Master's thesis

NTNU
Norwegian University of Science and Technology
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Department of Biotechnology and Food Science

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Microwave Assisted Extraction of Bioactive Compounds from *Alaria esculenta*, and its Antioxidant and Antimicrobial Properties

Master's thesis in Food Science, Technology and Sustainability
Supervisor: Jørgen Lerfall
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Trondheim, May 15th 2023

Synne H. Røsten

Synne Hylen Røsten

Abstract

Macroalgae is an abundant marine species known for being rich in bioactive compounds. However, extraction of high quality yields of these compounds has shown to be challenging. For this reason, novel and emerging green technologies have been gaining attention for their potential to produce high yields of bioactive compounds. Therefore, the aim of the present thesis was to investigate the potential of microwave assisted extraction (MAE), as a novel and emerging green technology, for its potential to extract high yields of bioactive compounds from the brown alga *Alaria escuelnta* and investigate its *in vitro* antioxidant activity and antimicrobial properties.

MAE has been optimized by testing the combination of the extraction temperatures: 40°C, 60°C, 80°C, 100°C, 120°C, and 140°C, and the holding times: 2, 5, and 15 minutes. As chemical characteristics, the total polyphenol content (TPC) in the extracts was determined using the Folin-Ciocaltue method, fucoxanthin was quantified using high performance liquid chromatography, and the pH in the extracts was measured. Further, the antioxidant activity was evaluated using the known *in vitro* colorimetric assays: 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), 2,2'-azin-bis(3-ethylbenithiazoline-6-sulfonic acid) assay (ABTS), and ferric reducing antioxidant power (FRAP). Furthermore, the relationship between the chemical characteristics and antioxidant activity was assessed using Pearson's correlation coefficient (r) and principal component analysis. Moreover, the antimicrobial effect of the extract towards the microorganisms *Pseudomonas fluorescence*, *Listeria innocua*, and *Listeria monocytogenes* was examined using a 96-well plate inhibition assay. Additionally, the difference in the chemical characteristics and antioxidant activity was evaluated for *A. esculenta* harvested in May 2021 and May 2022.

The results indicated the potential of MAE to extract bioactive compounds from the brown alga *A. esculenta*, as higher yields of the TPC and fucoxanthin content were found compared to no microwave treatment. The optimized extraction parameters differed depending on the chemical compound. For the total polyphenol content, the optimized parameters were an extraction temperature of 120°C with a holding time of 5 minutes. The optimized parameters for fucoxanthin are inconclusive, as the highest quantified fucoxanthin content was obtained by the highest tested extraction temperature and holding time. The pH in the extracts was affected by increasing extraction temperature. All extract exhibited *in vitro* antioxidant activity and was found to be strongly correlated with the fucoxanthin content and less correlated with the total polyphenol content. The extracts were found to have antimicrobial effects towards both *Listeria innocua* and *Listeria monocytogenes*. Difference in harvest year was only found for the TPC. In conclusion, MAE shows promise as an effective method for extracting bioactive compounds from *A. esculenta* with antioxidant and antimicrobial properties. Proposing bioactive compounds from *A. esculenta* as potential candidates for possible applications within the food industry.

Sammendrag

Makroalger er en rikelig marine art som er kjent for å være rik på bioaktive forbindelser. Imidlertid har utvinning av høykvalitets utbytte av disse forbindelsene vist seg å være utfordrende. Av denne grunn har nye og fremvoksende grønne teknologier fått oppmerksomhet for deres potensial til å produsere høye utbytter av bioaktive forbindelser. Derfor var målet med denne masteroppgaven å undersøke potensialet til mikrobølgeassistert ekstraksjon (MAE), som en ny og fremvoksende grønn teknologi, for dets potensial til å utvinne høye utbytter av bioaktive forbindelser fra brunalgen *Alaria esculenta* (*A. escuelnta*) og undersøke dens *in vitro* antioksidant kapasitet og antimikrobielle egenskaper.

En optimalisering av MAE ble gjennomført ved å teste ulike kombinasjoner av ekstraksjonstemperaturene: 40°C, 60°C, 80°C, 100°C, 120°C og 140°C med holdetidene 2, 5 og 15 minutter. For kjemisk karakterisering ble total polyfenol innhold målt ved bruk av Folin-Ciocaltue methoden, pigmentet fucoxantin ble kvantifisert ved high performance liquid kromatografi, og pH i ekstraktene ble målt. Videre ble den antioksidative kapasiteten evaluert ved ulike *in vitro* assays: 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), 2,2'-azin-bis(3-ethylbenithiazoline-6-sulfonic acid) assay (ABTS), and ferric reducing antioxidant power (FRAP). Den antimikrobielle effekten av ekstraktene mot mikroorganismene *Pseudomonas fluorescence* (*P. fluorescence*), *Listeria innocua* (*L. innocua*) og *Listeria monocytogenes* (*L. monocytogenes*) ble undersøkt ved bruk av et 96-brønnsplate inhiberende assay. I tillegg ble forskjellen i de kjemiske karakteristikene og den antioksidative kapasiteten undersøkt for *A. esculenta* høstes i mai 2021 og mai 2022.

