the shortest and longest run time where the 1:100 *w/v* illustrated statistically higher yields compared to the two lower ratios. This could have been as a result of the higher solvent volumes allowing for greater diffusion of the bioactives into the liquid phase. Sulaiman *et al.* described this effect of mass transfer coefficients increasing with solvent to solid ratio by 0.1% (547)

Table 5.6: Extraction yields of the methanol extracts of *P. lanosa* at different times and feed:solvent ratios for Soxhlet extraction.

Time (h)	**Average % yield**		
	1:25 *w/v*	**1:50 *w/v***	**1:100 *w/v***
2 h	6.6 ± 0.004^{ax}	7.6 ± 0.007^{ax}	9.3 ± 0.003^{ay}
4 h	8.2 ± 0.013^{abx}	8.6 ± 0.006^{abx}	9.3 ± 0.003^{ax}
6 h	9.1 ± 0.004^{bx}	10.5 ± 0.005^{bcx}	10.6 ± 0.013^{ax}
8 h	9.7 ± 0.001^{bx}	10.3 ± 0.013^{bcx}	10.9 ± 0.011^{ax}
10 h	9.1 ± 0.009^{bx}	11.7 ± 0.007^{cx}	11.6 ± 0.009^{ay}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different in terms of yield for the various times denoted by a, b, or c and for the three feed:solvent ratios by x, y or z at that specific extraction time (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

Figure 5.4 illustrates the main effects plot for these two variables with % yield increasing with time as shown by the relatively steep slope that continues beyond the tested time range and a ρ-value equal to 0.00 signifying that the different time intervals affects % yield. However, one-way ANOVA analysis found that although yield increases with time, the 6 h, 8 h and 10 h yields, regardless of the feed:solvent ratio's, were statistically the same, once again demonstrating the high efficiency of Soxhlet to achieve complete extraction of the *P. lanosa* bioactives within a 6 h

window further supporting the data in Table 5.6. The feed:solvent ratio was also found to have an effect on % yield (ρ<0.05) with a sharp slope between 1:25 and 1:50 *w/v* ratio before a shallower slope is observed between the 1:50 and 1:100 *w/v* ratios. Therefore, it was not unexpected that statistical analysis found that 1:100 *w/v* produced higher yields in comparison to 1:25 *w/v* due to the greater mass transfer via the higher solvent ratio.

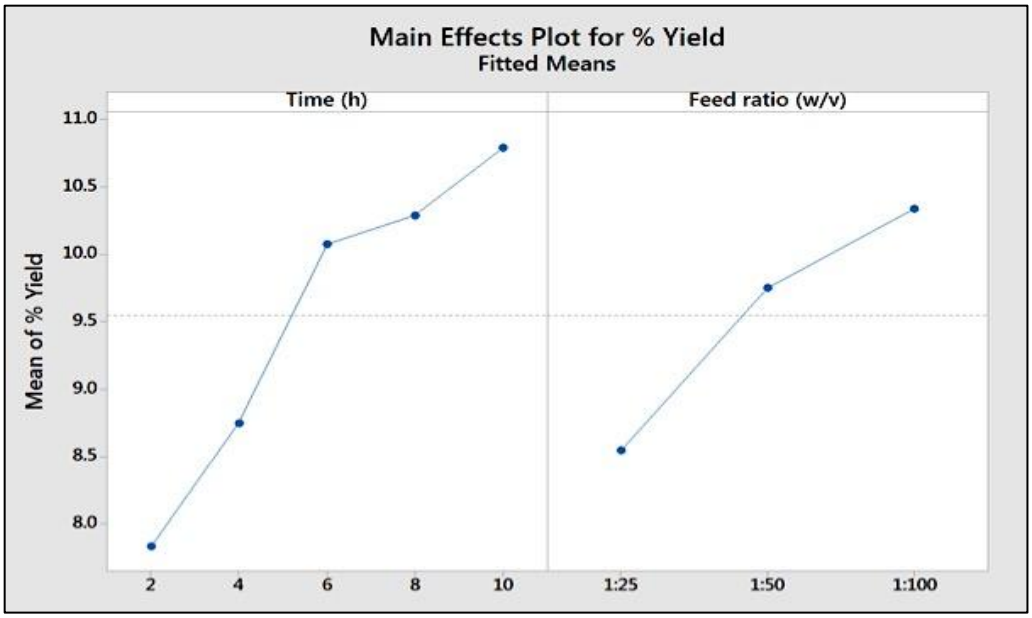


Figure 5.4: Main effects plot for % yield of *P. lanosa* using Soxhlet extraction for the categorical variables time (h) and feed:solvent ratio (*w/v*).

The interaction plot (Figure 5.5) illustrates the non-crossing of lines with a ρ-value of 0.242 and hence, an interaction effect was not present between the two independent variables. This meant that the % yield obtained for one factor e.g. 2 h does not depend on the value of the other factor e.g. 1:25 *w/v*.

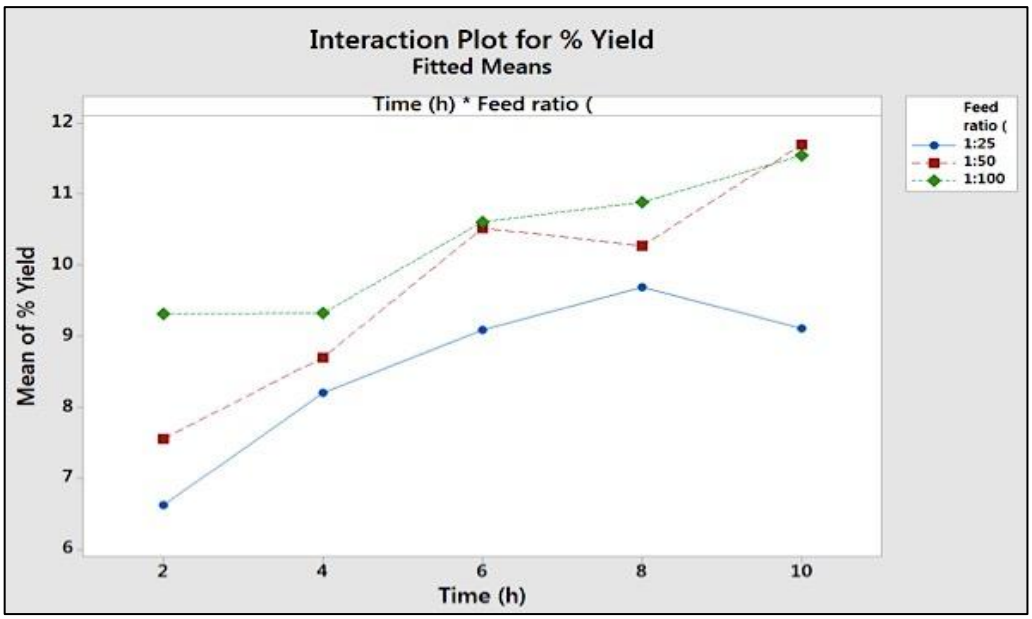


Figure 5.5: Interaction plot of the variables time and feed:solvent ratio on the % yield of *P. lanosa* extracts using Soxhlet extraction.

5.3.2.3 Ultrasound-assisted extraction

Ultrasound is an innovative food processing technology and has shown important advances over the last few years (548). This technology as mentioned before, has also been used in the extraction of bioactives from natural products (549, 550) including seaweeds (551). UAE can be conducted in two ways with the use of an ultrasonic probe or the use of an ultrasonic bath. This study utilised an ultrasound bath. The times selected were shorter than that for solvent extraction since previous studies have applied relatively short extraction times with Kadam *et al*. reporting a 25 min extraction time achieving the highest yields of bioactive compounds from *Ascophyllum nodosum* (551). In addition to this, studies have reported a fall in yield when a longer extraction time was used, as found by Melecchi *et al.* who investigated the effect of extraction parameters including time, sample polarity, sample volume and sample particle size on the ultrasonic extraction of *Hibiscus tiliaceus* with extraction time and sample polarity found to have the greatest influence (552). The extraction time above 300 min (5 h) resulted in a decrease in mass yield from 17.10% at 300 min to 14.60% after 340 min of extraction. This was suspected to be as a result of the decomposition of organic compounds by the sound

waves (552). Therefore, extraction times of 1 to 5 h were selected on the basis of achieving a high enough yield to be tested and also avoiding the possibility of time having a negative effect on % yield. The three feed:solvent ratios (1:25, 1:50 and 1:100 *w/v*) used were the same for all three techniques investigated.

Table 5.7 outlines the % yields obtained for *P. lanosa* due to the influence of extraction times and feed:solvent ratios. As with the previous two techniques, longer extraction times resulted in an increase in yield up to a point after which very little increase in yield was observed. This was due to dynamic equilibrium being reached as a result of the sonic waves rupturing the seaweed cells and extracting out all of the soluble compounds. Zheng *et al.* also investigated the effect of time on the polysaccharide yields obtained from *Artemisia sphaerocephala* (Krasch seeds), with dynamic equilibrium achieved after 120 min of extraction (553). The feed:solvent ratio had no effect on yield except for the longer sonication times of 4 and 5 h with the 1:100 *w/v* illustrating statistically higher % yields. It was suspected that the greater concentration difference between the exterior solvent and solid matrix caused this increase in yield coupled with a longer extraction time. Zheng *et al.* also investigated the effect of liquid:solid ratio on the polysaccharide yields with an increased from 4.12 to 10.96% reported when the liquid:solid ratio was increased from 20:1 to 60:1 (553). Xu *et al.* also investigated the effect of UAE parameters on the antioxidant activity from *Jatropha integerrima* extracts and it was found that as the solvent material ratio was increased from 15:1 to 40:1, antioxidant activity increased by 33% (554). This activity surge was also determined to be as a result of the higher solvent ratio accelerating mass transfer and facilitating the diffusion of the antioxidant compounds into the solvent phase until the mass transfer process reached its maximum (equilibrium).

Table 5.7: Extraction yields of the methanol extracts of *P. lanosa* at different times and feed:solvent ratio for ultrasound-assisted extraction.

Time (h)	**Average % yield**		
	1:25 *w/v*	**1:50 *w/v***	**1:100 *w/v***
1 h	2.7 ± 0^{ax}	2.9 ± 0.005^{ax}	3.0 ± 0.002^{ax}
2 h	3.3 ± 0.003^{bx}	3.6 ± 0.001^{abx}	3.7 ± 0.002^{abx}
3 h	4.0 ± 0.002^{cx}	3.5 ± 0.001^{abx}	4.1 ± 0.005^{bx}
4 h	4.1 ± 0.002^{cdx}	4.2 ± 0.004^{bcx}	5.1 ± 0.002^{cy}
5 h	4.9 ± 0.001^{dx}	4.5 ± 0.003^{cx}	5.4 ± 0.001^{cy}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different in terms of yield for the various times denoted by a, b, or c and for the three feed:solvent ratios by x, y or z at that specific extraction time (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

Figure 5.6 shows the main effects plot and illustrates that extraction time once again was shown to have a strong impact on the % yield compared to feed:solvent ratio. Both parameters did have an effect on yield with the ρ-values of extraction time and feed:solvent ratio below the significance level of 0.05, indicating that time and feed:solvent ratio are associated with different yields. Although the yield appears to continually increase with time, statistical analysis using one-way ANOVA, regardless of feed:solvent ratio, found that the 4 h and 5 h extractions were statistically the same clarifying that dynamic equilibrium had been reached. However, the feed:solvent ratio was much smoother with the line connecting the 1:25 and 1:50 *w/v* almost horizontal, demonstrating the little impact it had on % yields. A sharp increase was observed for the 1:100 *w/v* ratio, but one-way ANOVA analysis revealed that all three ratios were not statistically significant from one another (ρ>0.05).

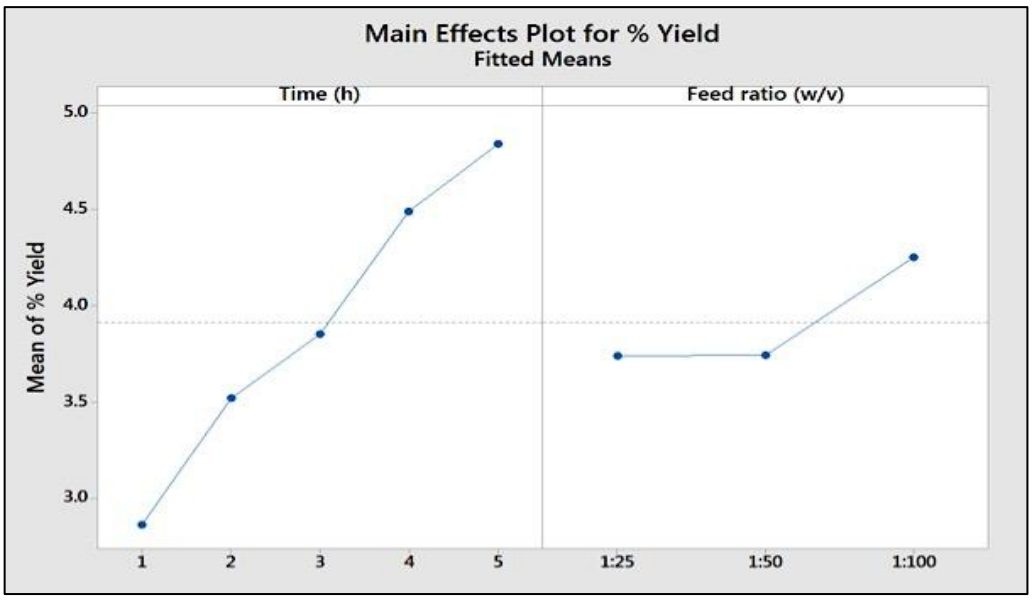


Figure 5.6: Main effects plot for % yield of *P. lanosa* using UAE for the categorical variables time (h) and feed:solvent ratio (*w/v*).

The interaction plot (Figure 5.7) shows non-parallel lines that cross at times and with an interaction effect was established (ρ=0.02) between the two variables. This meant that the response mean for the level of one factor does depend on the value of the other factor, hence, there was an interaction between time and feed:solvent ratio.

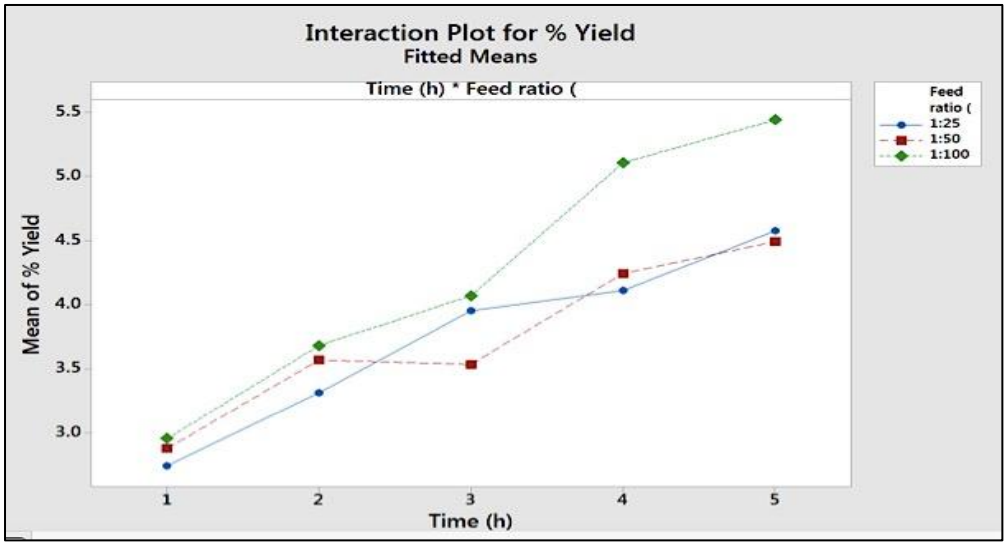


Figure 5.7: Interaction plot of the variables time and feed:solvent ratio on the % yield of *P. lanosa* extracts using UAE.

5.3.3 Determination of the effect of time and feed ratio on the extraction of antibacterial compounds from P. lanosa

5.3.3.1 Solvent extraction

Determination of the effect of extraction time and feed:solvent ratio on % yield is an important consideration in order to resolve the optimum experimental conditions that achieve the highest yields whilst operating at the shortest run times and lowest solvent consumption. Although sufficient yield is essential, a more important factor is the extraction of the desired bioactives. Longer extraction times may result in high yields but this can also result in reduced activity due to degradation of these bioactives in the organic solvent (78). This meant that a critical part of this study was to determine whether activity was affected by these parameters and for this reason the extracts generated at the shortest and longest times were tested against *X. fragariae,* i.e. the 1 h and 24 h extracts. Figure 5.8 demonstrates the interval plot of the antibacterial activity of *P. lanosa* extracts generated after 1 h and 24 h extractions with 95% confidence interval. A decrease in activity was observed over time with a statistically significant ρ-value of 0.012, indicating that extended exposure to the organic solvent methanol resulted in potential degradation of the desired bioactives. Rosell *et al.* also reported this influence of extraction time on the antibacterial activity of the acetone extracts from the brown seaweed *Nereocystis luetkeana* that had been extracted for 24 h, 48 h and 72 h at room temperature. The 72 h acetone extracts demonstrated a ++ inhibition zone corresponding to 21-30 mm against *Staphylococcus aureus* which was a smaller degree of activity compared to the 48 h extracts that exhibited a +++ inhibition zone corresponding to 31-40 mm against the same pathogen (363).

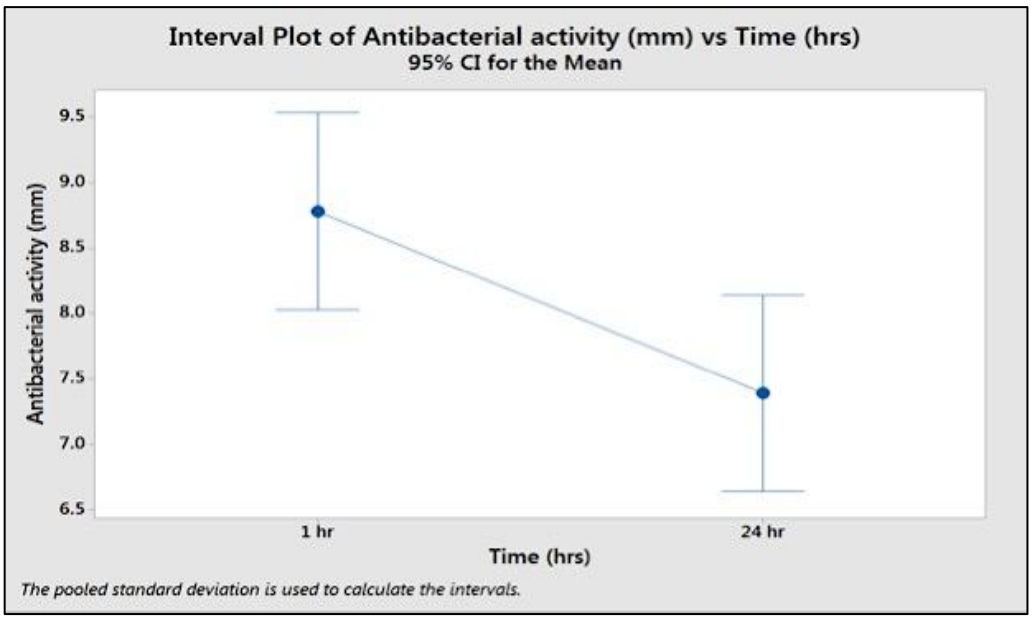


Figure 5.8: Interval plot of the antibacterial activity of *P. lanosa* extracts generated from 1 h and 24 h solvent extraction in methanol against *X. fragariae.*

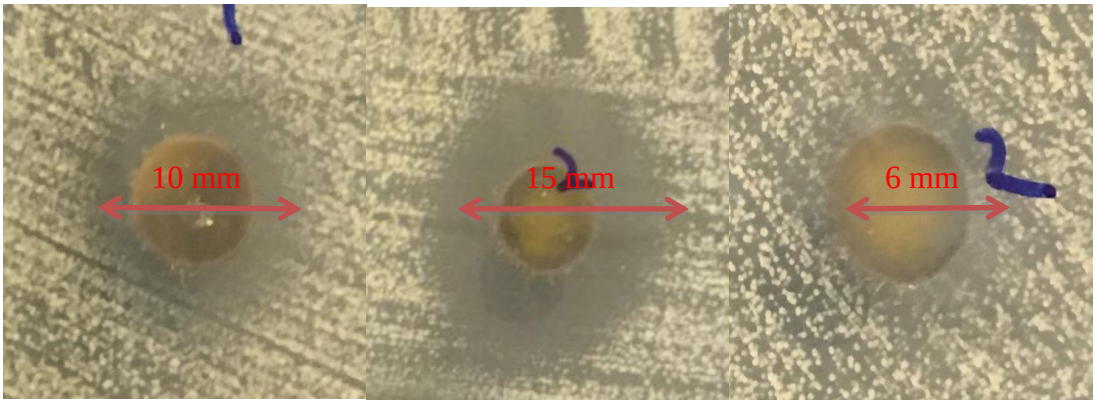
For this reason, all of the extracts generated at 2h, 6 h and 12 h were also tested to establish when this reduction in activity occurred with the results compiled in Table 5.8. It can be can be seen that activity increased with the 2 h, 6 h and 12 h extractions producing the same antibacterial activity with the exception of the 1:50 w/v ratio where the 12 h had reduced activity.