Resultatene viste at MAE har potensial til å ekstrahere bioaktive forbindelser fra brunalgen *A. esculenta*, da høyere utbytte av TPC og fucoxantin ble funnet sammenlignet med ingen mikrobølgebehandling. De optimale ekstraksjonsparametrene varierte avhengig av den kjemiske forbindelsen. For TPC var de optimale parameterne en ekstraksjonstemperatur på 120°C med en holdetid på 5 minutter. De optimale parameterne for fucoxantin var ikke entydige, da det høyeste kvantifiserte fucoxantin-innholdet ble oppnådd ved den høyeste testede ekstraksjonstemperaturen og holdetiden. pH-verdien i ekstraktene ble påvirket av økende ekstraksjonstemperatur. Alle ekstraktene viste *in vitro* antioksidantiv kapasitet og hadde sterk korrelasjon med fucoxantin innholdet og lavere korrelasjon med TPC. Ekstraktene viste også antimikrobiell effekt mot både *L. innocua* og *L. monocytogenes*. Det ble kun funnet forskjell i TPC mellom høstningsårene. Resultatene viser at MAE har et lovende potensial som en effektiv metode for å ekstrahere bioaktive forbindelser fra *A. esculenta* med antioksidant- og antimikrobielle egenskaper. Disse forbindelsene kan ha potentielle applikasjoner for bruk i matindustrien.

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Abbreviations

A. esculenta	*Alaria esculenta*
ABTS	2,2'Azin-bis(3-ethylbenzithiazoline-6-sulfonic acid)
ANOVA	Analysis of Variance
BHI	Brian Heart Infusion
BHIA	Brian Heart Infusion Agar
diH_2O	Deionized Water
DPPH	2,2-diphenyl-1-picrylhydrazyl
dw	Dry Weight
EAE	Enzyme Assisted Extraction
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalents
GLM	General Linear Model
HPLC	High Performance Liquid Chromatography
MAE	Microwave Assisted Extraction
OD	Optical Density
PCA	Principal Component Analysis
PEF	Pulsed Electric Field
ROS	Reactive Oxygen Species
RSA	Radical Scavenging Activity
S. latissima	*Saccharina latissima*
SDG	Sustainable Development Goals
SES	Seaweed Solution AS
SLE	Solid-Liquid Extraction
TE	Trolox Equivalents
TPC	Total Polyphenol Content
UN	United Nations

Chapter 1

Introduction

The ocean, making up 70% of the worlds surface (Skjermo et al., 2014), is a rich source of underutilized resources with a wide range of unexploited potentials. Macroalgae is a diverse group of low trophic marine organisms with a wide range of potential uses. The interest in macroalgae has rapidly increased over the past years, considering its substantial potential (Broch et al., 2019; FAO, 2021). Macroalgae can be applied as food, feed, medicine, fertilizers, bioenergy, biochemicals, and biomaterials (Skjermo et al., 2014).

To maintain food safety and quality, the food industry uses a variety of synthetic antioxidants (e.g., butylated hydroxyanixyquinone) and antimicrobial substances (e.g., sodium benzoate, sodium nitrite, and sorbic acid). However, these synthetic substances can have adverse effects on consumer health (Branen, 1975; Chen et al., 1992) and the environment (Bimpizas-Pinis et al., 2022). In addition, there is an emergence of multi-drug resistance (WHO, 2014). Consequently, consumers demand the use of natural preservatives. Marine macroalgae are among the richest sources of natural antioxidant and antimicrobial compounds among marine species. Moreover, bioactive compounds from macroalgae have shown strong antioxidant effects (Cox et al., 2010; Hermund et al., 2015) and antimicrobial activity against several microorganisms (Gupta et al., 2010; Rubiño et al., 2022), proposing them as good natural alternatives to synthetic preservatives.

Preserving microbial and oxidative deterioration is highly important in the food industry. In particular, these challenges lead to increased food waste. Considering the United Nations (UN) Sustainable Development Goals (SDGs) (United Nations, 2015) (Figure 1.1), finding natural and effective preservatives can lead to improved health (SDG 3) and a reduction of food waste (SDG 12). However, the method of obtaining these compounds also needs to be considered. Present methods to extract bioactive compounds from macroalgae have several limitations and negative drawbacks, such as the use of hazardous chemicals, high energy, and time consumption (Simoneau et al., 2000). Therefore, it is desirable to find more effective and sustainable methods (SDG 13). Furthermore, novel and emerging technologies, such as microwave assisted extraction, have shown to be good green alternatives for the extraction of bioactive compounds from macroalgae. All in all,

the search for bioactive compounds from macroalgae using novel green technologies is advantageous for all the three Ps of sustainability (people, planet, profit) (Van den Burg et al., 2021).

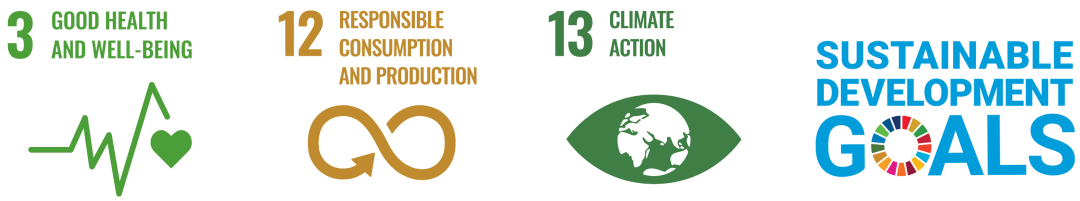

Figure 1.1: The Sustainable Development Goals the present thesis contributes the most towards, and include SDG 3, 12, and 13. Retrived from United Nations (2015)

1.1 Aim of the Thesis

The main aim of the present thesis is to investigate the potential of microwave assisted extraction, a novel, time-efficient, and emerging green technology, to extract bioactive compounds in regards to polyphenols and fucoxanthin from the brown alga *Alaria esculenta* as compared to no microwave treatment. Moreover, to investigate the bioactivity of *Alaria esculenta* extracts, with a focus on its antioxidant and antimicrobial properties. The following sub-objectives were established in order to achieve the main goal:

1. To optimize microwave assisted extraction on the brown alga *Alaria esculenta* by applying different microwave extraction temperatures and holding times. Moreover, to study the effect of the solvent on extraction.

2. To evaluate the optimized extraction parameters for microwave assisted extraction based on the total polyphenol content and fucoxanthin content and how the extraction affected the pH in the extracts.

3. To investigate the *in vitro* antioxidant activity of the extracts as affected by extraction parameters. In addition, to explore the relationship between the chemical characteristics and the antioxidant activity of the extracts.

4. To examine the extracts antimicrobial effect against the foodborne pathogenic bacteria *Pseudomonas fluorescence*, *Listeria innocua*, and *Listeria monocytogenes*.

5. To compare the chemical characteristics and antioxidant activity of *Alaria esculenta* harvested in May 2021 and May 2022.

Chapter 2

Background

2.1 Macroalgae

Macroalgae, also called seaweeds, are a diverse group of low-trophic marine organisms and a major component of the biosphere (Duarte et al., 2022). They are primary producers that form the basis of many aquatic food chains (Krause-Jensen and Duarte, 2016) and provide habitats for a range of marine organisms (Walker and Kendrick, 1998). Macroalgae are classified into three main phyla: Phaeophyta (brown algae), Chlorophyta (green algae), and Rhodophyta (red algae). The classification is based on the pigment distribution and chemical structure (Baweja and Sahoo, 2015). The largest and most complex group is the brown algae. They have a characteristic brown or olive color caused by carotenoids and chlorophyll in their plastids (Shannon and Abu-Ghannam, 2018). Macroalgae are inhabitants in the intertidal and subtidal marine ecosystems, which are constantly exposed to fluctuating and stressful environments. Factors such as temperature, humidity, salinity, irradiance, nutrient availability, and competitive biotic interactions play major roles in algal growth, development, reproduction, and productivity (Bedoux and Bourgougnon, 2015). Living in such conditions leads to good adaptation by the developing defense mechanisms and producing secondary metabolites that have potential bioactivities (Ibañez et al., 2012).

Since ancient times, macroalgae have been utilized as food, feed, fertilizers, and a source of pharmaceuticals. In 2019 the global wild harvest of macroalgae was 1.1 million tonnes, whereas macroalgae cultivation was approximately 35.9 million tonnes (Cai et al., 2021). Of the global production, 40% are utilized directly as food, where they are a huge component of Asian diets. As a result, the majority of cultivation takes place in Asia (Ferdouse et al., 2018), 40% is consumed indirectly through processed foods, and 20% are used in a range of industrial applications (Cai et al., 2021). In Europe, the brown algae *Alaria esculenta* and *Saccharina latissima* are the two most frequent cultivated species, characterized by their ability to reach high biomass yield and their abundance in valuable nutritional elements (Stévant et al., 2017). Nonetheless, the cultivation of macroalgae is gaining attention (FAO, 2021), and the cultivation of macroalgae has increased in

Europe over the last decades (Stévant et al., 2018). In addition, macroalgae are the raw material for the industrial production of carrageenan, agar, and alginate (Ferdouse et al., 2018). Carrageenan and agar are produced from red algae, and alginate is extracted from brown algae, *Laminaria hyperborea* are among the main species for alginate production, in which approximately 85 000 tons of algae are used for production annually (Abka-Khajouei et al., 2022).

2.2 Bioactivity of Brown Algae

Brown algae are found to be rich in substances that exhibit high bioactivity. Both antioxidant and antimicrobial effects are expressed by brown algae pigments (chlorophylls, xanthophylls, and carotenoids) (Karpiński and Adamczak, 2019; T. Wang et al., 2014), polysaccharides (Kadam et al., 2015; M. Liu et al., 2017; Miyashita, 2014), fatty acids (M. N. A. Khan et al., 2007) and phenolic compounds, including polyphenols (Farvin and Jacobsen, 2013; T. Wang et al., 2009) and flavonoids (Cox et al., 2010). The concentration of bioactive compounds in brown algae does vary after season, geographical area, and age. In addition, the distribution of these compounds in the algae varies (Connan et al., 2006; Gager et al., 2020; T. Wang et al., 2009). The major components contributing to this bioactivity have been identified as polyphenols, pigments, and polysaccharides.