Table 5.8: Antibacterial activity of the methanol extracts of *P. lanosa* using solvent extraction at 5 mg/disk against *X. fragariae.*

Time (h)	Antibacterial activity (mm)		
	1:25 *w/v*	**1:50 *w/v***	**1:100 *w/v***
1 h	8.2 ± 1.33^{ax}	9.8 ± 0.41^{ax}	8.3 ± 2.07^{ax}
2 h	13.2 ± 1.17^{bx}	15.3 ± 0.82^{by}	14.7 ± 1.37^{by}
6 h	14.2 ± 1.83^{bx}	14.5 ± 1.06^{bcx}	15.3 ± 1.64^{bx}
12 h	14.3 ± 2.50^{bx}	12.8 ± 1.47^{cx}	15.0 ± 0.63^{bx}
24 h	7.7 ± 1.86^{ax}	6.8 ± 0.98^{dx}	7.7 ± 1.86^{ax}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different in terms of antibacterial activity for the various times denoted by a, b, or c and for the three feed:solvent ratios by x, y or z at that specific extraction time (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

Therefore, it was concluded that 1 h extraction was an insufficient amount of time for the desired bioactives to be extracted, whereas the 24 h extraction resulted in the degradation of these bioactives. This can be further seen in Figure 5.9 with the zones of inhibition increasing from the 1 h to 6 h extracts before significantly reducing for the 24 h extracts.



1 h **6 h** **24 h**

Figure 5.9: The influence of extraction time on the antibacterial activity of the methanol extracts of *P. lanosa* at 1:50 *w/v* against *X. fragariae*. Data (n=3).

Therefore, the main effect plot demonstrated a steep slope from the 1 h to 2 h extracts, followed by a sharp drop off in activity for the 24 h extracts. Figure 5.10 also demonstrates the effect of the feed:solvent ratio on activity with a relatively parallel line indicating the little to no effect it had on activity. This was further clarified by the ρ-value of 0.478 above the significant level meaning varying feed:solvent ratio did not change antibacterial activity of *P. lanosa* extracts. The interaction effect was found to be non-significant (ρ=0.164) which meant the mean response for the level of one factor did not depend on the value of the other factor.

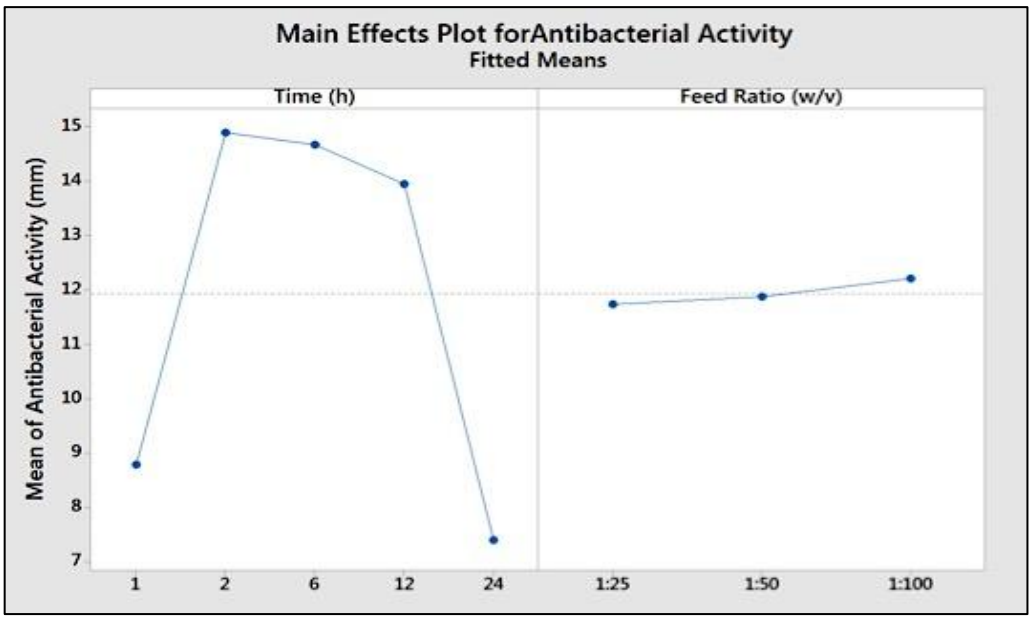


Figure 5.10: Main effects plot for antibacterial activity of *P. lanosa* using solvent extraction for the categorical variables time (h) and feed:solvent ratio (*w/v*).

These results, therefore, suggest that the optimum experimental conditions of time and feed:solvent ratio in solvent extraction are based on antibacterial activity rather than yield, since the activity of the extracts was effected in a negative manner specifically by too short or long a run time. A 6 h extraction time and a feed:solvent ratio of 1:100 *w/v* was concluded as the best experimental conditions since it achieved the greatest antibacterial activity and yield in the shortest time without compromising activity.

5.3.3.2 Soxhlet extraction

The crude extracts generated from Soxhlet extraction at the three feed:solvent ratios for the shortest time point of 2 h and the longest time point of 10 h were assessed for their antibacterial activity against the phtyopathogen *X. fragariae.* Table 5.9 demonstrates the zones of inhibition produced by the extracts and from statistical analysis it was established that neither extraction time nor feed:solvent ratio had any effect on the extraction of the antibacterial compounds from utilising Soxhlet extraction. This verified the high efficiency observed by this conventional extraction technique, which achieved complete extraction after 6 h for the 1:25 and 1:50 *w/v*

and required only 2 h for, 1:100 *w/v*. Numerous studies have also demonstrated this high level of efficiency for Soxhlet extraction with activity reported at a variety of extraction times, including pesticidal activity at 8 h (555), 10 h (64), 24 h (63) and 72 h (556). For instance, Anuradha *et al.* extracted *Halophila ovalis* in ethanol for 2 h using Soxhlet extraction and the extracts were found to exhibit repellent and adulticidal activities against *Culex quinquefasciatus* (557). This activity is believed to be as a result of the phytochemicals constituents such as hexadecanoic acid, glucobrassicin, ethanol, 2-(9-octadecenyloxy)-(Z), etc which were successfully extracted by the Soxhlet technique in a run time of only 2 h. This was also observed in this study with both the 2 h and 10 h extracts demonstrating the same degree of activity. These results also demonstrate the thermal stability of the bioactive(s) compounds present in *P. lanosa* since activity remained even after 10 h of extraction at 64.7 °C.

Table 5.9: Antibacterial activity obtained from the methanol extracts of *P. lanosa* using Soxhlet extraction at 5 mg/disk against *X. fragariae.*

Time (h)	**Antibacterial activity (mm)**		
	1:25 *w/v*	**1:50 *w/v***	**1:100 *w/v***
2 h	12.2 ± 1.60^{ax}	15.0 ± 1.90^{ax}	14.2 ± 2.14^{ax}
10 h	13.8 ± 1.47^{ax}	14.8 ± 1.47^{ax}	15.8 ± 2.48^{ax}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different in terms of antibacterial activity for the various times denoted by a, b, or c and for the three feed:solvent ratios by x, y or z at that specific extraction time (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

Although the main effect plot as shown in Figure 5.11 demonstrates a steep slope between the two points suggesting an effect was evident, analysis of the ρ-value was found to be below that of the significant level (ρ =0.103, ρ>0.05). This meant that time did not have an impact on the extraction of the antibacterial compounds from *P. lanosa*. Table 5.9 also summarises the statistical data between the feed:solvent ratios,

with activity remaining the same for all three feed:solvent ratios for each time point. However, the main effects plot (Figure 5.11) illustrating the statistical analysis on the feed:solvent ratios for both time points together found that a significant difference did indeed exist with a ρ-value of 0.022, particularly between the 1:25 *w/v* and 1:100 *w/v* ratios. This was most likely due to the higher volume of solvent increasing extraction capability of the desired bioactives. Zhang *et al.* also observed this effect where the ethanol extracts of grape leaves generated via Soxhlet extraction produced higher antibacterial activity regardless of increased solid-liquid ratio (558). The bacteriostatic effect on *Staphylococcus aureus* at 1:20, g/mL was 10.18 mm, whereas the 1:50, g/mL was 13.27 mm (558). There was no interaction effect established between these parameters. This was expected since extraction time itself did not affect the antibacterial activity of the seaweed extracts, meaning it did not rely on feed:solvent ratio.

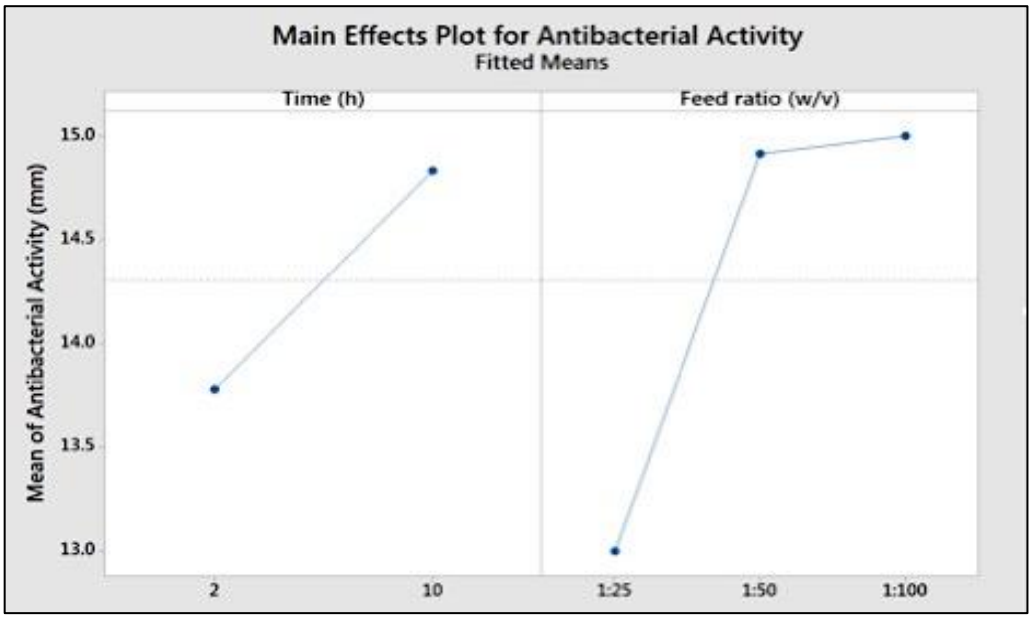


Figure 5.11: Main effects plot for antibacterial activity of *P. lanosa* using Soxhlet extraction for the categorical variables time (h) and feed:solvent ratio (*w/v*).

Therefore, from examining these results it can be seen that extraction time when using Soxhlet is not a defining factor on antibacterial activity compared to the feed:solvent ratio, with the higher the feed:solvent ratio the greater the activity with the 1:50 and 1:100 *w/v* giving statistically the same results.

5.3.3.3 Ultrasound-assisted extraction

The UAE generated extracts at both the shortest and longest extraction points were assessed using the disk diffusion assay to determine the influence that these two parameters had on extracting the desired bioactive compounds. Table 5.10 illustrates the inhibition zones exhibited by these extracts against *X. fragariae.* It can be seen that neither time nor feed:solvent ratio had an effect on the extract activity. This was not unexpected, since one of the advantages of UAE over other conventional methods is the shorter run times required for efficient extraction (559). For instance, Dang *et al.* also reported that the optimum ultrasonic extraction conditions to achieve the highest antioxidant activity included a run time of just 60 min (560).

Table 5.10: Antibacterial activity of the methanol extracts of *P. lanosa* using UAE at 5 mg/disk against *X. fragariae*.

Time (h)	**Antibacterial activity (mm)**		
	1:25 *w/v*	**1:50 *w/v***	**1:100 *w/v***
1 h	8.2 ± 1.47^{ax}	11.0 ± 3.46^{ax}	8.7 ± 1.21^{ax}
24 h	10.5 ± 2.17^{ax}	9.8 ± 1.83^{ax}	7.8 ± 1.72^{ax}

Data (n=3) are presented as the mean ± SD; Data that do not share a common superscript are statistically different in terms of antibacterial activity for the various times denoted by a, b, or c and for the three feed:solvent ratios by x, y or z at the specific extraction time (ρ<0.05; One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test).

The main effect plot (Figure 5.12) exhibited a shallow slope further confirming the limited effect extraction time had on bioactivity, with the main effect ρ-value (0.075) also found to be non-significant. However, the main effect plot showed a steep increase in activity from 1:25 *w/v* to 1:50 *w/v* extracts before another steep drop off was observed for the 1:100 *w/v* extracts. The ρ-value was greater than the significant level meaning that similar to time, the feed:solvent ratio did not change the degree of

activity exhibited by the *P. lanosa* extracts. This meant a 2-way interaction between the variables time and feed:solvent ratio did not exist (ρ=0.095, ρ>0.05).

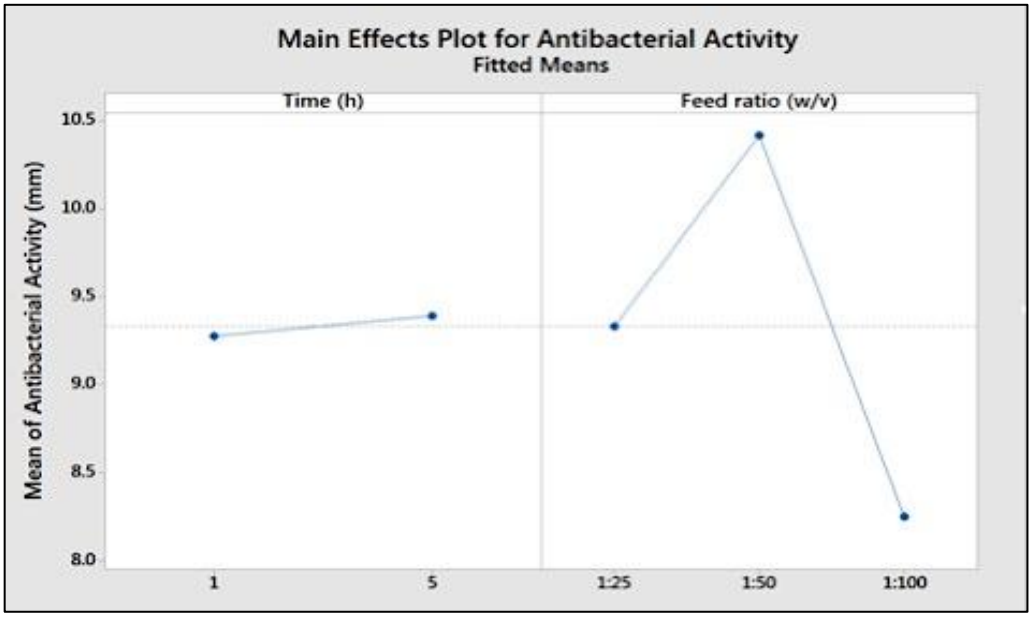


Figure 5.12: Main effects plot for antibacterial activity of *P. lanosa* using UAE for the categorical variables time (h) and feed:solvent ratio (*w/v*).

These results, therefore, suggest that the optimum experimental conditions of time and feed:solvent ratio in UAE are based on the highest yield that can be obtained rather than antibacterial activity, since the activity of the extracts remained unchanged. This further demonstrated the stability of the bioactives against sonic waves which have been previously reported to cause the formation of free radicals at irradiation above 20 kHz (instrument set to 40 kHz in this study) which may have an effect on the active phytochemicals (527).

5.3.4 Optimum extraction technique

From examination of the results obtained for each extraction technique the optimum conditions regarding extraction time and feed:solvent ratio of the *P. lanosa* extracts is shown in Table 5.11.

Table 5.11: Optimum experimental conditions for the extraction of bioactives from *P. lanosa* generated from solvent extraction, Soxhlet extraction and UAE.

Extraction technique	Extraction time (h)	Feed:solvent ratio (*w/v*)
Solvent extraction	12 h	1:100 *w/v*
Soxhlet extraction	6 h	1:50 *w/v*
UAE	4 h	1:100 *w/v*

The parameters for both Soxhlet extraction and UAE were based on optimal yield, since, activity was not affected by these conditions. It was only solvent extraction, specifically short and long extraction times, that had an influence on antibacterial activity and, therefore these conditions were selected on the basis of optimal yield without compromising activity.

For this reason, statistical analysis on the % yields of the extracts generated under each of these conditions was used to determine the optimal extraction technique. Figure 5.13 demonstrates the Tukey's comparison graph for the three techniques and it can be seen that solvent and Soxhlet extraction were statistically the same, producing a statistically higher yield compared to that of UAE.

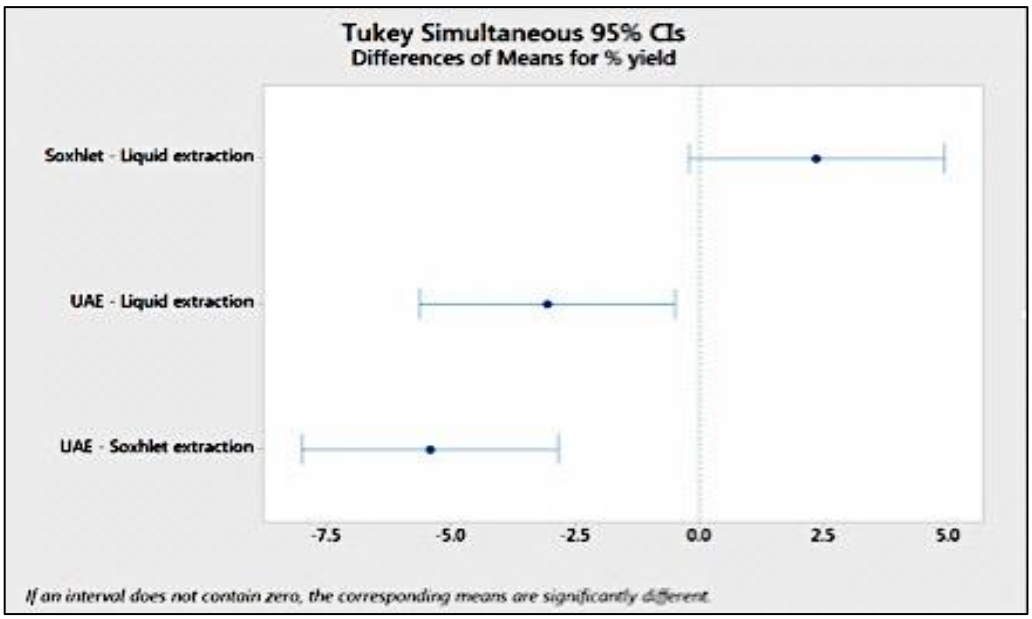


Figure 5.13: Tukey's comparison graph of the % yield generated under the optimum experimental conditions for solvent extraction, Soxhlet extraction and UAE.

Statistical analysis on the antibacterial activity of the generated extracts found that UAE extracts exhibited the lowest activity with the solvent and Soxhlet extracts demonstrating statistically the same activity (ρ>0.05). Therefore solvent extraction and Soxhlet extraction produce equivalent extracts in terms of yield and antibacterial activity.

5.4 Conclusions

A dose dependent effect of the methanol extract of *P. lanosa* against *X. fragariae* was determined in this research with the 1 and 2 mg/disk found to exhibit no activity and a dose response effect observed after this with the 10 mg/disk producing the highest activity, with an inhibition zone of 18.33 ± 1.2 mm. The extract concentration of 5 mg/disk was selected as the concentration to be applied for the remaining studies, since it is a relatively low concentration and results in a clear zone of inhibition to allow for easy determination of the degree of activity.

An investigation into the effect of extraction time and feed:solvent ratio on % yield and antibacterial activity of *P. lanosa* extract generated from three extraction techniques was conducted. Solvent extraction one of the most popular conventional techniques was first to be studied with five extraction times (1 h, 2 h, 6 h, 12 h, and 24 h) and three feed:solvent ratios (1:25, 1:50 and 1:100 *w/v*) investigated, while all other parameters remained constant. It was determined that a 12 h extraction at 1:100 *w/v* was the best since it achieved the maximum yield, whilst still retaining optimal antibacterial activity. Extraction time was found to have the greatest influence with yield increasing over time, but activity decreased at 24 h most likely as a consequence of the degradation of the target compounds.