Brown algae are known to be particularly rich in polyphenols, which are structural elements of the cell wall (Schoenwaelder and Clayton, 1999) and are found in specialized cytoplasmic vacuoles known as physodes (Schoenwaelder, 2002). The most abundant polyphenolic compound in brown algae is phlorotannins (T. Wang et al., 2009). Phlorotannins are hydrophilic polymers of phloroglucinol units generated as secondary metabolites in the acetate-malonate pathway (X. Liu et al., 2017). Phenolic compounds in macroalgae are produced for protection against various abiotic and biotic stressors, such as UV radiation, extreme temperature, salinity, pathogenic infections, and herbivory (Jimenez-Lopez et al., 2021).

Chlorophylls and carotenoids are the major pigments in brown algae (Shannon and Abu-Ghannam, 2018). Carotenoids in brown algae can be further classified into two groups, carotenes, and xanthophylls. Carotene is a pure hydrocarbon that does not contain oxygen, whereas xanthophylls are oxygenated carotenes. The presence of oxygen in xanthophylls makes them more polar and less hydrophobic than the carotenes (Gammone and D'Orazio, 2015). Xanthophylls can be divided into primary and secondary types. Primary xanthophylls are involved in light harvesting, while secondary xanthophylls are metabolites produced after environmental stimuli (Guedes et al., 2011). Fucoxanthin is a secondary metabolite located in the thylakoid membrane of the chloroplast. It is responsible for the brown color and the most abundant carotene in brown algae (Jin et al., 2003).

Polysaccharides in brown algae have several functions, such as being a food reserve, and provide strength and flexibility (Gupta and Abu-Ghannam, 2011a). Sulfated polysaccharides found in brown algae have been found to have high bioactivity, including compounds such as fucoidans and laminarin, both water-soluble (Gupta and Abu-Ghannam, 2011a), which mainly are located in the cell wall matrix and the intracellular space (T. Wang et al., 2014). The bioactivity of these compounds is dependent on molecular weight, sulfated ester content, glucuronic and fucose content, and the position of the sulfate group (Berteau and Mulloy, 2003; Li et al., 2005; Zhao et al., 2008).

2.2.1 Antioxidant Properties of Brown Algae

Antioxidants are molecules capable of suppressing oxidation processes (Gulcin, 2020). Their role is crucial in preventing oxidative stress in the human body and inhibiting lipid oxidation in food. Oxidative stress is caused by reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. They are generated as a product of cellular functions and oxygen metabolism (Valko et al., 2007). Lipid oxidation is caused by autoxidation, photooxidation, and enzymatic oxidation. Autooxidation is one of the most important oxidative mechanisms and consists of three phases: (1) Initiation, leading to an increase in free radicals, (2) propagation, by radical chain reactions and (3) termination by producing non-radical compounds (Choe and Min, 2009). Antioxidants can prevent or slow down these processes from occurring by their ability to free radical scavengers, by singlet oxygen quenchers, metal chelators, and inactivate photosensitizer and lipoxygenase (Choe and Min, 2009).

Polyphenolic substances can serve as chain-breaking antioxidants by donating hydrogen to free radicals, forming relatively unreactive antioxidant radicals (Phang et al., 2023). Phlorotannins capacity to scavenge ROS can be linked to their ability to their highly branched molecular structure with multiple phenol rings (up to eight) (T. Wang et al., 2009). One of the mechanisms in which polyphenols scavenge free radicals is illustrated in Figure 2.1. Upon the formation of free radicals, phenolic units donate their electron to these oxidizing agents when free radicals develop, producing an intermediate phenoxyl radical species (PhO•). The PhO• can then be stabilized through resonance delocalization of the unpaired electron to the benzene ring at the *orto* and *para* position. Additional mechanisms involve formation of hydrogen bonds with adjacent hydroxyl groups or dimerization (phenol coupling) to produce new compounds (Phang et al., 2023).

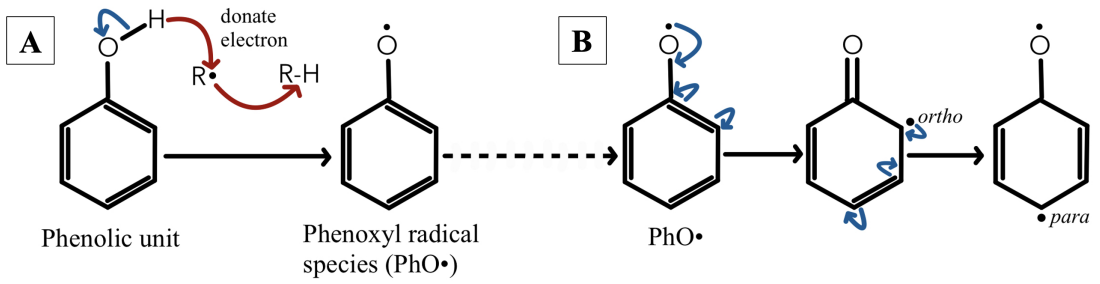

Figure 2.1: A mechanism in which polyphenols scavenge free radicals. [**A**] The phenolic unit donate their electron to the R• and transform into the phenoxyl radical species (PhO•). [**B**] PhO• are stabilized via resonance delocalization by shifting the unpaired electron to the *ortho* and *para* position of the benzene ring. Modified from Phang et al. (2023).