The second conventional technique to be investigated was Soxhlet extraction with the optimum conditions found to be a 6 h extraction at 1:50 *w/v*. These parameters were chosen on the basis that it was the shortest possible time and lowest solvent ratio with extraction time once again being the defining factor for % yield, although, equilibrium was achieved after 6 h. In addition to this, both extraction time and feed:solvent ratio were found to have no effect on antibacterial activity further demonstrating the stable nature of the bioactives as thermal degradation did not occur after 10 h of extraction at 64.7°C (boiling point of methanol). The non-conventional technique UAE was also examined with extraction time established to have a higher influence on yield compared to feed:solvent ratio. An extraction time of 4 h at 1:100 *w/v* was found to be the best parameters for achieving the highest yield in the shortest time, without impacting the antibacterial activity. This supported the fact that UAE offers the advantage of being applicable for the extraction of thermolabile and unstable compounds.

This extraction study, therefore, showed the ability of all three extraction techniques in extracting the desired bioactives from *P. lanosa*. Table 5.11 tabulates the optimum experimental condition for each technique regarding extraction time and feed:solvent ratio for achieving maximum yield whilst not jeopardizing antibacterial activity. However in order to determine which technique is the most useful in terms of extraction efficiency, cost, etc, a comparison was conducted on the yield and activity under the optimum conditions for each technique and it was found that both solvent extraction and Soxhlet extraction achieved the same yield and degree of activity compared to UAE. Soxhlet extraction however achieved this yield and activity in half the time and half the solvent ratio making it more efficient and environmentally friendly regarding reduced organic solvent consumption. Therefore, Soxhlet extraction was concluded as the optimum extraction technique for the extraction of antibacterial compounds from the red seaweed, *P. lanosa* against *X. fragariae*.

CHAPTER 6: PURIFICATION AND IDENTIFICATION OF THE BIOACTIVE COMPOUND(S) PRESENT IN *P. LANOSA* EXTRACTS

6.1 Introduction

Compounds sourced from natural resources became a popular concept after the development of the antibiotic penicillin in the 1940s which lead to a new era of research in the area of drug discovery from natural products (561). However, many of these products did not reach the market as companies are reluctant to invest in such research as even when new compounds are discovered, this is only the first step in a very long tedious process with low success rates (561, 562). Therefore, the control of plant diseases in the agri-sector relies heavily on the use of known chemical pesticides. As a result of the environmental pollution caused by the excessive misuse of these agrochemicals, it has led to many people changing their attitudes towards the use of these control measures (563). This is mainly as a result of their effects on non-target species and the development of genetic resistance by the pest to the pesticide (564). Stricter regulations on the usage of these chemical pesticides and government pressure to eradicate the most hazardous pesticides from the market has pushed efforts to develop alternative inputs including compounds sourced from natural products (563). Seaweeds are known to contain a wide variety of secondary metabolites and it is these metabolites that have been found to exhibit a broad spectrum of biological activities, including pesticidal activity (70, 228). This makes seaweeds a prime alternative to chemical pesticides and also has the added bonus of having a low impact on animal and human health with a long history of safe use (433-435).

Successful antimicrobial drug discovery requires the identification of the antibacterial compound(s) of interest and this consists of a number of different steps. The initial task of generating a crude extract and conducting an antibacterial screen provides insight into the polarity and classes of the bioactive compound(s) of interest. The second step involves the separation of the compounds present in the extract since the crude extract may contain hundreds of different compounds. This separation/purification step separates out the compounds, but this can be both a time consuming and tedious process with an appreciable amount of crude extract required. Chromatographic techniques including column chromatography or preparative thin layer chromatography (TLC) are commonly utilised with compounds separated out according to their different polarities typically on a silica-based stationary phase.

Column chromatography has been widely applied as a purification step (69, 186) with many different compounds of various polarities being isolated via this method such as polysaccharides (191), lipids (192) and halogenated compounds (bromophenols) (194). El-Shouny *et al.* used such a technique to purify the crude ethanol extract of *U. lactuca* which exhibited strong antibacterial activity (187). The responsible bioactive was chemically identified as diisooctyl phthalate through phytochemical analysis.

In previous studies, preparative TLC was also used to purify compounds from crude extracts such as hydrocarbons (565), polysaccharides namely fucoxanthin (118, 566), and fatty acids (193). Thirunavukkarasu *et al.* screened three seaweed species for antibacterial activity against fish pathogens with the brown seaweed *Sargassum wightii* methanol extracts found to exhibit the strongest activity (208). For this reason, preparative TLC was conducted on this crude extract as a method of purification with two separate fractions obtained. Fraction 2 demonstrated the lowest MIC value and was analysed via GC-MS with the fraction 2 found to contain a high quantity of *n*-hexadecanoic acid (59.44%). Fatty acids present in seaweeds have been previously reported for their antimicrobial activities (194, 363). Ismail *et al.* purified the methanol extract of *Ulva rigida* using preparative TLC and identified a number of fatty acid constituents namely mainly oleic, linoleic, palmitic, and stearic acid, which all demonstrated antibacterial activity (193).

However, a number of studies have incorporated sequential purification steps to ensure complete segregation of the antibacterial compound(s) of interest, such as preparative TLC followed by column chromatography (122). Solomon *et al.* investigated the purification of the antibacterial compounds present in *Dictyota acutiloba* J. Ag. by silica column chromatography, TLC and reverse phase HPLC (484). Two active fractions were obtained and phytochemical analysis of these fractions suggested that they were related to terpenoid secondary metabolites.

Once purification is completed, the purity of the fractions is generally evaluated with analytical TLC to ensure a relatively pure fraction has been acquired. The next step is to analyse this purified fraction through various spectroscopic techniques such as GC-MS, liquid chromatography electrospray ionisation mass spectroscopy (LC-ESI-MS), NMR and FTIR for the identification of the responsible bioactives. However, since these compound(s) are typically unknown it makes the process much more

difficult with the vast majority of published literature requiring further work on the elucidation and chemical characterisation of bioactive compounds (42, 65, 567, 568). These long and tedious studies are essential however in novel drug development with a number of studies successfully elucidating the specific antibacterial compound(s) (187, 200). For instance, Nylund *et al.* identified a polyhalogenated 2-heptanone compound: 1,1, 3, 3 – tetrabromo-2-heptanone as the bioactive responsible for the antibacterial activity in the red seaweed *Bonnemaisonia hamifera* against bacterial colonization (569). This metabolite was identified following a number of purification steps with an initial bioassay-guided separation of the crude extract using normal phase vacuum liquid chromatography followed by TLC on normal phase silica gel plates and final purification conducted on normal phase HPLC. The molecular structure of the purified fraction was determined by ^{1}H, ^{13}C NMR and electron impact mass spectra on GC-MS.

In this study, the results from Chapter 4 and Chapter 5 highlighted the strong antibacterial activity of the *P. lanosa* methanol extract against *X. fragariae* in a dose response manner. Therefore, this chapter of work aimed to develop separation, purification and identification procedures for the bioactive compound(s) present in the methanol extract of *P. lanosa*. This was the first study to attempt to purify and identify the bioactives from *P. lanosa* against this particular phytopathogen.

6.2 Experimental Procedures

6.2.1 Production of crude Polysiphonia lanosa extract

The *P. lanosa* collected in June 2017 was extracted in the solvent methanol at the ratio 1:50 *w/v* under magnetic stirring conditions for 2 h at room temperature. This was the same extraction conditions used in Chapter 4 to ensure consistency. Additionally, Chapter 5 reported extended extraction times as having a negative effect on antibacterial activity. The solid seaweed was removed from the solvent containing the bioactives by filtration and the solvent removed via rotary evaporation as outlined in section 2.2.2. The dried methanol extract was stored at -20 °C until further analysis.

6.2.2 Analytical TLC separation of crude P. lanosa extract

The dried extract of *P. lanosa* was re-dissolved in methanol to give a concentration of 100 mg/mL. The extract was spotted onto normal phase silica TLC plates (TLC silica gel 60 F_{254}, Merck, Darmstadt, Germany) at three different concentrations: 1, 2.5 and 5 mg/spot at a 10 µL rate. Chloramphenicol served as the positive control (10 mg/spot). The compounds were separated in equilibrated developing tanks with a variety of mobile phases: Hexane/ethyl acetate (8:5, 6:4 and 4:6 *v/v*), chloroform/methanol (10:1, 9:1, and 4:1 *v/v*), hexane/diethyl ether/1% acetic acid (5:4:1 *v/v*), n-Butanol/acetic acid/water (4:1:5 *v/v*), chloroform/ethanol/acetic acid/water (98:10:2:2 *v/v*) and dichloromethane/methanol (19:1 *v/v*). The developed plates were allowed to dry under aseptic conditions before being placed in sterile petri dishes. All experiments were carried out in triplicate.

6.2.3 Direct bioautography for antioxidant activity

Direct bioautographic evaluation was conducted on the separated compounds on the TLC plates for antioxidant activity as described by Rajauria *et al.* (118). The developed plates were uniformly sprayed with a methanolic solution of 2.54 mM 2,2-diphenyl-1-picryl-hydrazyl (DPPH) antioxidant reagent and allowed to air dry.

Bands demonstrating antioxidant capacity were observed as yellow bands on a purple background. The experiment was carried out in triplicate for each solvent system.

6.2.4 Overlay bioautography for antibacterial activity

For the screening of antibacterial activity, overlay bioautographic analysis was carried out on the separated compounds against *X. fragariae* as described by Rajauria *et al.* (118). A 1% inoculation of *X. fragariae* was prepared in BHI broth and incubated overnight at 27 °C under static conditions. Sterile nutrient agar supplemented with 1% glucose below 50 °C was inoculated with this overnight culture (1 mL of bacteria into 100 mL of agar) in order to avoid overheating and killing the bacteria. This media was then poured over the front facing TLC plates and allowed to set before being incubated at 27 °C overnight. The plates were sprayed with 2.5 mg/mL solution of 2, 3, 5-triphenyl-tetrazolium chloride (TTC) and incubated for a further 6 h. Bands of inhibiton were observed as clear areas against a red-coloured background on the TLC plate. The retention factor (R_f) of the bands exhibiting antibacterial activity was calculated using Equation 6.1. The experiment was conducted in triplicate.

Equation 6.1:

$$R_f = \frac{\textbf{Distance travelled by the compound}}{\textbf{Distance travelled by the solvent front}}$$

6.2.5 Specialised TLC stain profiles for compound classification

The *P. lanosa* extract was separated on the analytical TLC plate via the optimum mobile phase composition determined in section 6.2.1. The separated compounds were stained with specialised TLC stains (Table 6.1) in order to classify the antibacterial compounds, with experiments conducted in triplicate.

Table 6.1: Specialised staining procedures used for the visualization and detection of different classes of compounds in the *P. lanosa* extract (570, 571).

Compounds/functional groups	Reagents and procedures	Expected result
Unsaturated and aromatic compounds	TLC tank containing a number of crystals of iodine.	Dark brown spots on a light brown background
Phenols	Prepare a 1% solution of ferric (III) chloride in 50% aqueous methanol.	Blue spots
Aldehydes/ketones	Prepare a solution of 20 g of 2,4-dinitophenylhydrazine in 60 mL of concentrated sulfuric acid.	Yellow to orange spots
Alkenes/alkynes/alcohols/aldehydes (compounds sensitive to oxidation)	Prepare a solution of 1.5 g of potassium permanganate, 10 g of potassium carbonate in 1.25 mLof 10% sodium hydroxide and 200 mL water.	Bright yellow spots on a bright purple background
Carboxylic acids	Prepare a solution of 0.04 g of bromocresol green. A 0.1M solution of aqueous sodium hydroxide.	Bright yellow spots
Amino acids	Prepare a solution of 1.5 g ninhydrin in 100 mL of *n*-butanol and 3 mL of acetic acid.	Deep blue or purple spots
Terpenes	Prepare a solution of 1.4 g vanillin in 40 mL of methanol and 250 μL of concentrated sulfuric acid. Incubate at 100 °C for colour development.	Blue or green spots

6.2.6 The total phenolic content (TPC) of the crude P. lanosa extract

The total phenolic content of the methanol extract of *P. lanosa* was determined using a modified Folin-Ciocalteu method described by Ainsworth *et al.* (572). A concentration of 5,000 μg/mL of crude extract was prepared in methanol. A 10% *v/v* Folin-Ciocalteu reagent was prepared by 1:10 dilution in deionised water. A 700 mM solution of sodium carbonate was also prepared in deionised water. In the TPC assay, 100 μL of crude extract and 200 μL of 10% *v/v* Folin-Ciocalteu reagent were added into 2 mL eppendoffs and vortex mixed thoroughly. A 100 μL aliquot of methanol in place of the crude extract served as the blank. After 4 min, 800 μL of the 700 mM sodium carbonate solution was added and the assay tubes were incubated at room temperature for 2 h. After the 2 h period, the tubes were centrifuged for 4 min at 15,000 RPM and 200 μL of the supernatant was transferred into a 96 well microtiter plate. The absorbance was measured at 765 nm using a BioTek EL×800 Absorbance Microplate Reader. A standard curve was generated from blank corrected gallic acid standards in the concentration range of 0.001 - 5000 μg/mL. Although the standard curve was constructed from 1 - 200 μg/mL (Figure 6.7) since above and below this concentration was found to be outside the Beer Lambert Law. The TPC of the *P. lanosa* extracts were calculated using the regression Equation 6.2 of the gallic acid standards and expressed as gallic acid equivalents (GAE), in milligrams per gram of sample. Determination of the TPC was performed in triplicate and repeated in duplicate.

Equation 6.2: $\text{Absorbance} = 0.0044x + 0.0041$

6.2.7 Purification with preparative TLC

The dried *P. lanosa* crude extract was purified using normal phase preparative TLC (silica gel GF, 2,000 μm, 20 × 20 cm, Sigma Aldrich, Dublin, Ireland). The concentrated extract (100 mg) was applied across the TLC plate using a pipette tip 2 cm from the bottom and 2 cm in from each side. The separation was performed in a latch-lid TLC developing chamber with the mobile phase chloroform/methanol (8:1 *v/v*) at room temperature based on the analytical TLC results. The developing chamber was equilibrated prior to separation and the separation was allowed to develop to 2 cm from

the top of the plate. The plate was removed from the chamber and allowed to dry with eight fractions observed. These fractions were based on the bioautography results and the colour of the bands. The fractions were scrapped off and re-extracted in 30 mL of methanol, via vortexing for 3 min and re-extraction was repeated in triplicate. The silica was removed via centrifugation at 4000 RPM for 15 min (118). The supernatant was collected and re-concentrated through rotary evaporation.

The fractions were assessed for purity using analytical TLC followed by overlay bioautography to determine the presence of antibacterial compound(s) against *X. fragariae*. The most active fraction was evaluated to determine whether activity had enhanced or reduced through synergistic effects as a result of this purification procedure. Activity was assessed using the disk diffusion assay as described in section 4.2.3. The preparative TLC was completed a total of ten times in order to obtain enough purified extract to conduct spectroscopic analysis.

6.2.8 Identification of the semi-pure fraction

6.2.8.1 LC-ESI-MS analysis

Preliminary identification of the semi-pure fraction collected from preparative TLC was performed first with LC-ESI-MS analysis on an Agilent 1200 series LC/MSD Trap XCT Ultra ion trap mass spectrometer (Agilent Technologies, Cork, Ireland). Separation was achieved on an Eclipse XDC-C_{18} non-polar column (Agilent 5 μm, 4.6 × 150 mm) at 25 °C with a mobile phase composition of 95:5 *v/v* acetonitrile/water, with 0.1% formic acid at 0.5 mL/min. A 1 mg/mL filtered (0.45 μm) sample of the semi-pure fraction prepared in methanol was injected at an injection volume of 5 μL with a run time of 25 min. Ions were generated using electrospray ionisation (ESI), with scans performed from 200 to 800 *m/z*, since the molecular ion was unknown.

6.2.8.2 GC-MS analysis

The semi-pure fraction was analysed via GC-MS on a Variant 220-MS Ion Trap Mass Spectrometer coupled to a Variant 450-GC containing a capillary column (DB-5ms, 0.25 mm × 30 m, 0.25 μm, temperature range of -60 to 325/350 °C, and phase

composition of 5% phenyl and 95% dimehylpolysiloxane) (Agilent Technologies, Cork, Ireland). A temperature gradient was implemented starting at 50 °C for 3 min followed by 20 °C/min gradient up to 280 °C and held for 10.5 min producing a total run time of 25 min. Helium was used as the carrier gas in the electron-ionisation (EI) mode at 1 mL/min. A 1 mg/mL filtered (0.45 μm) sample of the semi-pure fraction prepared in methanol was manually injected at an injection size of 1 μL with split ratio of 1:40. The mass spectroscopy was scanned from 40 to 350 *m/z* and the mass spectrum obtained was compared to the National Institute of Standards and Technology (NIST) database for a compound match.

6.2.8.3 NMR analysis

Further identification of the collected fraction was conducted using NMR analysis on a Joel ECX-400 MHz NMR system (Tokyo, Japan). The semi-pure fraction was re-dissolved in deuterated methanol (D4 >99.8%, Fluorochem Ltd, UK) at a concentration of 50 mg/0.75 mL. A number of NMR experiments including; ^{1}H NMR, ^{13}C NMR, 2D COSY, DEPT and NOESY were carried out on the sample. The data obtained was compared to that of published literature on isolated compounds from seaweeds in order to elucidate a possible structure of the purified antibacterial compound.

6.2.8.4 FTIR-ATR

The types of functional groups present in the semi-pure fraction were analysed on a Variant 660-IR FTIR Spectrometer (Agilent Technologies, Cork, Ireland) equipped with liquid nitrogen cooled mercury cadmium telluride (MCT) detector and KBr beam splitter. The infrared spectroscopy (IR) was combined with attenuated total reflectance (ATR) technology for minimal sample preparation and involved placing the sample on a zinc selenide crystal that was subject to an IR beam. The crystal containing no sample served as the blank. The analysis was conducted at room temperature in the mid-infrared range from 600 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. A total of 64 scans were performed for both the blank and dried semi-pure fraction. The resultant spectra were displayed in terms of transmission with baseline correction applied.

6.2.8.5 Ultraviolet-visible analysis

UV-visible analysis of the semi-pure fraction was conducted on a Varian Cary 50 UV-Visible spectrophotometer (Agilent Technologies, Cork, Ireland) equipped with a single beam Xenon flash lamp. A full scan (190 – 800 nm) was performed on a 0.5 mg/mL filtered sample in order to determine the λ_{max} of this active compound(s) and/or identify possible chromophores present in the semi-pure fraction. Methanol was used to zero the instrument.

6.2.9 Statistical analysis

Data obtained for the dose response studies on the antibacterial activity of the semi-pure fraction of *P. lanosa* were performed with repeated measures using one-way ANOVA followed by a post-hoc analysis using Tukey's multiple comparison tests. These analyses were performed by Minitab 17 Statistical Software using a 5% statistical significant level ($\rho<0.05$). The results were said to be statistically different if $\rho<0.05$ and were designated with different superscripts. The antibacterial activity of the semi-pure fraction was also compared to that of the crude extract. All experiments were performed in triplicate and presented as the mean ± standard deviation (SD), unless otherwise stated.

6.3 Results and Discussion

6.3.1 Separation of crude methanol extract of P. lanosa using TLC

TLC is considered a quick and easy method used to separate compounds into smaller fractions depending on their polarity (181). The selection of mobile phase is critically important to ensure satisfactory separation of this crude extract. Polar solvent systems such as water, ethanol, methanol and ethyl acetate can be used to separate out polar compounds including polyphenols, fucoidans, laminarans and ulvan (5). Non-polar solvents such as hexane and petroleum ether can be used to separate non-polar compounds including chlorophyll, lipids, fatty acids, sterols (5). The selected mobile phases are typically a mixture of polar and non-polar solvents to ensure the optimum separation of the specific compound(s), with sequential separation conducted if required. Since the identity of the bioactives in *P. lanosa* were unknown, a large variety of solvent systems were used as illustrated in Figure 6.1. The selected mobile phases were based on previous studies that separated methanol extracts from natural products and also the polarity of the extract itself. For instance, Jayabarath *et al.* screened for potential phytochemical compounds present in the methanol extract of the brown seaweed, *Turbinaria conoides* using TLC analysis (573). The solvent system n-Butanol/acetic acid/water 4:1:5 *v/v* was used and a spot with a R_f value of 0.66 was observed and identified as a flavonoid (573) which was previously reported for its antibacterial activity (574, 575).