Most carotenoids contain an extended system of conjugated double bonds, which is responsible for their antioxidant activity, with singlet oxygen quenching being their main antioxidant mechanisms, but they are also capable of inhibiting free radical reactions (Sies and Stahl, 1995). Sachindra et al. (2007) investigated the singlet oxygen quenching and radical scavenging activity of marine fucoxanthin and reported fucoxanthin as a potent singlet oxygen quencher and as an effective free-radical scavenger. In the case of the antioxidant ability of polysaccharides, it is related to their structural features, such as the degree of sulfating, molecular weight, type of major sugar, and glycosidic branching (Qi et al., 2005; Zhang et al., 2003).

A range of studies has been conducted for the antioxidant properties of macroalgae for many different species, as reviewed by Balboa et al. (2013). However, the use of macroalgae antioxidants in food products/systems and more limited but existing, as reviewed by Roohinejad et al. (2017).

2.2.2 Antimicrobial Effect of Brown algae

Brown algae have been found to have higher antimicrobial properties compared to green and red algae (Cox et al., 2010). The antimicrobial effect of isolated compounds from macroalgae or macroalgae extracts has been reported on both gram-positive and gram-negative bacteria. Including the food borne pathogenic bacteria; *Listeria monocytogenes* (Cox et al., 2010; Rajauria and Abu-Ghannam, 2013), *Staphylococcus aureus* (Eom et al., 2014; Pierre et al., 2011, *Pseudomonas aeruginosa* (Cox et al., 2010; Karpiński and Adamczak, 2019; M. Liu et al., 2017), and *Escherichia coli* (Nagayama et al., 2002; Y. Wang et al., 2009), among others. The expression of antimicrobial activity of macroalgae components is only moderately understood (Gupta and Abu-Ghannam, 2011b). However, the investigation of isolated compounds has provided a greater understanding of the expressed antimicrobial activity of macroalgae. Possible investigated mechanisms for antimicrobial activity are related to stasis (growth inhibition) or cidal (destruction) actions. These include the disruption of cell membranes (Y. Wang et al., 2009), causing a release of intracellular components, binding to surface proteins (Pierre et al., 2011), disruption of electron transport (Eom et al., 2014), nucleic acid synthesis (Karpiński et al., 2022), and enzyme activity (Nagayama et al.,

2002). These various mechanisms have been demonstrated by a number of isolated macroalgae compounds, as illustrated in Figure 2.2. However, a synergistic effect between these mechanisms and compounds is likely the cause of the antibacterial activity (Shannon and Abu-Ghannam, 2016).

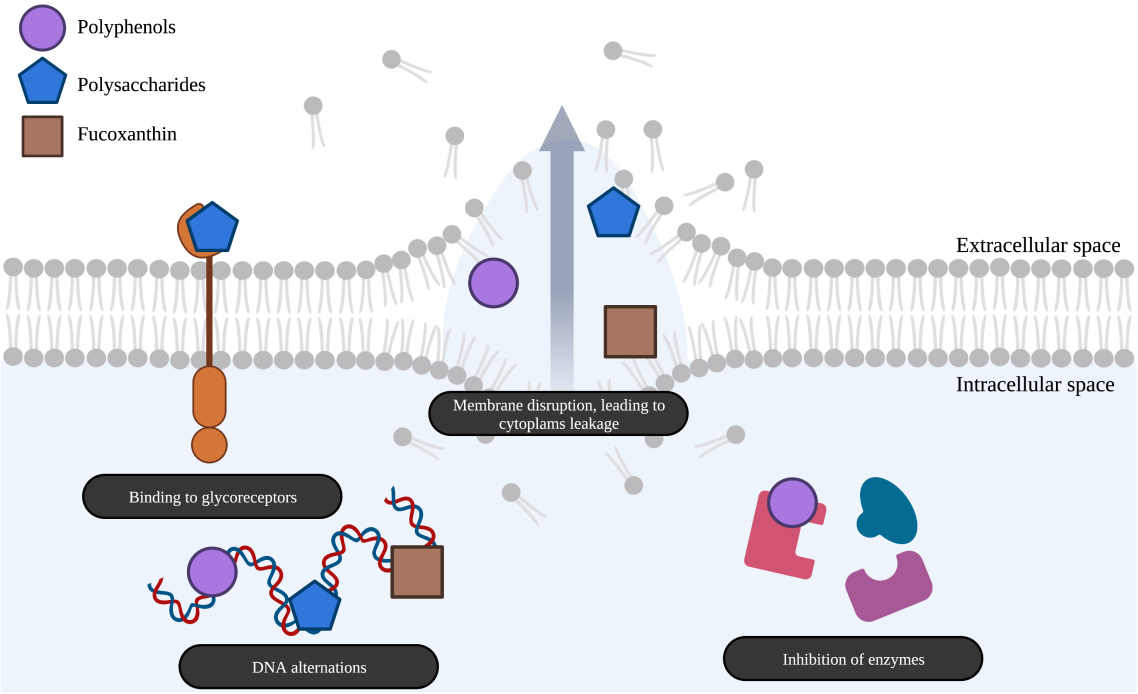

Figure 2.2: Illustration of the main action mechanisms of antimicrobial compounds extracted from macroalgae, with a focus on the bioactive compounds polyphenols, polysaccharides, and fucoxanthin. Modified from Silva et al. (2020).

Polyphenols have been associated with their ability to alter membrane permeability (leading to cell lysis) (Eom et al., 2014; Y. Wang et al., 2009; Y. Wei et al., 2016). Y. Wei et al. (2016) reported that phlorotannins from brown algae damaged the cell membrane and cell wall of *Vibrio parahaemolyticus*, causing cytoplasm leakage and deconstruction of membrane permeability. In addition, algal polyphenols have been found to bind proteins, enzymes (Nagayama et al., 2002), and DNA (Cherian et al., 2019) and inhibit oxidative phosphorylation (Shannon and Abu-Ghannam, 2016). Phlorotannins isolated from different species of macroalgae have been found to have antimicrobial effects (Bogolitsyn et al., 2019; Nagayama et al., 2002; Y. Wang et al., 2009).

The antimicrobial mechanism of fucoxanthin and other pigments is little known. However, the mechanism is suggested to be similar as for other carotenoids (Karpiński et al., 2022). Suggested mechanisms involve membrane disruption and binding to DNA (Karpiński and Adamczak, 2019). The antimicrobial effect of fucoxanthin on several microorganisms has been documented (Karpiński and Adamczak, 2019; Rajauria and Abu-Ghannam, 2013). Karpiński and Adamczak

(2019) reported that fucoxanthin has a better antimicrobial effect against gram-positive bacteria than gram-negative bacteria.

Polysaccharides antimicrobial properties involve the interaction between glycoprotein receptors of the bacterial cell wall of the membrane (Amorim et al., 2012) and nucleic acids and polysaccharides (F. He et al., 2010; Pierre et al., 2011). These interactions lead to the disruption of membrane stability, and cellular functions have been studied and described F. He et al. (2010) and Pierre et al. (2011). The antimicrobial effects of isolated sulfated polysaccharides from macroalgae have been investigated and documented for fucoidans (M. Liu et al., 2017) and laminarin (Kadam et al., 2015).

2.3 *Alaria esculenta*

The species of interest in the present MSc project, *Alaria esculenta* (Linnaeus) Greville (*A. esculenta*), commonly known as winged kelp or Atlantic wakame, is an edible brown alga belonging to the family Alariaceae and the order Laminariales, also known as kelp (Verma et al., 2015). Its thallus is green-yellow-brown in color and can be primarily divided into three parts: holdfast, stripe, and lamina (Altin and Solem, 2003). The species are characterized by its clearly defined midrib and chipped ends of the lamina (Morrissey et al., 2001), as shown in Figure 2.3. The lamina can be between one and three meters long. Sporophylls are found in the upper regions of the stripe. The species primarily grows on rocks from the upper sublittoral zone to a depth of 10 m (Altin and Solem, 2003). Typically found in areas exposed to harsh weather conditions, such as strong waves and wind (Indergaard, 2010). *A. esculenta* can be found in cold waters of the Northern Hemisphere and are most abundant on the coast of Norway, Greenland, the United Kingdom, Ireland, Canada, and Iceland (Kraan, 2020).

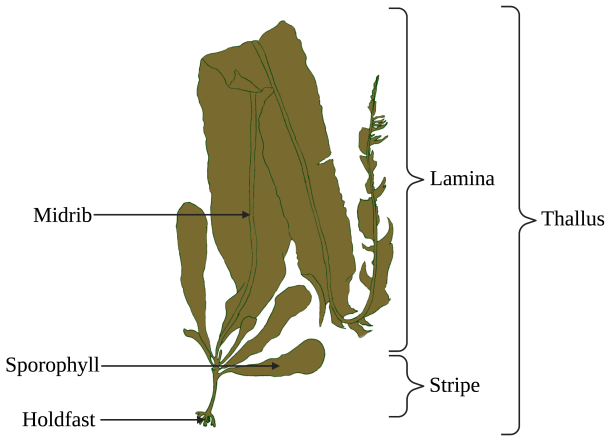

Figure 2.3: Morphology of *Alaria esculenta*. Modified from Altin and Solem (2003) and Kraan and Guiry (2000)

A. esculenta have fluctuating growth phases throughout the year. As a result, seasonal differences in its chemical composition have been documented by both Roleda et al. (2019) and Schiener et al. (2015). With the optimal time for harvest before summer, at the end of the growth season, and before epiphytic fouling becomes a problem (Mæhre et al. (2014) and Walls et al. (2017)). The market price for the species was reported in 2014 to be 1200 NOK per kilogram of fresh weight (Skjermo et al., 2015), and they are mostly used as food and feed. The chemical composition has been previously investigated, as summarized in Table 2.1. The performed studies documented high moisture, carbohydrate, and ash content. Among the carbohydrates, alginate is the major component, and the species potential for alginate extracting has been explored (Nøkling-Eide et al., 2023), other carbohydrates include mannitol, laminarin, fucoidan, and cellulose (Schiener et al., 2015).

Table 2.1: Chemical composition of *Alaria esculenta*. Moisture content is given in percent of wet weight, all other values are given in percent of dry weight. Saturated- (S), monounsaturated- (MU), and polyunsaturated- (PU) fatty acids (FAs) are given as percentage of total lipids.