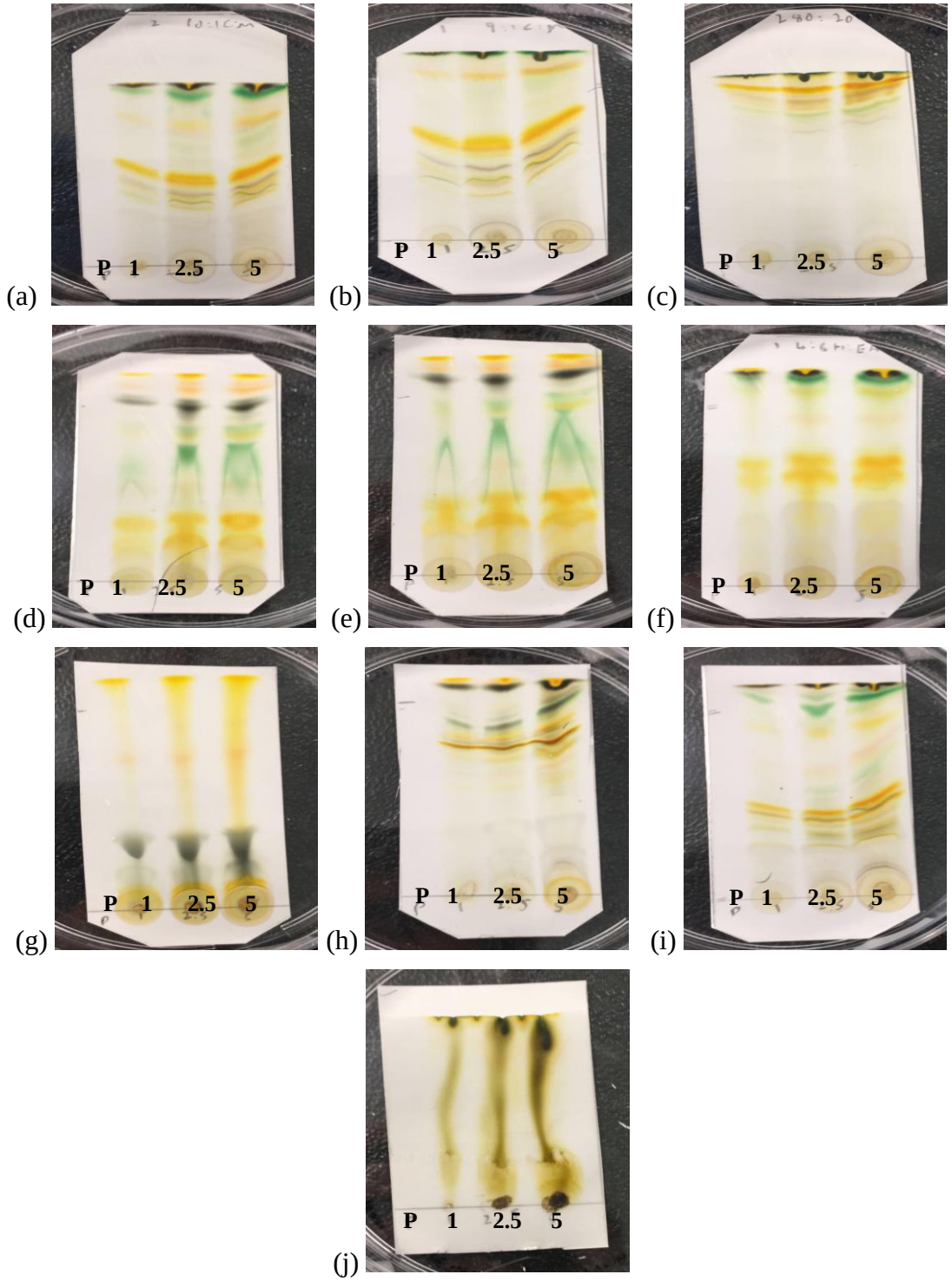


Figure 6.1: Separation of the compounds present in *P. lanosa* at 1, 2.5 and 5 mg/spot for six different mobile phases. (a) chloroform/methanol (10:1 *v/v*), (b) chloroform/methanol (9:1 *v/v*), (c) chloroform/methanol (4:1 *v/v*), (d) Hexane/ethyl acetate (8:5 *v/v*) (e) Hexane/ethyl acetate (6:4 *v/v*), (f) Hexane/ethyl acetate (4:6 *v/v*), (g) n-Butanol/aceticacid/water (4:1:5 *v/v*), (h) chloroform/ethanol/aceticacid/water (98:10:2:2 *v/v*) (i) dichloromethane/methanol (19:1 *v/v*) and (j) hexane/diethylether/1% acetic acid (5:4:1 *v/v*). Data (n=3).

The most commonly used solvent system for the separation of methanol extracts consisted of chloroform and methanol (208, 436, 574) and due to this three different ratios were investigated: 10:1; 9:1; 4:1 *v/v*. For example, Saravanan *et al.*, investigated various solvents including methanol, chloroform, acetic, n-butanol, n-hexane and water in different proportions in order to determine the best solvent system for separating the antimicrobial compounds present in mangrove leaves (576). It was found that the solvent system methanol/chloroform (60:40 *v/v*) showed the optimum separation of the methanol extract with two spots observed, with the first spot demonstrating activity against the test organisms. The solvent system consisting of chloroform and methanol utilised in this research also produced sufficient separation of the crude extract with over ten bands observed (Figure 6.1a-c). The 10:1 and 9:1 *v/v* solvent system resulted in better separation compared to the 4:1 *v/v*. This was as a result of the higher ratio of methanol present leading to the extract exhibiting less interaction with the stationary phase and hence, poorer separation. High quality separation was also observed by the solvent system consisting of dichloromethane and methanol in a ratio of 19:1 *v/v*, demonstrating the effectiveness of moderately non-polar systems.

It was found that once methanol was removed from the solvent system the quality of chromatography fell dramatically. For instance the n-Butanol/acetic acid/water 4:1:5 *v/v* and hexane/diethyl ether/1% acetic acid 5:4:1 *v/v* (Figure 6.1g and j) demonstrated no degree of separation with the extract appearing to smear as it migrated up the TLC plate. This illustrated the importance of the solvent system containing some ratio of the extracting solvent. Tan *et al.* also reported this, by employing the extracting solvent ethyl acetate into their solvent system and successfully separating six bands from the crude *U. lactuca* extract, with the second band demonstrating the strongest antimicrobial activity compared to the others (122). Therefore, the chloroform/methanol and dichloromethane/methanol systems were found to produce the best separation and were selected for further study to determine the antibacterial bioactives present in *P. lanosa* in section 6.3.3. All of the TLC plates produced in this investigation into the optimum solvent system were also utilised in section 6.3.2 with the aim of determining the antioxidant potential of the compounds present in the *P. lanosa* extract.

6.3.2 DPPH radical scavenging activity of methanol P. lanosa extract

Bioautography is a simple, rapid and inexpensive method for the chemical and biological screening of natural extracts in conjunction with TLC (111). In this procedure, the TLC plates of the separated *P. lanosa* compounds were subject to the antioxidant agent DPPH in order to determine the specific fractions that exhibited such activity. This is the most commonly used method as it is simple, efficient, relatively inexpensive and quick. DPPH is a stable free radical that exhibits a deep purple colour which absorbs strongly at 517 nm. In the presence of antioxidant compounds in the crude extract the DPPH is converted to a more stable DPPH molecular product through the donation of an electron or a hydrogen (577, 578). This results in the DPPH colour changing to a pale yellow as it is reduced (579). The TLC separated compounds from section 6.3.1 were tested for their antioxidant activity in order to identify the specific compounds that exhibited such a biological property through direct bioautography. The separated compounds were sprayed with a DPPH solution with Figure 6.2 demonstrating the antioxidant activity of the separated compounds of *P. lanosa* for three solvent systems.

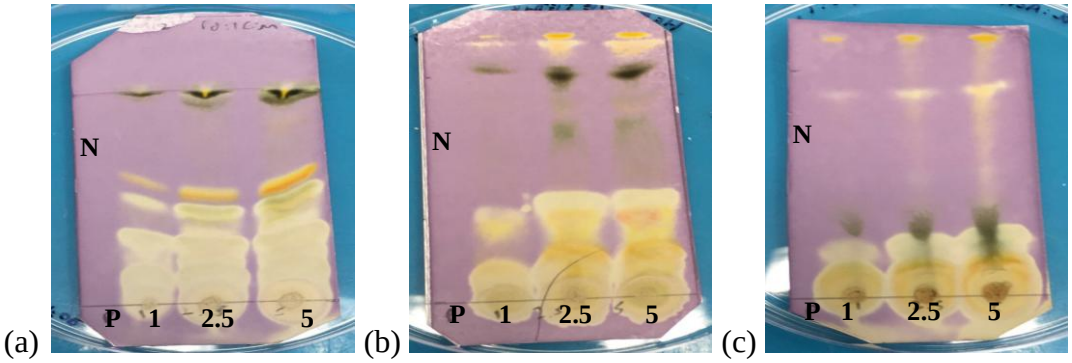


Figure 6.2: DPPH radical-scavenging capacity of the separated compounds of the *P. lanosa* extract using direct bioautography for three different solvent systems: (a) chloroform/methanol (10:1 *v/v*), (b) hexane/ethyl acetate (8:5 *v/v*) and (c) n-Butanol/acetic acid/water (4:1:5 *v/v*). Concentration of extract: 1, 2.5 and 5 mg/spot. Positive control (P) was 2 µL chloramphenicol (10 mg/mL); Negative control (N) was 50 µL of methanol. Data (n=3).

Antioxidant activity was observed for all TLC plates with a dose response effect, as the 5 mg/spot produced relatively larger areas of activity (yellow spots) compared to the 1 and 2.5 mg/spot. The majority of bands were very intense meaning there was a high amount of radical scavengers present demonstrating the strong antioxidant potential of *P. lanosa.* It can also be seen that due to the different solvent systems, the compounds exhibiting this radical-scavenging activity vary in position between plates. For example, the solvent system, chloroform/methanol 10:1 *v/v* (Figure 6.2a) demonstrated a number of antioxidant compounds separated up along the plate compared to the TLC bioassay produced by the solvent system n-Butanol/acetic acid/water 4:1:5 *v/v* (Figure 6.2c), with the majority of compounds responsible for this activity residing at the baseline. This further supports the conclusion drawn in section 6.3.1 that the chloroform/methanol solvent system produced optimum separation of target bioactives. This observed activity was not surprising since seaweeds have been widely reported to posses antioxidant activity with Rajauria *et al.* observing such activity from TLC separated compounds of the brown seaweed *Himanthalia elongate* with two bands out of the six exhibiting this activity (118).

A number of bioautography assessments on seaweeds have been performed on High-Performance Thin Layer Chromatography (HPTLC) which is based on the full capabilities of TLC with enhancements to improve resolution of separation and allow for quantitative analysis (580, 581). Agatonovic-Kustrin *et al.* evaluated the antioxidant potential of ethanol and ethyl acetate extracts of ten marine algae using HPTLC coupled with DPPH as the detection agent. Images of the plates were captured using a TLC-visualiser with quantitative analysis performed on the images using the digital image analysis software Sorbfil TLC Videodensitometer and the CAMAG TLC scanner III. It was found that the ethyl acetate extract of *Caulerpa racemosa* and *Padina minor* produced moderately high antioxidant activity (582). The majority of studies investigating the antioxidant activity of seaweeds is completed by spectrophotometry (506, 583-585) which has the added advantage of being capable of quantifying the antioxidant response of an extract at a specific concentration. Zubia *et al.* assessed the DPPH radical scavenging activity of four red seaweed species including *P. laonsa* 80% methanol extract and it was found that *P. lanosa* exhibited high antioxidant activity with % inhibition of $80.88 \pm 0.72\%$ at 500 mg/L in a dose response manner (586).

Regardless, of the method applied, the DPPH radical-scavenging capacity reported in this study and other literature demonstrated the antioxidant potential of seaweeds with the TLC bioautography-guided separation shown as an effective technique in screening for antioxdant compounds in natural products. For example, Gu *et al.* applied TLC bioautography-guided separation for the isolation of antioxidant compounds present in the perennial plant *Perilla frutescens* var. *acuta* extracts (587). Four antioxidant compounds were isolated and identified as rosmarinic acid, luteolin, apigenin and chrysoeriol. In the case of this study, the observed activity could potentially be as result of phenolic compound(s) present in the methanol extract, since phenolics are widely known for their antioxidant activity in seaweeds (588-590). Kang *et al.* isolated and identified a phenol derivative 2,7-phloroglucinol-6,6-bieckol from the brown seaweed *Ecklonia cava* as a new antioxidative compound (591).

6.3.3 Detection of antibacterial compounds by overlay bioautography

Preliminary separation of the crude *P. lanosa* extract in section 6.3.1 identified that the mobile phase compositions chloroform/methanol and dichloromethane/methanol produced the best separations. Therefore, these compositions were selected for the detection of the antibacterial compounds with an additional composition of chloroform/methanol in the ratio 8:1 *v/v* also investigated. It was hoped that the slightly greater volume of methanol would further improve separation. Figure 6.3 demonstrates the bioautography assay results obtained for each specific mobile phase. However, the indicator spray was very faint making zones of inhibition difficult to observe.

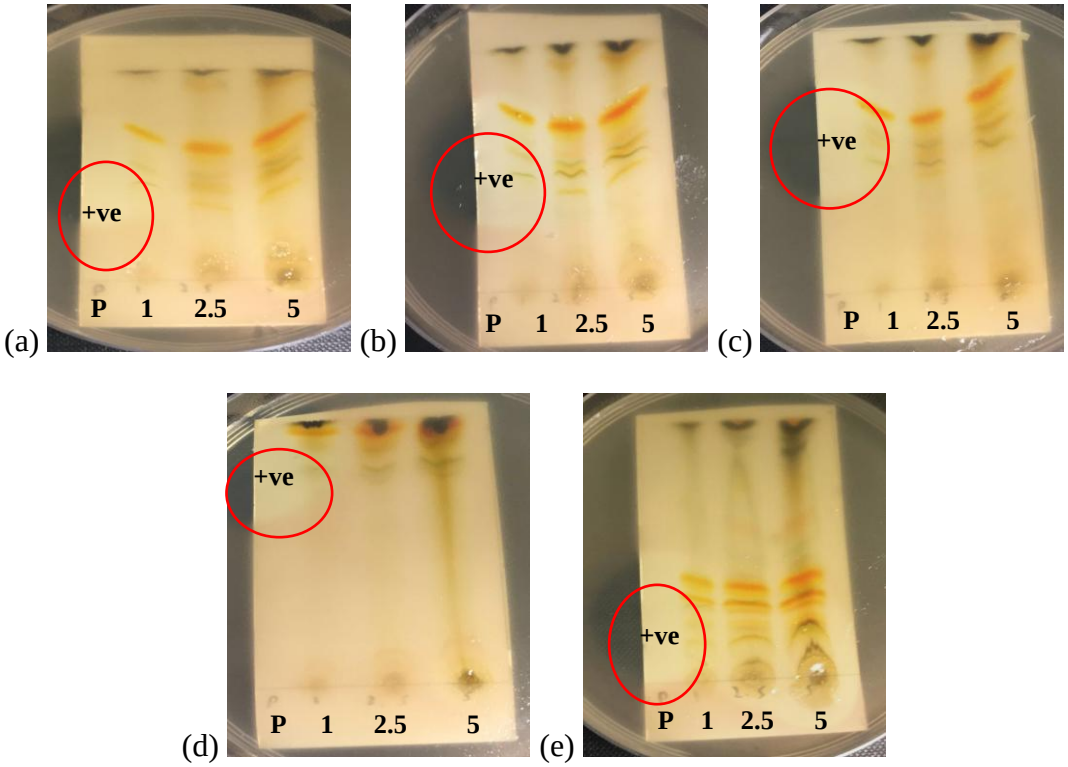


Figure 6.3: Antibacterial activity of the separated compounds of the *P. lanosa* extract against *X. fragariae* for different solvent systems: (a) chloroform/methanol (10:1 *v/v*), (b) chloroform/methanol (9:1 *v/v*), (c) methanol (8:1 *v/v*), (d) chloroform/methanol (4: 1 *v/v*) and (e) dichloromethane/methanol (19:1 *v/v*). Concentration of extract: 1, 2.5 and 5 mg/spot. Positive control was 2 µL chloramphenicol (10 mg/mL). Data (n=3).

Since the indicator spray TTC was very faint it meant that optimisation of the method was required. For this reason, the experiment was repeated with a number of modifications including the concentration of TTC increased to 2 g/100 mL and the quantity of bacteria halved i.e. 1% reduced to 0.5%. These alterations proved effective with a clear zone of inhibition observed around the specific antibacterial compound(s), specifically for the mobile phases chloroform/methanol 8:1 *v/v* and 4:1 *v/v* (Figure 6.4). The active compounds were not highly coloured appearing as one large zone of inhibition. Therefore, the R_f value for each development system was calculated as the centre spot of the zone of inhibition ± the lower and higher end of the zone. The resulting R_f values were calculated as 0.36 ± 0.24 and 0.52 ± 0.19 for 8:1 *v/v* and 4:1 *v/v* chloroform/methanol, respectively. The difference in R_f values was not unexpected since R_f is defined as the ratio of the distance travelled by the centre of the spot to the

distance traveled by the solvent front (181) with the distance travelled by the compound(s) differing between solvent systems. Hence, the 4:1 *v/v* solvent system being more polar and, therefore, more capable of displacing the bioactives from the silica stationary phase resulting in actives moving further up the TLC plate (leading to a larger R_f values). Suleiman *et al.* investigated the antimicrobial potential of leaves from selected South African tree species (592). The leaves were extracted in methanol, acetone, dichloromethane and hexane with bioautography used to evaluate the antibacterial compounds present in the various leaf extracts. It was reported that the R_f values for the same extract differed between the various solvent systems. This was due to the differences in polarity of the solvent systems. For example, compounds that had relatively high R_f values in polar solvent systems such as ethyl acetate/methanol/water (40:5.4:5 *v/v*), had low R_f values in non-polar solvent systems e.g. benzene/ethanol/ammonia hydroxide (18:2:0.2 *v/v*) (592).

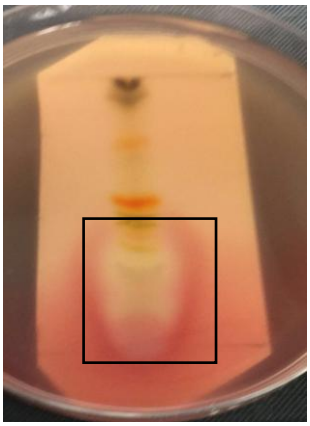
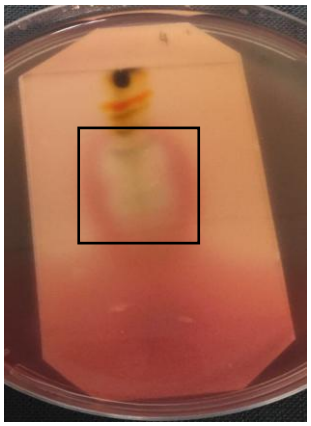
(a)  (b) 

Figure 6.4: Antibacterial activity of the separated compounds of the *P. lanosa* extract at 5 mg/spot against *X. fragariae* for the solvent systems: (a) chloroform/methanol (8:1 *v/v*) and (b) chloroform/methanol (4:1 *v/v*). Data (n=3).

From examination of the chromatography in Figure 6.4, it can be seen that the solvent system 8:1 *v/v* produced a better degree of separation with the compounds more evenly spaced compared to the 4:1 *v/v* solvent system in which the majority of the separation takes place in the upper part of the TLC plate. For this reason the 8:1 *v/v* composition was selected as the optimum mobile phase and used for the rest of the study. These results also proved that the compound(s) responsible for this activity did not share a

synergistic relationship, since the separated crude extract still retained activity. Synergistic activity by seaweeds has been reported with Cortés *et al*. evaluating the antimicrobial activity of dichloromethane extracts from the red seaweed *Ceramium rubrum* and the individual compounds including ethyl myristate, palmitic acid, stearic cid, etc identified within this extract through GC-MS analysis against the bacteria *Yersinia ruckeri* and the oomycete *Saprolegnia parasitica* (593). It was found that the dichloromethane extract exhibited greater activity compared to the individual compounds, for example, the dichloromethane extract demonstrated a % inhibition of 14.7 ± 0.6% compared to the % inhibition of 8.0 ± 0.0% exhibited by the palmitic acid against *Y. ruckeri* (593). Synergistic activity can be used to aid activity (594), for instance, Morán-Santibañez *et al.* found that a combination of polyphenol rich extracts from *Ecklonia arborea* and *Solieria filiformis* with a low concentration of sulphated polysaccharides from *S. filiformis* exhibited the best synergistic activity against the measles virus (595).

6.3.4 Phytochemical analysis of the compounds present in the crude P. lanosa extract

The crude *P. lanosa* extract was found to contain a number of compounds as outlined in section 6.3.1. Such compounds can be sprayed with specific dyes that react and change colour depending on the phytochemicals present. Table 6.1 summarises the different stains applied on the separated *P. lanosa* compounds using the chloroform/methanol 8:1 *v/v* solvent system with a particular interest in the compound(s) present at the R_f 0.36 ± 0.24 since antibacterial activity was observed within this specific R_f range. The iodine stain was first applied as it is non-destructive (compounds remain unchanged) technique that has a high affinity for unsaturated and aromatic compounds (596). These compounds appear as dark brown spots on a light brown background, as the iodine forms reversible coloured complexes with these organic compounds including phenolic compounds. In this study, dark brown spots were observed with four bands with R_f values of 0.16, 0.26, 0.39 and 0.48 identified within the zone of the antibacterial compounds (0.36 ± 0.24) indicating the presence of such compounds (Figure 6.5a). Ferric chloride ($FeCl_3$) is a highly specific stain and is mainly used to visualize phenols but some carbonyl compounds with high enol content can also be visualized (596). The

Fe (III) forms a colour complex with the phenols by the reduction of ferric (III) to ferric (II) in a redox-linked colorimetric reaction that involves a single electron transfer (597). Similar to the iodine stain four bands produced a positive result for phenols within the specific zone of antibacterial activity with R_f values of 0.18, 0.28, 0.38 and 0.53 (Figure 6.5b). These are relatively similar to the R_f values detected in the iodine stain suggesting these are as a result of the aromatic functional groups of a phenolic compound.

The potassium permanganate stain is a strong oxidizing agent that readily illustrates functional groups which are oxidative sensitive as well as alkenes and alkyenes (596). A positive result was observed with the appearance of yellow bands against a purple background (Figure 6.5c). This was not surprising since the crude extract was found to exhibit antioxidant activity, with a zone of yellow with R_f range of 0.42 – 0.63 observed which is within the region for the compounds of interest. The presence of these oxidative sensitive compounds, as mentioned in section 6.3.2 such as phenolic compounds have been reported for this antioxidant activity observed in seaweeds (588-590).

The presence of terpenes was also detected as a small blue band with an R_f value of 0.38 and once again was within this zone of antibacterial activity identifying the presence of an unsaturated compound (Figure 6.5d). Further staining revealed the presence of aldehydes/ketones (Figure 6.5e), carboxylic acids (Figure 6.5f) and amino acids (Figure 6.5g) but these compounds were present at the origin spot i.e. had not been separated and were, therefore, not suspected to be responsible for the antibacterial activity. It must be noted that the black boxes on the TLC images indicate the location of the compounds that exhibited positive results for a specific stain within the R_f range of the antibacterial compounds. The black circles indicate positive results for stains outside of this range i.e. compounds on the baseline.

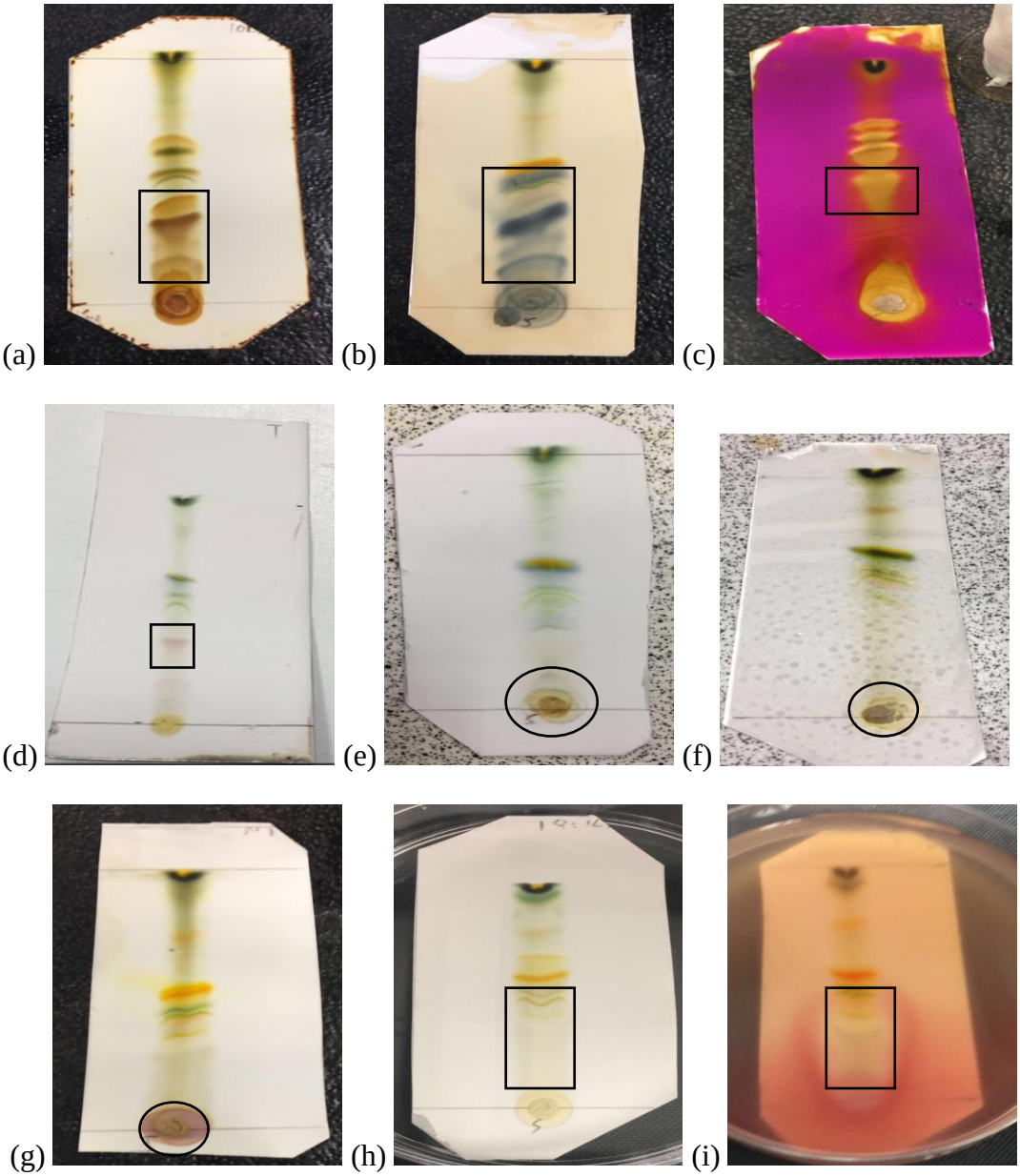


Figure 6.5: Classification of the separated compounds of *P. lanosa* using specialised TLC stains. (a) iodine stain for unsaturated and aromatic compounds, (b) ferric chloride stain for phenols, (c) potassium permanganate stain for compounds sensitive to oxidation, (d) vanillian stain for terpenes (e) dinitophenylhydrazine stain for aldehydes/ketones, (f) bromocresol green stain for carboxylic acids and (g) ninhydrin stain for amino acids, (h) control separation without staining and (i) bioautography of the separated compounds indicating the antibacterial compounds (R_f value of 0.36 ± 0.24). Data (n=3).

This staining procedure, therefore, indicated the presence of phenolic compounds as the possible antibacterial compounds. Phenolic compounds represent hundreds of molecules found in seaweeds and are considered secondary metabolites since they are not directly involved in primary processes such as cell division or photosynthesis of seaweeds (5, 15). They are characterized by an aromatic ring with at least one substituted hydroxyl group. Polyphenols are a wide class of compounds namely, flavonoids, lignins, tocopherols, tannins and phenolic acid (598). These compounds play an important part in plant defence mechanisms against pathogenic bacteria and other types of potential environmental factors including wounding, drought or UV radiation (599, 600). A number of studies have reported the antibacterial activity of phenolic compounds obtained from seaweeds (601, 602). Hierholtzer *et al.* investigated the mode of action of phenolic compounds extracted from the brown seaweed, *Laminaria digitata* against mixed microbial cultures (603). Phloroglucinols, a division of phenols, were found to significantly induce extra- and intra-cellular effects with the disruption of cell membranes in the majority of cases. Additionally, phenolic compounds are soluble in polar organic solvents such as the extracting solvent methanol applied in this study, with Cox finding that the methanol extract of the red seaweed *P. palmata* demonstrated the highest phenolic content compared to the ethanol and acetone extracts (15).

However, this staining process is only a general guideline with further testing required by LC-ESI-MS, GC-MS, NMR and FTIR-ATR to confirm the identity of these antibacterial compound(s) once separation and purification was successfully completed.

6.3.5 Determination of total phenolic content of methanol P. lanosa extracts

Classification of the separated *P. lanosa* compounds determined the presence of phenol functional groups for the compound(s) that exhibited antibacterial activity. For this reason, a total phenol content assay was conducted to quantitatively estimate the amount of phenol present in the crude extract. This was measured using the Folin-Ciocalteu method and is the most commonly used method for determining phenolic content in seaweeds (506, 604-606). The Folin-Ciocalteu reagent contains a mixture of phosphotungstic and phosphomolybdic acids which are reduced to tungsten and molybdene (blue oxides) by electron transfer under alkaline conditions (addition of

sodium carbonate) (607). This blue complex can be spectrophotometrically detected with the intensity of blue directly reflecting the quantity of phenol in the sample (607) i.e. the reaction relies on the oxidation of phenolic rings present in the seaweed extract. Figure 6.6 demonstrates this, with the colour intensity reducing as the concentration of gallic acid standard decreases. Since the Folin-Ciocalteu reagent rapidly decays in the alkaline solution, an excess of the reagent is required for the reaction to go to completion (608). This excess resulted in a precipitate and high turbidity, making spectrophotometric analysis impossible, which meant the sample and controls had to be centrifuged to remove this precipitate. The addition of lithium salts could also be used to prevent such turbidity (608).

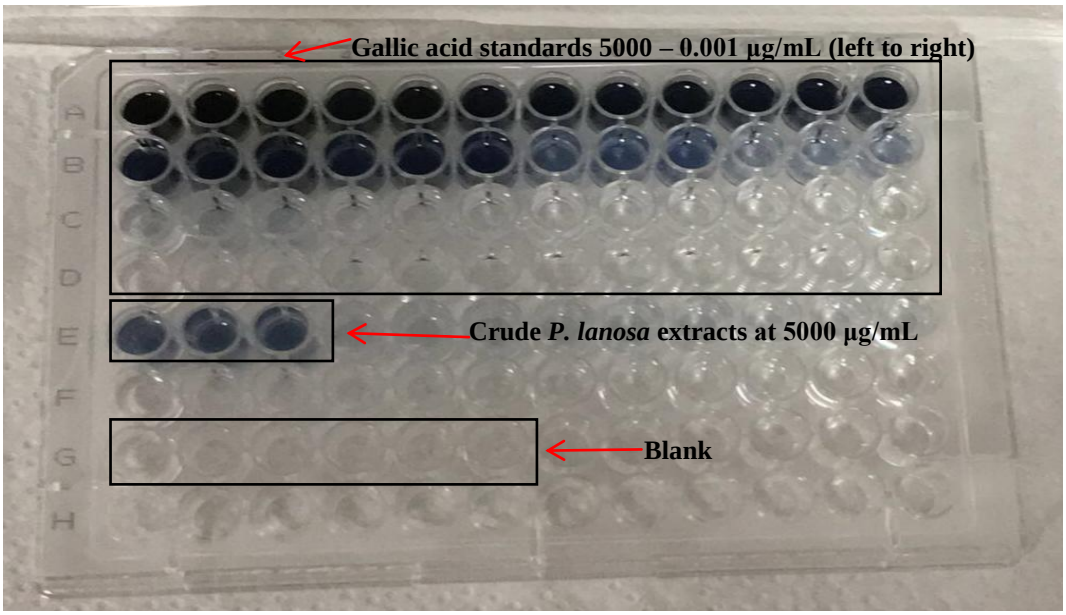


Figure 6.6: TPC of the crude methanol extract of *P. lanosa* at 5000 µg/mL determined by the Folin-Ciocalteu method. Standard: gallic acid (0.001-5000 µg/mL) and blank: Folin-Ciocalteu reagent, sodium carbonate and methanol. Data (n=3).

Gallic acid was selected as the standard on the basis that it is found in almost all plants (607). A linear standard curve (R^2 = 0.99942) was constructed in the concentration range 1 – 200 µg/mL (Figure 6.7) as these concentrations followed the Beer Lambert Law. The regression Equation 6.2 obtained from this gallic acid standard curve was used to calculate the TPC of the crude extract as gallic acid equivalents.

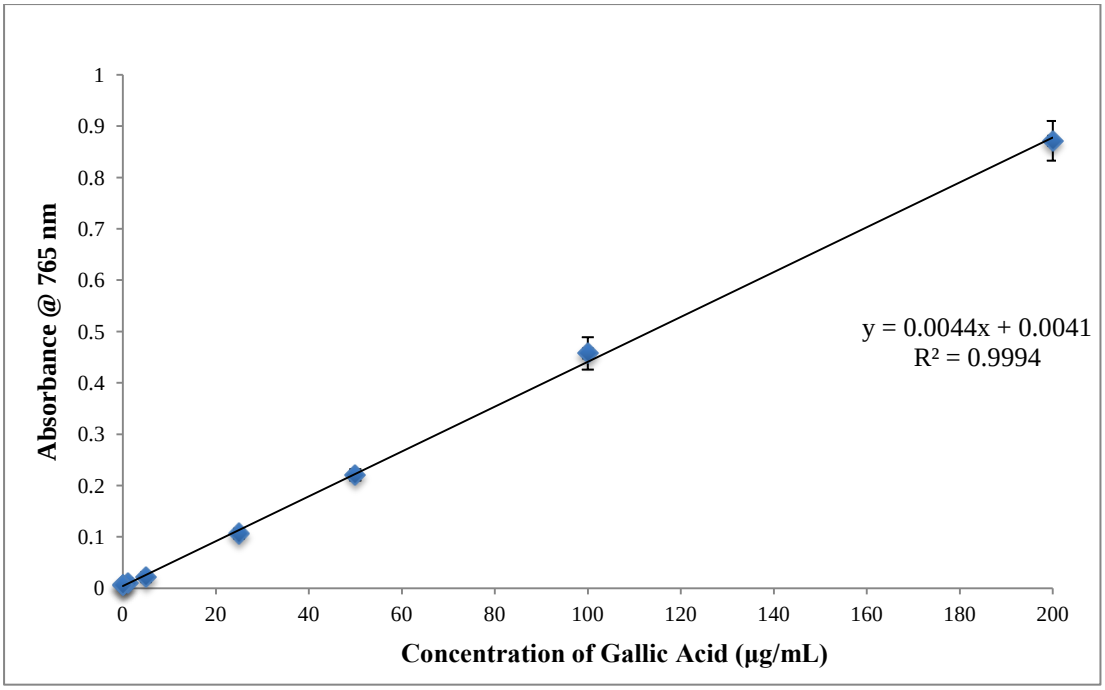


Figure 6.7: Calibration curve of the standard gallic acid used to estimate the total phenol content of the *P. lanosa* extract. Data (n=3).

The phenolic content of the *P. lanosa* extract was found to be 2.1 ± 0.013 mg GAE/g at 5000 µg/mL. Chakraborty *et al.* recorded slightly higher TPC for the methanol extracts of the red seaweeds *Hypnea musciformis*, *H. valentiae*, and *Jania rubens* with TPC values of 9.84 ± 0.03, 6.91 ± 0.06 and 4.95 ± 0.06 mg GAE/g, respectively (505). This relatively low TPC obtained for the crude *P. lanosa* extract also supported the fact that red seaweeds have been previously reported to contain lower phenolic content compared to brown and green seaweeds. For example, Iiknur *et al.* found that the brown seaweeds *Cystoseira barbata* and the green seaweeds *Enteromorpha intestinalis* demonstrated significantly higher phenolic content compared to the red seaweed *Gigartina acicularis* (583). Jiménez-Escrig *et al.* (609) and Matanjun *et al.* (355) also reported similar findings with brown seaweeds containing higher phenolic content than the red seaweeds. Ahmad *et al.* estimated the total phenolic content of 15 seaweeds using the Folin-Ciocalteu method with significant differences reported among the different seaweed species ranging from 9.41 to 51.87 mg/g phloroglucinol equivalents (PGE) of dried sample (344). The study indicated that both green and brown seaweeds contained higher amounts of polyphenols compared to red seaweeds. For example the green seaweed, *Caulerpa racemosa* exhibited a TPC of 47.88 ± 0.53 mg PGE/g dried

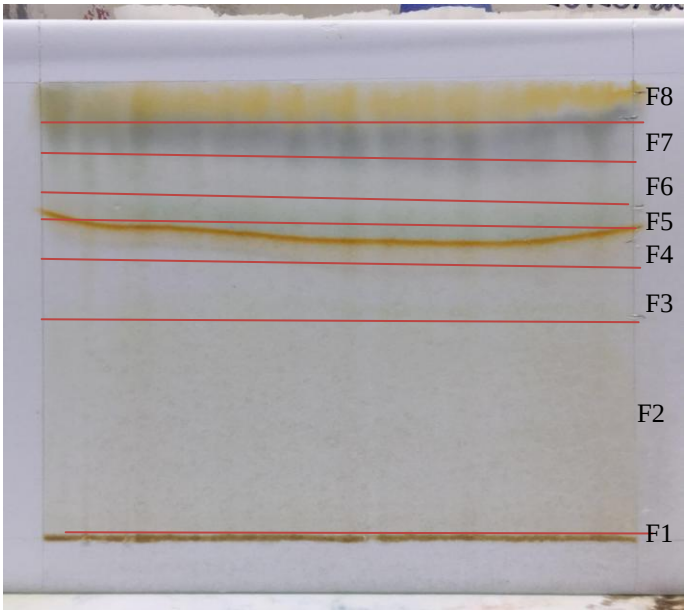
sample compared to TPC of 12.08 ± 2.35 mg PGE/g dried sample reported for the red seaweed *Gracilaria verrucosa* (344).

Neethu *et al.* found that the solvent methanol was the best at extracting phenols from the red seaweed *Asparagopsis taxiformis* compared to the ethyl acetate, chloroform and petroleum ether solvents (610). This result data further supports the hypothesis that phenol containing compounds were responsible for the antibacterial activity, with methanol being capable of maximum phenol recovery from *P. lanosa*. Zubia *et al.* methanol extract also exhibited strong antioxidant activity with a correlation between phenolic content and antioxidant activity reported in numerous studies (604, 611-613). This was also observed in this study with compounds present in the *P. lanosa* extract demonstrating DPPH radical-scavenging activity by direct bioautography, indicating the importance of polyphenols as antioxidants. As mentioned previously, phenols have also been reported to exhibit antimicrobial activity. However, the Folin-Ciocalteu assay is not specific for TPC determinations, as other types of compounds present in high quantities such as reducing sugars and amino acids can also reduce the Folin-Ciocalteu reagent, skewing results (614). Therefore, further purification of the *P. lanosa* extract is required in order to purify these possibly responsible phenolic compounds.

6.3.6 Purification of the antibacterial compound(s) using normal-phase preparative TLC

Purification of the crude *P. lanosa* extract was completed through preparative TLC using the mobile phase chloroform/methanol 8:1 *v/v* that was found to be the optimum solvent system in section 6.3.3. Eight fractions were collected from the preparative TLC as presented in Figure 6.8. The R_f range 0.36 ± 0.24 identified as the antibacterial zone in analytical TLC was used as a guide to isolation. Since the R_f value is the ratio a compound travels under the same experimental conditions (181) with the thicker silica stationary phase on preparative TLC potentially causing a slower solvent migration up the plate compared to analytical TLC.

(a)



(b)

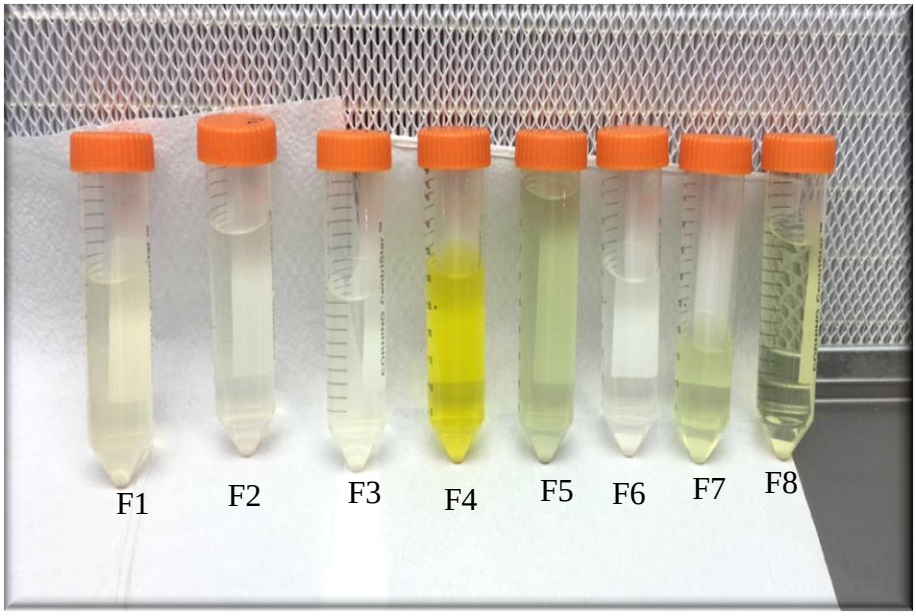


Figure 6.8: Purification of the antibacterial compounds present in the crude methanol extract of *P. lanosa* (a) preparative TLC separation using the solvent system chloroform/methanol 8:1 *v/v*, (b) eight fractions collected from preparative TLC.