Moisture	**Ash**	**Protein**	**Carbohydrate**	**Lipid**				**Reference**
				Total	*SFA*	*MUFA*	*PUFA*	
-	-	-	-	1.6	32.3	13.5	49.4	Afonso et al. (2021)
-	37.6	10.2	46.2	1.94	-	-	-	Cebrián-Lloret et al. (2022)
78.83	26.0	11.30	50.72	0.82	49.5	26.7	23.8	Lytou et al. (2021)
-	30.06	11.8	-	2.03	-	-	-	Mohammed et al. (2021)
82.6	24.6	9.1	-	1.5	37.4	25.4	33.2	Mæhre et al. (2014)
85.5	25.3	-	72.1	-	-	-	-	Schiener et al. (2015)
-	24.2	10.5	40.7	-	-	-	-	Stévant et al. (2017)

2.3.1 Bioactivity of *Alaria esculenta*

In *A. esculenta*, the most prominent bioactive components are polyphenols, fucoxanthin, and polysaccharides. The species are richer in these compounds than the other abundant kelp species in Norway; *Saccharina latissima*, *Laminaria digitata*, and *Laminaria hyperborea* (Schiener et al., 2015). However, the detected presence of bioactive compounds is affected by the extraction method, the origin of raw material, and part of the thalli investigated, which leads to variations in the reported presence of these compounds. The differences are displayed in Table 2.2, for total polyphenol content, and Table 2.3, for fucoxanthin content in *A. esculenta*, using different extraction methods, geographical locations, and harvest times. The distribution of these compounds is additionally different within the thalli for polyphenols (Gager et al., 2020; T. Wang et al., 2009) and fucoxanthin (Table 2.3).

Table 2.2: Reported total polyphenol content (TPC) in *Alaria escuelnta*, obtained using different extraction methods, including solid-liquid extraction (SLE), enzyme-assisted extraction (EAE), and pulsed electric field (PEF) supported extraction, and harvested at different geographical locations and times.

TPC [g GAE/100g dw]	Extraction Method	Location and Time of Harvest	Reference
0.04	SLE, water	Trøndelag, Norway, March 2011	Afonso et al. (2021)
0.10	SLE, ethanol	Trøndelag, Norway, March 2011	Afonso et al. (2021)
1.04	PEF, water	Breidafjord , Iceland, May 2018	Einarsdóttir et al. (2021b)
0.75	SLE, water	Breidafjord , Iceland, May 2018	Einarsdóttir et al. (2021b)
0.66	Hot water	Cork, Ireland, June 2019	Sapatinha et al. (2022)
1.12	EAE, viscozyme	Cork, Ireland, June 2019	Sapatinha et al. (2022)

Table 2.3: Reported fucoxanthin content in *Alaria escuelnta*, for different parts of thalli, using extraction methods used, including solvent-liquid extraction (SLE) and enzyme-assisted extraction (EAE), and harvested at different geographical locations and times.

Fucoxanthin [mg/100g dw]	Part of Thalli	Extraction Method	Location, and Time of Harvest	Reference
66.1	Whole algae	SLE, 96% ethanol, 5 min	Trøndelag, Norway, March 2017	Afonso et al. (2021)
82.2	Blade	EAE, viscozyme	Donegal, Ireland, July 2015	Shannon and Abu-Ghannam (2018)
50.0	Stripe	EAE, viscozyme	Donegal, Ireland, July 2015	Shannon and Abu-Ghannam (2018)
4.0	Holdfast	EAE, viscozyme	Donegal, Ireland, July 2015	Shannon and Abu-Ghannam (2018)
87.0	Blade	SLE, 62.2% acetone	Donegal, Ireland, July 2015	Shannon and Abu-Ghannam (2017)
55.0	Stripe	SLE, 62.2% acetone	Dongegal, Ireland, July 2015	Shannon and Abu-Ghannam (2017)
6.0	Holdfats	SLE, 62.2% acetone	Donegal, Ireland, July 2015	Shannon and Abu-Ghannam (2017)
87.1	Whole algae	SLE, 60% ethanol, 2h	Brittany, France, May 2015	Stévant et al. (2017)

The high content of bioactive compounds gives the foundation for strong bioactivity. The bioactivity of *A. esculenta* have been documented for its antioxidant (Zubia et al., 2009), antimicrobial (Hornsey and Hide, 1985; Lerfall et al., 2018), anti-inflammatory (Afonso et al., 2021), anti-diabetic (Sapatinha et al., 2022), and anti-hypertensive (Sapatinha et al., 2022) potential. The antimicrobial properties of *A. esculenta*, considering its high abundance of bioactive compounds, is little investigated compared to other kelp species (Cox et al., 2010; Shannon and Abu-Ghannam, 2016). The interest in bioactive compounds from *A. esculenta* and macroalgae, in general, is not recent. Already 1985, reported Hornsey and Hide (1985) that *A. esculenta* have inhibitory effects against *Staphylococcus aureus*, this was later confirmed by Lerfall et al. (2018), whom additionally reported inhibitory effect towards *Listeria innocua*, *Shewanells putrefaciens*, and *Aeromonas salmonicidia*.