Table 6.2 summarises the R_f values obtained in the preparative TLC for the eight fractions with ± values for the broader bands.

Table 6.2: The R_f value for each of the eight fractions attained from the preparative TLC using the solvent system chloroform/methanol 8:1 *v/v*.

Fraction	R_f value
Fraction 1 (baseline)	0
Fraction 2	0.24 ± 0.23
Fraction 3	0.53 ± 0.06
Fraction 4	0.63 ± 0.04
Fraction 5	0.71 ± 0.04
Fraction 6	0.78 ± 0.04
Fraction 7	0.86 ± 0.04
Fraction 8	0.95 ± 0.05

From examination of the R_f values obtained it can be seen that fractions 2 and 3 were within the R_f zone 0.36 ± 0.24 of the antibacterial compounds identified by analytical TLC bioautography. For this reason fractions 2 and 3 were tested for activity via overlay bioautography which also demonstrated the purity of the two fractions (Figure 6.9b-c). All of the other six fractions were also tested to ensure the antibacterial compounds had not migrated further up the plate or resided on the baseline due to differences in silica thickness. Figure 6.9 demonstrates the bioautography results obtained for each fraction with some degree of activity exhibited by all of the fractions except fraction 1, illustrating that all of the bioactive compounds had migrated off of the baseline.

Fractions 2 and 3 exhibited activity (Figure 6.9b-c) which was to be expected since the bioactive compound(s) identified in analytical TLC (section 6.3.3) were recorded within their R_f regions (Table 6.3). The activity exhibited by fractions 4 to 8 (Figure 6.9d-h) had not been witnessed (by these compounds) in the initial bioautography assessment (Figure 6.4a) particularly for fraction 5 and 7. Additionally, activity resided on the

baseline with a mass of other compounds separated out. This was a very interesting result and was speculated to be one of three things. The compounds had been degraded possibly by the silica into smaller polar biologically active compounds which would explain their non-movement from the baseline. Another reason is that these compounds were biologically active but were too dilute to exhibit activity until concentrated through repetitive preparative TLC. A final theory was the separation of compounds in preparative TLC removed antagonist compounds that were preventing such antibacterial activity prior to this. Compared to fraction 5 and 7 the much smaller degree of activity observed in fraction 4, 6 and 8 may have been caused by a small concentration of compounds from fraction 5 or 7 being introduced during the scraping off process of the preparative TLC plate. It must be noted that this investigation was conducted in triplicate with these results recorded for each bioautographic plate confirming this was not a once off phenomenon.

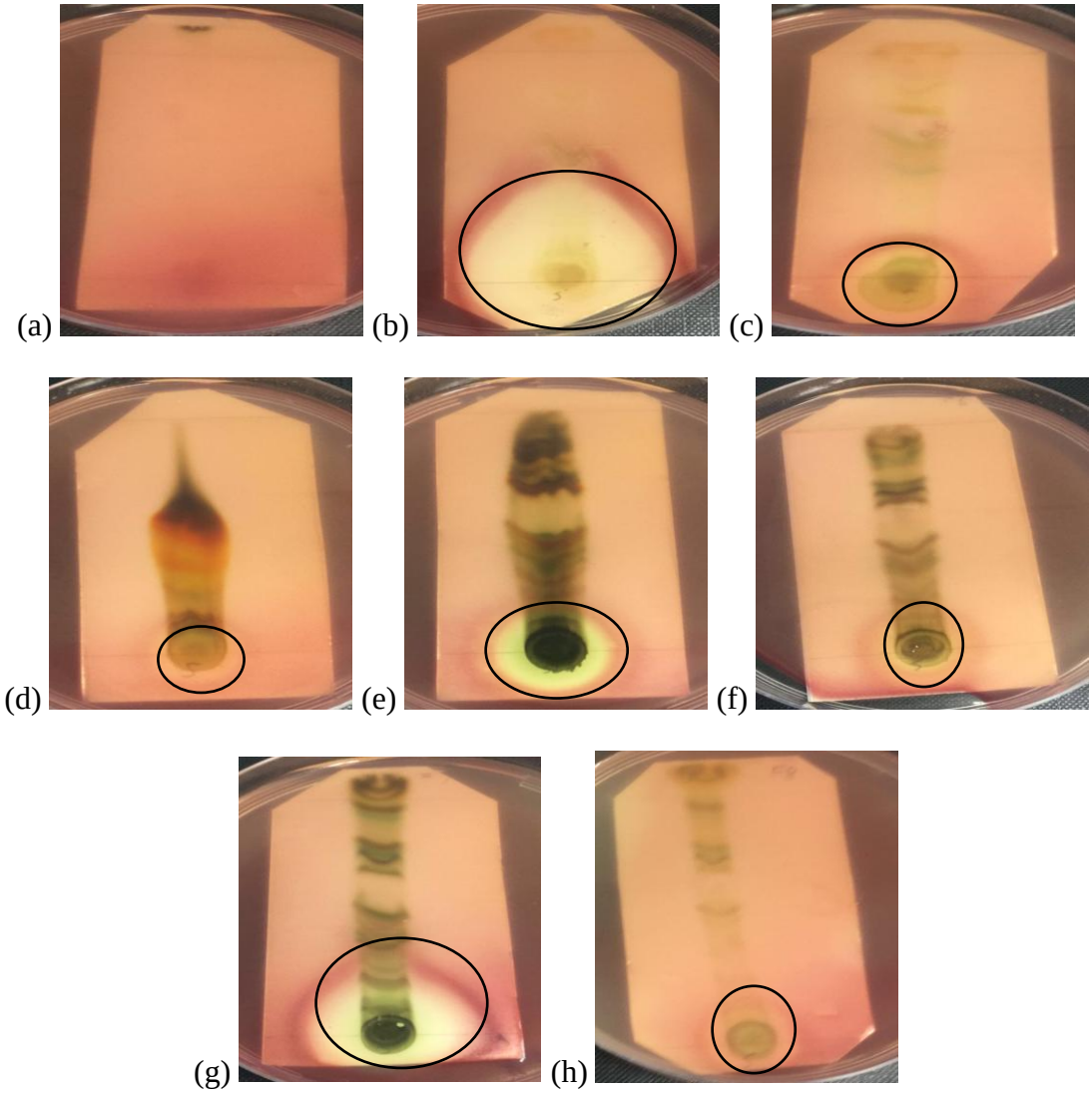


Figure 6.9: Bioautographic profiles of the compounds from different fractions of the crude *P. lanosa* extract against *X. fragariae* at 5 mg/spot using the solvent system chloroform/methanol 8:1 *v/v*. (a) fraction 1, (b) fraction 2, (c) fraction 3, (d) fraction 4, (e) fraction 5, (f) fraction 6, (g) fraction 7 and (h) fraction 8. Data (n=3).

Fraction 2 was found to be the most potent antibacterial fraction with a large zone of inhibition (Figure 6.9b). An R_f value ranging from 0 (baseline) to 0.47 was recorded, which is within the R_f region 0.36 ± 0.24 of the identified antibacterial compounds in analytical TLC. Fraction 3 on the other hand produced a low degree of activity with a hazy zone of inhibition observed (Figure 6.9c). Therefore fraction 2 was selected for further study and furthermore, appeared to be relatively pure with a reduced number of bands observed confirming this was a semi-pure fraction (Figure 6.10) making structural elucidation that bit easier.

Figure 6.10: Analytical TLC separation of the semi-pure fraction 2 collected from preparative TLC (5 mg/spot) using the solvent system chloroform/methanol 8:1 *v/v* selected for further study.

A study was conducted to determine whether antibacterial activity had been improved through this purification step. The semi-pure fraction was found to exhibit enhanced antibacterial activity in a dose response manner compared to its crude extract counterpart (Table 6.3). This was concluded to be the product of a more concentrated extract of the desired bioactives with the removal of possible antagonistic compounds. This has also been reported by other studies (419, 484). The difference in activity was substantial with activity almost triple that of the crude extract. These results, therefore, signify the importance of purification of these bioactive(s) not only in terms of characterization of the compound(s) but also to determine the full potential of this pure compound(s) from an activity perspective.

The successful purification of the crude *P. lanosa* extract demonstrates the effectiveness of preparative TLC as a purification method capable of separating out the active compound(s) from non-active compounds. Preparative TLC has been used in other studies either in combination with another purification techniques (122, 615) or as a stand alone method in the successful isolation of antibacterial bioactives from seaweeds (118). Yuvaraj *et al.* investigated the antibacterial effect of the green seaweed *Cladophora glomerata* against the multidrug resistant human pathogen *Acinetobacter baumannii* and fish pathogens (565). TLC was used to purify the crude methanol extract with five separate fractions obtained. Three fractions exhibited activity with preliminary GC-MS analysis revealing the presence of major compounds including octacosane, tridecane 8-hextl- and heptadecane 9-hexyl-.

Table 6.3: Antibacterial activity of the purified fraction 2 compared to the crude extract of *P. lanosa* against *X. fragariae* using the disk diffusion assay.

Extract concentration (mg/disk)	Crude *P. lanosa* extract (mm)	Semi-pure *P. lanosa* fraction (mm)
1	0^{a}	15.0 ± 1.00^{d}
2	0^{a}	25.0 ± 0^{e}
3	8.3 ± 1.20^{b}	29.0 ± 1.41^{f}
4	10.2 ± 0.40^{bc}	31.0 ± 0^{f}
5	14.3 ± 1.00^{c}	35.0 ± 1.41^{g}

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

6.3.7 Elucidation of the semi-pure fraction of P. lanosa

Prior to the spectroscopic analysis of this semi-pure fraction a short study was conducted to determine the best method for removing any potential particulates whose presence could block the chromatographic columns. This is usually achieved through the filtration of the sample but, as found in Chapter 4, filtering (0.2 μm) of the extract resulted in a loss of antibacterial activity. Therefore, both centrifugation and filtration was investigated using the crude extract so as to avoid the usage of the semi-pure fraction. The antibacterial activity of each centrifuged and filtered crude extract was assessed to determine whether there was a loss in activity due to the bioactive(s) remaining either in the centrifuged pellet or on the filter itself. Table 6.4 presents the results acquired for each method and it can be seen that centrifugation regardless of conditions applied did not affect activity. Filtration with a 0.45 μm nylon filter also resulted in retention of activity similar to the untreated extract. However, the extracts

filtered with a 0.45 μm polytetrafluoroethylene (PTFE) filter exhibited reduced activity. This data suggested that the bioactives were absorbed onto the surface of the PTFE filter. Based on these results, the semi-pure fraction was selected to be filtered with the 0.45 μm nylon filter prior to analysis as it is the most commonly used method for sample preparation and allowed for high sample throughput.

Table 6.4: Effect of centrifugation and filtration on the antibacterial activity of the crude *P. lanosa* extract at 10 mg/disk using the disk diffusion assay against *X. fragariae* (g=gravity).

Clean-up step	Antibacterial activity (mm)
Untreated extract (control)	18.5 ± 1.23^{a}
10,000 g for 10 min	17.3 ± 0.82^{a}
3,000 g for 5 min	17.2 ± 0.98^{a}
0.45 μm nylon filter	17.2 ± 0.84^{a}
0.45 μm PTFE	13.75 ± 0.50^{b}

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

Preliminary identification of the semi-pure fraction was first conducted on LC-ESI-MS as it was suitable for non-volatile molecules and had the ability to scan over a wide molecular range allowing for the detection of large molecules. This was critically important since the identity of this bioactive(s) was unknown. In addition, LC-ESI-MS is an extremely sensitive technique with good selectivity (616). Unfortunately, the data proved inclusive as it revealed a large number of broad peaks. This was suspected to be either the fact that this was a semi-pure fraction, i.e. requiring further purification or from ghost peaks primarily caused by a dirty pre-column or column acquired from previous analysis. The semi-pure fraction was then subjected to GC-MS analysis with

the hope of obtaining the molecular ion and a distinctive fragmentation pattern in order to determine a potential structure for the antibacterial compound (Figure 6.11). The data revealed a relatively clear chromatogram as presented in Figure 6.11a with a number of small peaks followed by one large peak at 17.308 min that was suspected to be the antibacterial compound. This was a semi-pure fraction and hence contained a higher concentration of this specific bioactive and also exhibited enhanced activity compared to its crude counterpart (Table 6.3). The resultant mass spectra of this peak detected a molecular ion with *m/z* of 279.2 and a major fragment with *m/z* of 149.2. Other smaller fragments were also detected with *m/z* of 167.2, 150.2, 104.2, 71.1 and 41.2.

(a)

(b)

(c)

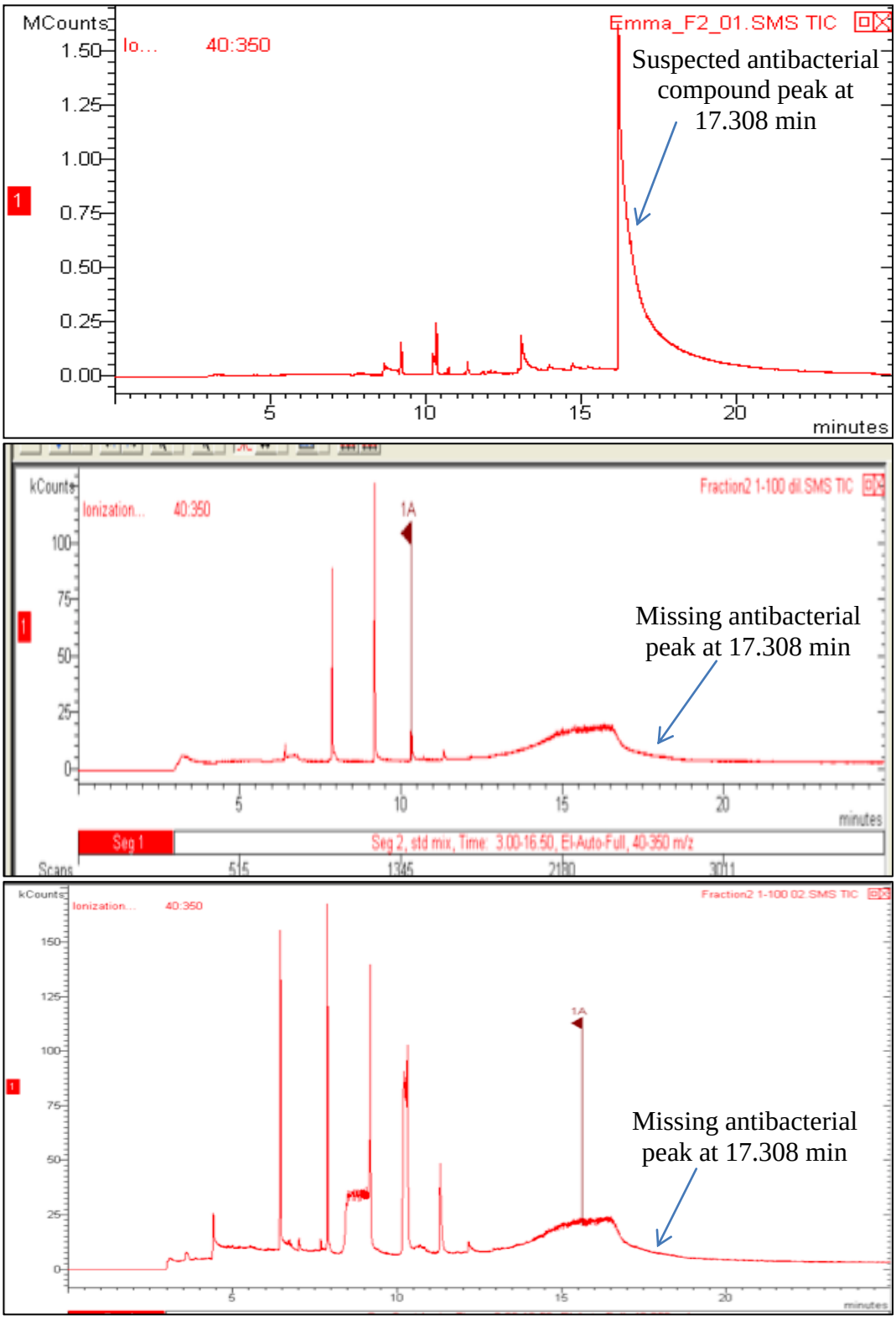


Figure 6.11: GC separations of the semi-pure fraction over a number of injections.

GC-MS has been widely used to identify components present in seaweed extracts (207-209). Hassan *et al.* investigated the chemical composition of crude *Corallina officinalis* and *Colpomenia sinuosa* extracts that exhibited antibacterial activity using GC-MS and revealed six major components including n-nonadecane, 1,2,3- propanetricarboxylic acid 2-(acetyloxy)- tributyl ester, 2-methylhexadecan-1-ol, 1-docosene, 1-eicosanol and chloroacetic acid octadecyl ester (617). Unfortunately, when the fraction was diluted (1:100) and re-analysed at a lower concentration i.e. kCounts as opposed to MCounts it was found that the GC separation had changed significantly with the suspected antibacterial peak disappearing (Figure 6.11b) and even when the sample was re-prepared fresh to ensure the presence of methanol had not caused the degradation of the compound(s), a different chromatogram with extra peaks was observed (Figure 6.11c). The concentration of the semi-pure fraction was also increased to determine whether this dilution factor was too low but once again a different separation was observed with the antibacterial peak at 17.308 min still missing.

Therefore, this change was suspected to be as result of the degradation of the compounds most likely a consequence of silica residues which are known for their degradation properties on natural products (618). This is due to silica being slightly acidic causing the possible breakdown of acid-sensitive compounds such as alkenes and alkynes (619). In order to confirm this, the semi-pure fraction was tested to determine whether activity had diminished but activity was still found to be present and was statistically the same. Additionally, these non-reproducible separations were concluded to be not as a result of contamination from a previous GC run since the suspected antibacterial peak had disappeared and a blank (methanol) was injected after each sample analysis to ensure all compounds had migrated off the column. Therefore, this indicated that the antibacterial compound had not degraded or was not contaminated but instead the compounds present in the semi-pure fraction were not stable enough to withstand the extreme conditions of GC-MS analysis leading to these non-reproducible GC chromatograms.

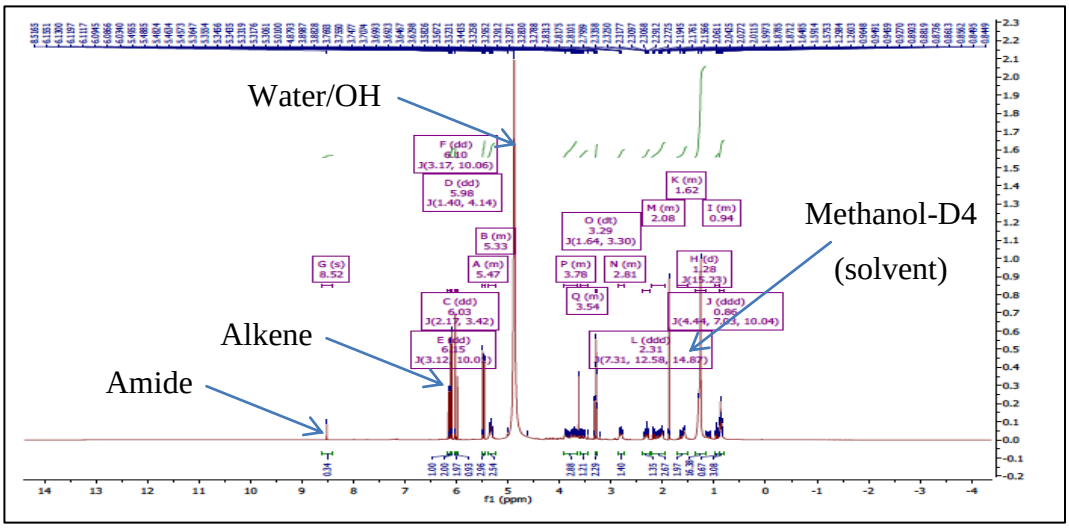
Since the mass spectroscopy analysis on the semi-pure fraction proved inclusive, the semi-pure fraction was subjected to NMR (Figure 6.12) analysis with the aim of determining a definitive structure through the identification of the carbon-hydrogen framework. The compound was also analysed by FTIR-ATR to identify other possible functional groups. Rodrigues *et al.* characterized both *Sargassum muticum* and

Osmundea pinnatifida extracts obtained by Alcalase and Viscozyme assisted extraction, respectively, through the use of ^{1}H NMR and FTIR-ATR analysis and confirmed the presence of important polysaccharides namely fucoidans from *S. muticum* and agarans as sulfated polysaccharides from *O. pinnatifida* (214). The results obtained in section 6.3.4 on the specialised stains identified the antibacterial compound as a polyphenol from the positive result for four compounds within the identified R_f 0.36 ± 0.24 antibacterial zone for the iodine and ferric chloride stains. This indicated the presence of unsaturated/aromatic compounds and phenols indicative of polyphenols.

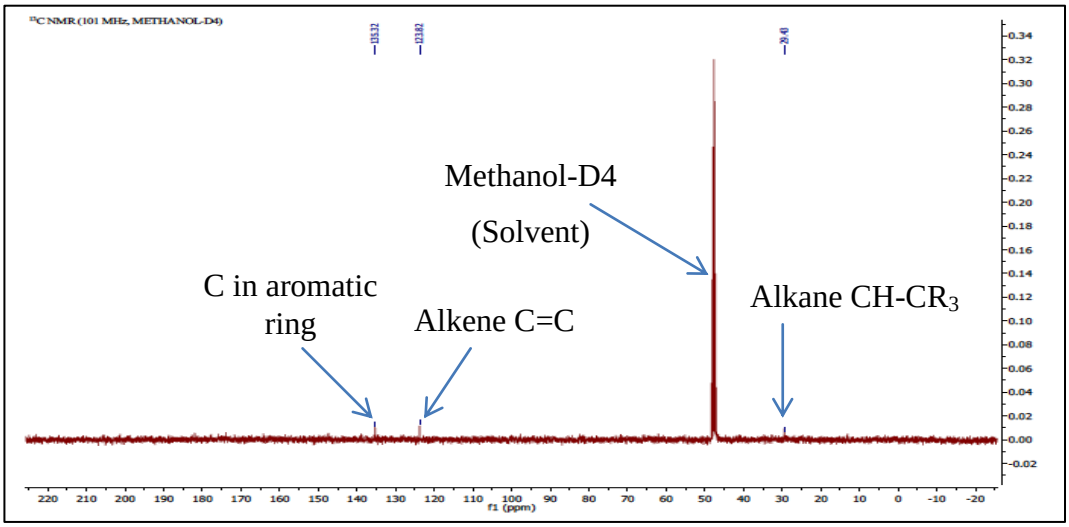
The ^{1}H NMR data demonstrated a relatively complicated spectrum with the solvent peak at δ 1.94 ppm and a number of minor peaks such as at δ 3.5 – 3.9 ppm suspected to be impurities since this was only a semi-pure fraction (Figure 6.12a). However from initial analysis of the data it was clear that the semi-pure fraction supported the proposal of a polyphenol with a set of doublet of doublets at 6.10 ppm (dd, J = 10.1, 3.2 Hz, 30H) and 6.03 ppm (dd, J = 3.4, 2.2 Hz, 29H) which corresponded to alkene groups from their characteristic proton chemical shifts and spin-spin splitting patterns (620, 621). A significant peak at δ 1.94 ppm corresponded to water but could have potentially masked an OH peak relating to an alcohol functional group.

Although a distinct feature with polyphenols is the presence of aromatic rings with the typical chemical shift for an aromatic ring in ^{1}H NMR found between δ 6.5 - 8.2 ppm (622) but unfortunately no such peaks were observed. This was most likely as a result of a concentration issue with the peaks corresponding to an aromatic ring too weak compared to the other peaks in the ^{1}H NMR data and hence lost in the system noise. It must also be noted that a singlet at δ 8.52 ppm (s, 5H) in the ^{1}H NMR data corresponding to an amide was identified as a result of its chemical shift (212) but since this was a semi-pure fraction it was suspected to be functional group of another compound.

(a)



(b)



(c)

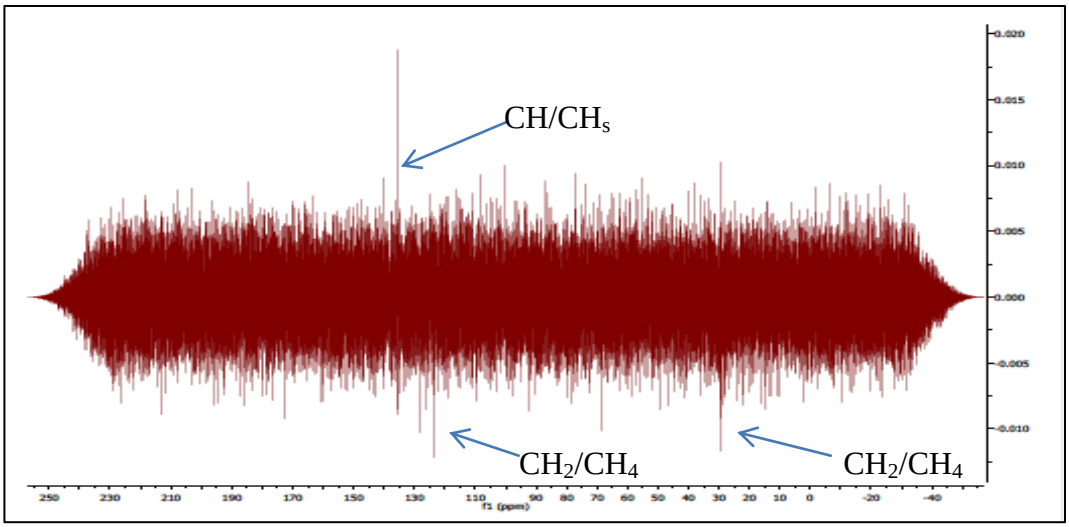


Figure 6.12: (a) ^{1}H NMR spectrum of the semi-pure fraction and the predicted proton structure with respect to their chemical shifts. (b) ^{13}C NMR spectrum of the different carbon environments at δ 135.32, 123.82 and 29.43 ppm. (c) DEPT spectrum of the identified carbons.

This concentration issue of the semi-pure fraction was also reported for the ^{13}C and DEPT NMR data too with weak signals also observed. For this reason the number of ^{13}C scans were increased from 1000 to 10000 in order to improve the sensitivity of the ^{13}C NMR data, however the peaks remained relatively small. The ^{13}C NMR spectrum identified three different carbon environments with small peaks at δ 135.32, 123.82 and 29.43 ppm (Figure 6.12b). The peaks at δ 135.32 was typical of carbon present in aromatic ring indicative of polyphenol, 123.82 ppm were identified as alkenes and the peak at 29.43 ppm corresponded alkanes based on their ^{13}C chemical shifts and supported the data obtained for the ^{1}H NMR (213). This low number of carbon environments could be caused by the symmetry exhibited by the unsaturated compound or the low concentration of the semi-pure fraction causing peaks to be lost within the system noise. DEPT was used to distinguish between primary, secondary, tertiary and quaternary carbon. Three peaks were observed in the DEPT spectrum (Figure 6.12c) which corresponded to the three carbons identified in the ^{13}C NMR spectrum. The peak at δ 135.32 ppm is positive so therefore related to either a primary or tertiary carbon, whereas the peaks at δ 123.82 and 29.43 ppm were negative corresponding to either a secondary or quaternary carbon which indicated a symmetrical unsaturated compound with an OH functional group and carbon related to aromatic ring as observed in polyphenols.

COSY and NOESY analysis were also completed on the semi-pure fraction in order to indicate which hydrogen atoms are coupling with each other and identify which signals arise from protons that are close to each other in space, even if they are not bonded. Correlation was found between the major different peaks confirming that they were all part of the same compound and not part of some impurity. These techniques are generally applied on a pure compound as a form of confirmation and ensuring the various hydrogen's, carbons and functional groups are located in the correct region on the compound.

The semi-pure fraction was also analysed using FTIR-ATR to identify the functional groups based on their peak values in the region of IR radiation with Figure 6.13 demonstrating the resultant spectrum. Sudha *et al.* also investigated the bioactive compounds in the brown seaweed *Padina pavonica* using FTIR analysis which confirmed the presence of phenols, alcohol and aromatic compounds with the major

functional groups reported to be five phenolic compounds and were identified using HPLC analysis (623).

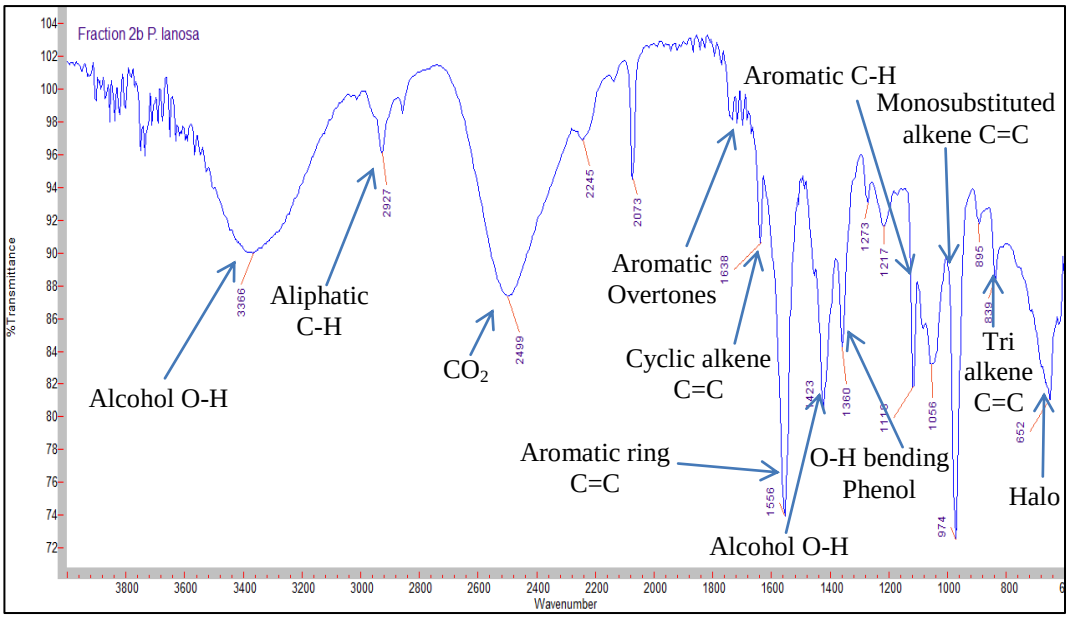


Figure 6.13: FTIR-ATR spectrum of the semi-pure antibacterial compound and its associated functional groups. Data (n=2).

The spectrum revealed the presence of a number of peaks with Table 6.5 outlining the distinctive functional groups and their corresponding wavenumbers (624). Since this was a semi-pure fraction a number of peaks were suspected to be from other compounds present in the fraction such as the peak at 2499 cm^{-1} corresponding to CO_2. However, this data supports the proposed structural characteristics of a polyphenol with OH group detected at 3366 cm^{-1} and overtones also detected at 1850-1700 cm^{-1} which are a distinct characteristic of aromatics with the presence of alkenes (double bonds) likely to be originating from these aromatics making this an unsaturated compound. Rajauria *et al.* reported similar findings when completing qualitative identification of phenolics in the methanol extract of the brown seaweed *Himanthalia elongata* using FTIR analysis with the presence of a hydroxyl group also around 3431 cm^{-1} and peaks relating to an aromatic ring detected around 1465, 1505 and 1624 cm^{-1} which suggested the presence of phenolic compounds in the extract (579).

Table 6.5: FTIR peak values of the semi-pure fraction of *P. lanosa* extract.

Peak value (cm^{-1})	Bond	Functional group
3366	O-H stretch	Alcohol
2927	Aliphatic C-H stretch	Alkenes
1850-1700	Overtones (C-H bending)	Aromatic
1638	Cyclic C=C stretch	Alkene
1556	C=C stretch	Aromatic
1423	O-H bending	Alcohol
1360	O-H bending	Phenol
1116	C-H-in-plane bending	Aromatic
974	Monosubstituted C=C	Alkene
839	Trisubstituted C=C	Alkene
652	C-X	Halogenated compound

Although it must be noted that in IR interpretation, the bands detected between 4000 – 1500 cm^{-1} relate to specific types of bonds such as the OH stretch, C-H stretch and C=C stretch and, therefore, can be used to identify the presence of specific functional groups (625). The 1500 – 400 cm^{-1} region is known as the fingerprint region and can be very difficult to pick out any specific functional groups. However, the pattern of absorbance peaks in this region is unique to every molecule and, therefore, can be used to identify a compound, particularly through comparison with a standard (625). A peak identified as a phenolic compound was also detected and more significantly a peak corresponding to that of a halogenated compound was also identified at 652 cm^{-1}.

Red seaweeds are regarded as the main producers of halogenated compounds particularly bromophenols. For this reason, the halogenated compound identified in the

FTIR-ATR analysis was believed to be that of a bromine which was indicative of a bromophenol. Bromophenols share one or several benzene rings and a varying degree of bromine and hydroxyl-substituents with these compounds reported to possess promising antibacterial activity (626). Many bromophenol compounds have been isolated and identified from *P. lanosa* (518) including a 2,3-dibromobenzyl alcohol 4,5-disulfate, dipotassium salt (Figure 6.14) elucidated from the aqueous-alcoholic extracts of *P. lanosa* (200). Although no peak/band relating to sulfate was detected in the spectroscopic analysis so confirmed it was not this compound but instead a similar compound.

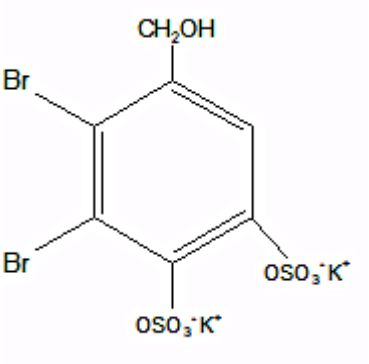


Figure 6.14: The bromophenol compound 2,3-dibromobenzyl alcohol 4,5-disulfate, dipotassium salt isolated from the aqueous-alcoholic extracts of *P. lanosa* (200).

A full spectrum analysis of the semi-pure fraction was also conducted on UV-visible spectroscopy (200 – 800 nm) and revealed strong absorption below 300 nm. This was concluded to be as a result of absorption by methanol through a comparison of the blank spectrum (methanol only). Unfortunately, the λ_{max} of the antibacterial compound could potentially absorb within this wavelength region and, therefore, be masked by the solvent peak. Rajauria *et al.* also completed qualitative identification of phenolics on the methanol extracts of *H. elongate* using UV-visible spectrophotometry analysis with the spectral data exhibiting absorption maxima at 205 and 260 nm (579). Further, increasing the possibility that the λ_{max} of this polyphenolic antibacterial compound was masked in the solvent peak.

However, analysis between 400 – 800 nm revealed a weak chromophore at 665 nm which related to its light yellow colour (Figure 6.15) and is indicative of a system of

extensively conjugated pi-electrons (π-electrons) such as an unsaturated compound. As mentioned previously, Sudha *et al.* investigated the bioactives present in *P. pavonica* using HPLC and FTIR techniques with phenolic compounds found to be the major functional group (623). UV-visible analysis was also conducted on the extract with nine absorption peaks identified with a peak at 664.5 nm possibly corresponding to the phenolic compounds.

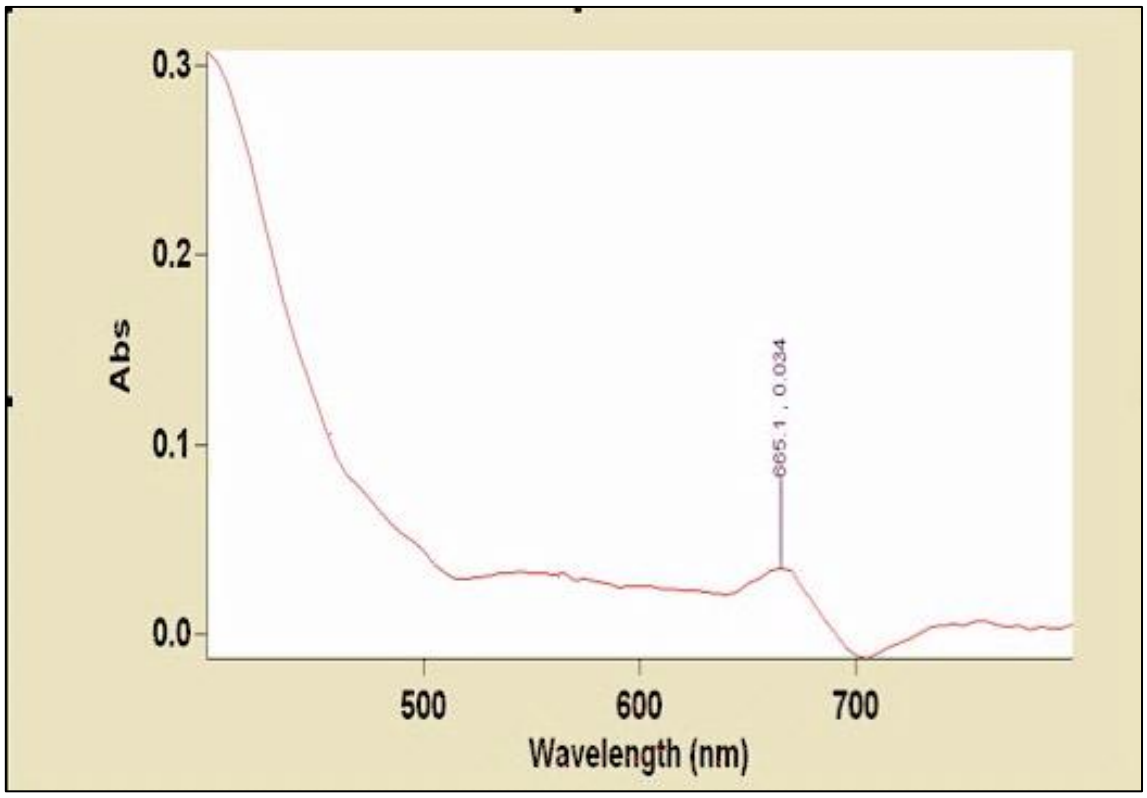


Figure 6.15: UV-visible spectrum of the semi-pure fraction scanned from 400 – 800 nm with an identified chromophore at 665.1 nm.

The information acquired in this research points to a polyphenol specifically a bromophenol as the antibacterial compound present in *P. lanosa*. The antioxidant activity observed in section 6.3.2 gave the first indication of the possible presence of phenols since phenolics are widely known for their antioxidant activity in seaweeds (588-590). The specialised stains in section 6.3.4 confirmed the presence of phenolic compounds through positive results for the iodine (unsaturated and aromatic compounds) and ferric chloride (phenols) stains. Furthermore four compounds within

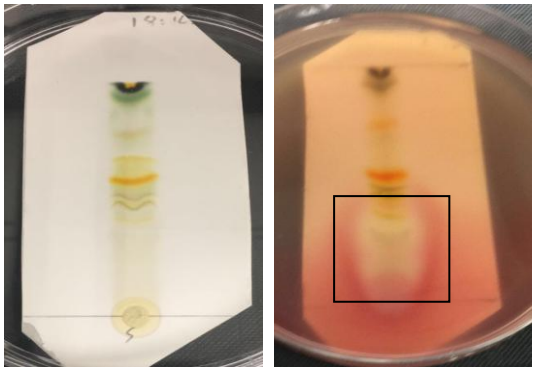
the antibacterial R_f range 0.36 ± 0.24 exhibited these positive results illustrating the fact that the antibacterial activity was from polyphenols. The TPC assay quantitatively demonstrated the presence of phenolic compounds in this red seaweed. The NMR, FTIR and UV-visible analysis on the semi-pure fraction also supported the hypothesis that the antibacterial compound responsible for such activity was a polyphenol with peaks and absorption bands related to OH groups and aromatic rings. The FTIR analysis produced the greatest depth of information with peaks corresponding to a phenolic functional group and the halogenated compound bromine concluding the antibacterial compound was indicative of a bromophenol.

Additionally, the presence of phenolic compounds was previously reported for their antimicrobial activity in seaweeds (601, 602, 627). Polyphenols (including bromophenols) are easily extracted in polar solvents such as the extracting solvent methanol used in this study (10) and have been described as stable compounds that can withstand high temperatures. For example, a study on the thermostability of the polyphenol compound dieckol isolated from the brown seaweed *Ecklonia cava* showed stable scavenging activities towards DPPH and hydroxyl radicals after 7 days at 90 °C (628). Polyphenols were also reported to exhibit strong activity against Gram-negative bacteria (575) which was also reported in Chapter 4 for the crude *P. lanosa* extract.

Further purification is required followed by spectroscopic analysis to allow for complete elucidation of this bromophenol compound. Nonetheless, it is a starting point for the determination of the structure of this potent antibacterial compound that is capable of controlling a destructive pathogen. Figure 6.16 illustrates a flow diagram of the optimised separation and purification methods established in this work for the bromophenol antibacterial compound(s) present in *P. lanosa*.

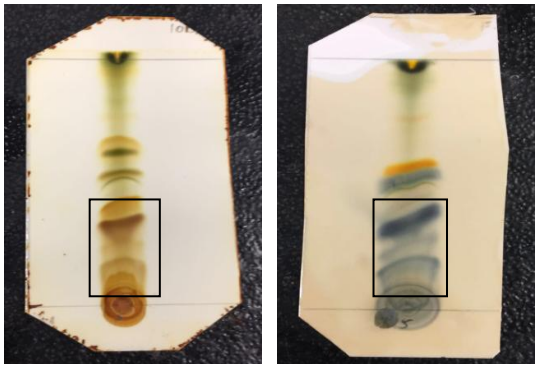
(A) Crude extract

Solvent extraction 1:50 *w/v* for 2 h under continuous magnetic stirring



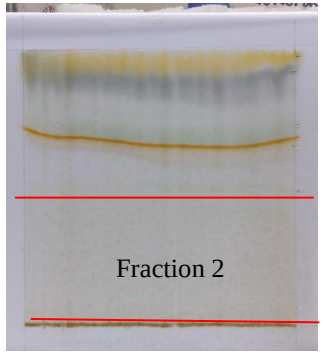
(B) Separation and identification of the bioactive(s)

Optimum mobile phase composition 8:1 *v/v* chloroform/methanol using overlay bioautography



(C) Specialised TLC profile stains

Aromatic/saturated and phenolic compounds identified, respectively



(D) Preparative TLC separation

8:1 *v/v* chloroform/methanol mobile phase

(E) Semi-pure antibacterial compound

Fraction 2 from preparative TLC separation

Structure elucidation on the semi-pure fraction with:

- LC-ESI-MS analysis
- GC-MS analysis
- NMR analysis
- FTIR-ATR analysis
- UV-visible analysis

Figure 6.16: Flow diagram of the optimised separation and purification methods for the antibacterial compound(s) present in *P. lanosa*.

6.4 Conclusions

The crude methanol extract of *P. lanosa* was first separated with analytical TLC using a variety of solvent systems. The crude *P. lanosa* extract was found to contain many compounds that separated out into smaller fractions depending on their polarity, which made the selection of mobile phase critically important. The optimum mobile phase was found to be chloroform/methanol 8:1 *v/v* which produced the best separation between the bands. Direct bioautography was conducted on the separated compounds and it was found that multiple compounds present in *P. lanosa* exhibited radical scavenging activity and demonstrated the significant antioxidant potential of seaweeds. Overlay bioautography was used to identify the antibacterial active compounds with a large zone of inhibition observed corresponding to a R_f range of 0.36 ± 0.24 on analytical TLC. Specialised TLC stains (Table 6.1) were used to profile the constituents of the crude extract, particularly for the compounds in the R_f range 0.36 ± 0.24. A positive result for four compounds in this R_f range for the iodine and ferric chloride stains indicated the presence of aromatic/unsaturated compounds and phenolics indicative of polyphenols. The stains also revealed the presence of terpenes, aldehydes/ketones, carboxylic acids and amino acids in the crude extract. Based on the specialized stain profiles, the TPC of the crude extract was established using the Folin-Ciocalteu method with phenolic content found to be present in a relatively low dose and corresponding to reports of red seaweed species typically exhibiting the lowest phenolic content compared to brown and green seaweeds.

Preparative TLC carried out under the same chromatographic conditions was used to separate out the active and non-active compounds from one another with eight fractions obtained. The fractions were tested for antibacterial activity against *X. fragariae* with fraction 2 found to exhibit the strongest antibacterial activity and was within the R_f antibacterial zone. Subsequently, a dose response study on this fraction found that the purified fraction exhibited much greater activity compared to the crude extract identifying the postive effects of purification suspected to be either due to pre-concentration and/or removal of antagonistic compounds. Therefore, fraction 2 was selected for spectroscopic analysis and was identified as semi-pure based on TLC analysis of the fraction which showed the presence of a number of bands. The LC-ESI-MS and GC-MS data proved inclusive. The NMR and FTIR data showed the presence of OH and aromatic peaks indicative of polyphenols with FTIR further exhibiting peaks

for a phenol and a halogenated compound believed to be that of bromine. This concluded the antibacterial compound as a bromophenol which have been reported to posses antibacterial activity and have been previously isolated from *P. lanosa*. UV-visible analysis of the fraction detected a chromophore at 665 nm which relates to a system of extensively conjugated pi-electrons in an unsaturated compound likely from the presence of aromatic rings. However, no full structure could be derived for this bromophenol antibacterial compound due to the semi-pure nature of this fraction. Further purification and phytochemical analysis of this fraction are needed to fully identify this bromophenol antibacterial compound(s).

CHAPTER 7: CONCLUSIONS & FUTURE WORK

7.1 Conclusions

The potential of seaweed extracts to be utilised as biopesticides has gathered much interest in the last number of years as a result of synthetic pesticides coming under scrutiny including the indiscriminate use of these broad-spectrum pesticides that can damage human health and the environment. Furthermore, pressure on pesticide use has increased amidst growing concerns expressed by governments and consumers about the safety of pesticide residues in food. In order to evaluate the biopesticide potential of seaweeds, a number of species commonly found along the Irish coastline were screened against a range of significant plant pathogens.

Initial research focused on two fungal pathogens which cause considerable damage to the Irish forestry industry, *Armillaria mellea* (Vahl) P. Kumm and *Heterobasidion annosum sensu stricto* (Fries) Brefed. Seaweed species from the three major marcoalgae groups were selected *Fucus serratus* and *Ascophyllum nodosum* (Phaeophyceae), *Polysiphonia lanosa* (Rhodophyta) and *Ulva lactuca* (Chlorophyta) and were collected from the Irish coastline. The seaweeds were extracted in solvents ranging in polarity in order to identify the seaweed with the best fungicidal properties. The seaweeds were extracted using conventional solvent extraction techniques at a low ratio (1:50 *w/v*) at two varying time points of 2 h and 24 h to ensure all of the bioactive compounds were extracted. An initial investigation into the stirring methods applied to solvent extraction found that magnetic stirring generated a higher yield of bioactives compared to automatic shaking and was, therefore, selected as the extraction method for the entire study. The extracting solvents water, methanol, ethanol and acetone were found to have an influence on the resulting yields with water producing the highest yield and acetone the lowest; most likely caused by the high composition of polar constituents present in seaweeds namely, carbohydrates such as polyphenols, phlorotannins. The extraction period also had an influence on yields, with the longer the extraction period (24 h) the greater the yield. The newly generated crude extracts were screened for antifungal activity using the poisoned food technique commonly used to assess the antifungal activity of natural products. Unfortunately, crude extracts exhibited no antifungal activity against the problematic fungus *A. mellea.* However, the seaweed extracts were found to produce a potential hormetic dose response on both tested fungi, highlighting a possible novel application as a biocontrol agent. The methanol extract of the green seaweed, *U. lactuca* exhibited the strongest percentage inhibition against *H. annosum*

(50.0 ± 5.3% at 5 mg/mL) in a dose response manner. Ethanol also proved to be the superior solvating solvent compared to water and the aprotic solvent DMSO. As the methanol extract of *U. lactuca* proved the most promising for use as a biofungicide from the initial screen (Chapter 2) it was selected for further study.

An investigation into other antifungal test methods namely disk diffusion assay, well diffusion assay, broth dilution assay and agar plate test was conducted in order to determine whether a more efficient method could be developed for assessing the antifungal capacity of crude seaweed extracts against *H. annosum*. Although the poisoned food technique is a renowned method of choice, it suffers from a number of drawbacks including the use of larger volumes of extract compared to other methods, results take a week to be obtained and it is not accurate enough to determine an MIC value. However, it was revealed that none of the four methods were effective with both disk and well diffusion exhibiting problems at the adjustment stage of the *H. annosum*. The solvating solvent ethanol proved toxic in the broth dilution assay and diffusion issues caused problems with the agar plate test. Therefore, the optimum antifungal test method for *H. annosum* remains that of the poisoned food technique.

The second part of this study involved assessing the potential of the methanol extract of *U. lactuca* as a natural fungicide *ex vivo*. A method was successfully developed to qualitatively assess the protectant activity of the crude extract against *H. annosum* on disks of the susceptible plant species, Sitka spruce. The optimised method involved the application of autoclaved disks soaked in extract for a specific period of time before being dried and sandwiched to a pre-inoculated plate of *H. annosum* for one week at 20 °C. The crude extract at a concentration of 5 mg/mL soaked for 3 h proved ineffective with strong mycelia growth observed on the disks. However, an increase in concentration to 20 mg/mL and extended soaking time of 18 h or 24 h resulted in strong protective activity with the majority of disks displaying no visual signs of infection. The commercial plant protection product (PPP) urea currently used to control *H. annosum* in the forestry industry was compared to that of the crude extract both *in vitro* and *ex vivo*. The extract was found to compete favorably with the commercial product, however field trials would allow for a direct comparison. These results, therefore, highlighted the potential of seaweed extracts for use as a naturally derived pesticide against problematic fungal plant pathogens.

In order to fully establish the novel application of seaweed extracts as biopesticides a second screen was conducted on quarantine bacterial plant pathogens in order to determine whether the seaweeds were also effective against bacterial pathogens. The four seaweed species used in the initial screen in Chapter 2 were used again and extracts were generated by applying the same method and extracting solvents. The water extract of the brown seaweed *Fucus vesiculosus* was also investigated due to a previous researcher in the Eco-Innovation Research Centre (EIRC) group reporting strong antibacterial activity by this extract against human clinical pathogens. The antibacterial activities of the crude extracts were tested against three Gram-positive and six Gram-negative pathogens using the standardised disk diffusion assay at 5 mg/mL. All of the seaweed species were found to exhibit antibacterial activity at varying degrees, except for *U. lactuca* which exhibited no activity which was unexpected since it demonstrated the strongest antifungal activity. The methanol extract of *P. lanosa* exhibited the broadest range and strongest activity specifically against Gram-negative bacteria including; *Xanthomonas arboricola* (15.83 ± 0.41 mm), *Xanthomonas hyacinthi* (7.83 ± 0.98 mm), *Xanthomonas fragariae* (14.83 ± 1.47 mm) and *Xanthomonas campestris* (8.0 ± 1.0 mm). Therefore, this *P.lanosa* extract was studied further with a short investigation conducted on sterilisation methods with autoclaving proving to be the superior method. This study also demonstrated the thermostability of the bioactive compound(s) present in the methanol extract with activity still retained after autoclaving. The phytopathogen, *X. fragariae* was selected as the bacteria for further study for the rest of this research due to the fact that it was found to be the most susceptible bacteria from the initial screen and is also an international quarantine pathogen of considerable concern to the strawberry industry with no effective control measure available. The MIC and MBC of this sterilised extract was determined using a colorimetric assay. The MIC was found to be 6.25 mg/mL with the MBC found to be greater than the tested concentration range of 25 mg/mL.

Since the vast majority of bacteria form biofilms as a method of survival, including plant pathogens, an effective biopesticide must possess antibiofilm properties. Fortunately, the crude extract was found to exhibit biofilm prevention in a dose response manner with greater than 80% prevention at 6.25 mg/mL against *X. fragariae.* The *P. lanosa* extract was also found to be capable of disrupting an established biofilm with an MBEC50 and MBEC90 of 3.125 mg/mL and 6.25 mg/mL, respectively. This

demonstrated the methanol extract of *P. lanosa* as a potential antibiofilm agent capable of inhibiting biofilm formation and/or disruption of an already established biofilm. A phytotoxicity study using the root hair assay was also conducted on this promising extract to identify potential phytotoxic properties. The crude *P. lanosa* extract was found to be phytotoxic against *A. thaliana* in a dose response manner with isolation and purification of the bioactive(s) required to allow for a more accurate phytotoxicity study.

The extraction of bioactive(s) is a critical part in assessing the antimicrobial potential of seaweed with a vast range of techniques available. This led to an investigation in Chapter 5 on the effect of extraction time and feed:solvent ratio on % yield and antibacterial activity of the *P. lanosa* extract generated from three extraction techniques; solvent extraction, Soxhlet extraction and UAE. Extraction time was found to have a greater influence, particularly on % yields for all techniques. Soxhlet extraction was found to be the superior method with regards to optimum % yields and activity, with optimum extract properties achieved with an extraction time of 6 h and feed:solvent ratio of 1:50 *w/v*. The effectiveness of the crude *P. lanosa* extract was also found to be dose dependent with 1 mg/mL exhibiting no activity compared to 10 mg/mL which demonstrated a relatively large zone of inhibition (18.33 ± 1.2 mm).

As mentioned previously, in order to fully establish the potential of the *P. lanosa* extract as a biopesticide against *X. fragariae*, it is essential to isolate and purify the responsible bioactive(s). The first step was the development of an efficient analytical TLC method to allow for maximum separation of the various compounds present in *P. lanosa* based on their polarities. The solvent system chloroform/methanol 8:1 *v/v* was found to produce optimum separation of the crude extract. An investigation into the radical-scavenging capacity of these separated compounds via direct bioautography (DPPH agent) identified the strong antioxidant activity exhibited by specific bioactives present in *P. lanosa*. Overlay bioautography was conducted in order to identity which compound(s) were responsible for this antibacterial activity with a zone of inhibition observed and a R_f range of 0.36 ± 0.24 recorded. Since the compound(s) responsible had not been identified, a number of specialized stains were sprayed onto the TLC plate and were used to identify various functional groups depending on the specific colour change. Aldehydes/ketones, carboxylic acids, terpenes and amino acids were found to be present in *P. lanosa* with four compounds producing positive results for phenolic and

aromatic/unsaturated compounds within the R_f range of the identified antibacterial activity, indicating the possible presence of polyphenols. Since these results revealed the presence of phenols, TPC of the crude methanol extract was conducted using the standardised Folin-Ciocalteu method, with phenolic content quantatively determined and typical to red seaweeds was found to be low compared to reported brown and green seaweed values.

After identifying the antibacterial compound(s) present in *P. lanosa*, the next step was purification which involved preparative TLC. A total of eight fractions were obtained and tested for antibacterial activity with seven out of the eight fractions demonstrating some degree of activity. Fraction 2, which was in within the R_f range 0.36 ± 0.24 as the initially identified antibacterial compounds in analytical TLC and showed the strongest degree of activity making it the most potent antibacterial compound(s). For this reason, fraction 2 was chosen to undergo preliminary characterisation via LC-ESI-MS, GC-MS, NMR, FTIR-ATR and UV-visible analysis with the aim of elucidating a polyphenol structure for this bioactive compound(s). From examination of the spectroscopic data, the LC-ESI-MS and GC-MS data proved inconclusive. However, the NMR and FTIR data supported the results in the specialized stain profiles with the detection of phenolic groups, aromatic rings and alkenes all of which are indicative of polyphenols. FTIR also detected the peresence of a phenol functional group and a halogenated compound believed to be that of bromine indicative of bromophenol. However, complete elucidation of this antibacterial compound could not be proposed since this was only a semi-pure fraction and required further purification to allow for full characterisation of the compound(s).

Completion of this research identified novelty in a number of areas including:

1. This is the first study on the fungicidal activity of seaweed extracts against *Armillaria mellea* (Vahl) P. Kumm and *Heterobasidion annosum sensu stricto* (Fries) Brefed.
2. This is the first report of strong antifungal activity by the methanol extract of *U. lactuca* against *H. annosum*.
3. This is the first report of potent bactericidal and antibiofilm activity exhibited by the methanol extract of *P. lanosa* against the phytopathogen *X. fragariae.*
4. This is the first phytotoxicity study on the crude *P. lanosa* extract using the root hair assay on the model plant species *Arabidopsis thaliana.*
5. This is the first attempt to purify and identify the anti-*X. fragariae* bioactive(s) present in *P. lanosa.*

7.2 Future work

This body of work represents one of the first uses of seaweed extracts as potential biopesticides against fungal and bacterial plant pathogens that are currently causing devastating effects to both the forestry and agri-food sector. The investigations into the effectiveness of the most promising extracts provided comprehensive data on the capability of these extracts with respect to their potential as an attractive alternative to conventional synthetic pesticides.

This initial section of the study demonstrated the potential novel use of methanol extracts of *Ulva lactuca* as a biopesticide against *Heterobasidion annosum*. However, there are a number of areas in which more work could be done to add to the research including the isolation and identification of the responsible bioactive(s) to aid in developing a commercially viable biopesticide product. Some of the future potential directions for this research include:

- Phytotoxicity study of the *U. lactuca* extract using the root hair assay to identify any possible phytotoxic effects that would affect non-target species.
- Study of the mechanisms of action of *U. lactuca* against *H. annosum* including; inhibition of respiration, inhibition of polysaccharide biosynthesis, disruption of cell membrane, etc, (629).
- Completion of a field trial on a Sitka spruce-felling site in order to assess the ability of *U. lactuca* extracts in preventing *H. annosum s.s.* basidiospores germination over the period of susceptibly, typically one month.
- Study of the competitive ability of the methanol extract to that of the PPP urea in field trials to determine the potential of eradicating the use of this controversial PPP or reducing its current approved concentration (37% *w/v*) through a synergistic relationship.
- Separation and identification of the active compound(s) through various chromatography and spectroscopic techniques.
- Study of the possibility of synthetically producing the active compound so as to overcome the issue of low yields.

Promising results for the methanol extract of *P. lanosa* as a biopesticide in controlling *X. fragariae* infection were identified in Chapter 4 – 6. However, similar to the antifungal work more research related to this antibacterial activity could be carried out especially in relation to the isolated compound of *P. lanosa*. Therefore the aims would be:

- To study sequential purification techniques including column chromatography, solvent partitioning (630) and semi-preparative HPLC (631) to obtain a pure compound.
- To elucidate the structure of this purified antibacterial compound through UV, FTIR, LC-MS and NMR analysis.
- To purify enough compound(s) to allow for the determination of MIC, MBC and antibiofilm activity.
- To study the phytotoxicity and cytotoxicity of this pure compound(s) to ensure its safety to non-target species and human health including genetic and biochemical work on the cytotoxicity assessments in order to identify the source of this cytotoxic activity if observed (632).
- To study the potential of synthetically producing such compound(s) to allow for streamline production of the compound without the need for mass removal of seaweed from the coastline.
- To study the *in vivo* potential of the *P. lanosa* compound(s) in protecting *Fragaria x ananassa* (strawberry plants), a susceptible plant with great economic importance, against *X. fragariae* infection.
- To compare the antibacterial activity of *P. lanosa* against copper-based products currently used as a control with a possible synergistic application.
- To develop an effective biopesticide formulation containing the antibacterial compound and allowing for maximum protection.

Regardless of the specific seaweed species, to be a viable alternative it must be capable of meeting the demands of the global pesticide market. In order to achieve this industrial scale up, a vast quantity of seaweed is required but such a large removal of seaweed can have destructive effects on the ecology of surrounding areas (239). A number of possible solutions exist and include: aquaculture of the specific seaweed

species or identification of these bioactive(s) to allow for large-scale production of these novel compounds.

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APPENDIX I

RESEARCH OUTPUTS

LIST OF OUTPUTS

Papers:

1. **O' Keeffe, E.,** Hughes, H., McLoughlin, P., Tan, S. P. and McCarthy, N. 'Methods of analysis for the *in vitro* and *in vivo* determination of the fungicidal activity of seaweeds: a mini review', Journal of Applied Phycology. 2019 doi: 10.1007/s10811-019-01832 (Impact Factor: 2.401).
2. **O' Keeffe, E.,** Hughes, H., McLoughlin, P., Tan, S. P. and McCarthy, N., 'Antibacterial activity of seaweed extracts against plant pathogenic bacteria'. Journal of Bacteriology and Mycology. 2019;6(3): 1105 (Internal Impact Factor: 3.724).

Published abstracts:

Oral presentation

1. **O' Keeffe, E.,** Hughes, H., McLoughlin, P., Tan, S. P. and McCarthy, N. (2018) 'Investigating the use of seaweed extracts as biopesticides', *In 20th International Conference on Biopesticides and Human Health*, 20th – 21st August 2018, Barcelona, Spain.

Poster presentation

1. **O' Keeffe, E.,** Hughes, H., McLoughlin, P., Tan, S. P. and McCarthy, N. (2017) 'Investigating the use of seaweeds as a novel source of pesticides in forestry', *In 14th International Phytotechnologies Conference*, 25th – 29th September 2017, Montréal, Canada.
2. **O' Keeffe, E.,** Hughes, H., McLoughlin, P., Tan, S. P. and McCarthy, N. (2017) 'Investigating the potential use of seaweed extracts as pesticides in forestry', *In Waterford Institute of Technology Research Day*, 18th May 2017, Waterford, Ireland.

3. **O' Keeffe, E.,** Hughes, H., McLoughlin, P., Tan, S. P. and McCarthy, N.. (2016) 'Investigating the potential use of seaweed extracts as pesticides in forestry', *In Waterford Institute of Technology Research Day*, 4th May 2016, Waterford, Ireland.

Workshop:

1. **O' Keeffe, E.,** (2016) 'Fungal taxonomy and identification using traditional (i.e. not molecular) techniques', *In Instytut Badawczy Leśnictwa (IBL)*, 22nd - 24th November 2016, Sękocin Stary, Warsaw, Poland.