2.4 Potential Applications of Macroalgae Extracts

As mentioned, macroalgae are currently utilized in several areas. However, macroalgae extracts can have numerous applications. Macroalgae are, as described, a rich source of bioactive compounds, and these can be applied in several manners, as illustrated in Figure 2.4. These include use as a functional ingredient, in packaging materials, as preservatives, aquaculture, agriculture, cosmetics (Hermund et al., 2018), and pharmaceuticals/nutraceuticals (Thomas and Kim, 2011).

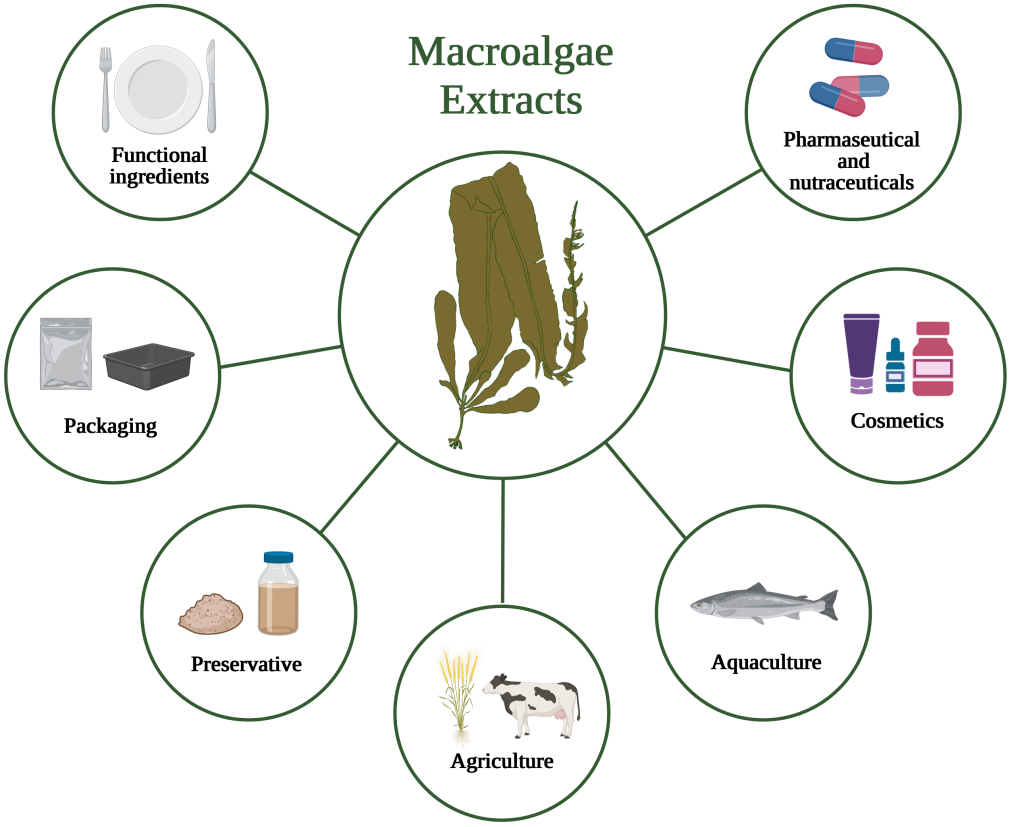

Figure 2.4: Possible applications of macroalgae extracts in the food industry.

With a focus on the food industry, the use of bioactive compounds from macroalgae can have multiple applications, such as functional ingredients, preservatives, and used in packaging. The application of bioactive compounds from macroalgae as functional foods has been extensively reviewed by Holdt and Kraan (2011), with a special focus on human health. Holdt and Kraan (2011) points to phlorotannins being a prime candidate for the development of a natural antioxidant for industrial applications. Polysaccharides are beneficial for human health, including improving the intestinal environment. Moreover, the bioactivity of pigments has shown to be effective against cancer, obesity and decrease blood glucose.

Furthermore, bioactive compounds can be directly added to food products as a preservative. Preservatives are added to food products to restrain and maintain the products quality and shelf-life. Microbial spoilage leads to the formation of undesirable compounds, causing changes in the foods characteristics (Petruzzi et al., 2017). Quality deterioration caused by lipid oxidation during storage and processing of foods is problematic. Both chemical and physical changes occur, such as the production of rancid odors, unpleasant flavors, reduced nutritional value, and textural changes (Vercellotti et al., 1992). This is particularly problematic in lipid-rich foods, especially those with a high content of polyunsaturated fatty acids (Jacobsen, 2015), such as salmon (Lerfall et al., 2016) and mackerel (Standal et al., 2018). Poveda-Castillo et al. (2018) added fucoidan isolated from *Fucus vesiculosus* as an ingredient to an apple beverage and found a reduction in microbial counts. Furthermore, Miranda et al. (2021) investigated the preservative effect (microbial and oxidative) from the brown algae *Bifurcaria bifurcata*, on European hake (*Merluccius merluccius*). Reported results included inhibited microbial effect and inhibition in lipid oxidation. These studies support the potential macroalgae have as a preservative in food products.

The incorporation of macroalgae extracts into food packaging has also demonstrated promise. Surendhiran et al. (2019) investigated the encapsulation of phlorotannins in alginate/polyethylene oxide blended nanofibers to protect chicken meat from Salmonella contamination. Phlorotannin was found to have strong antimicrobial activity on *Salmonella enteritidis*. In addition to the observed antimicrobial effect, antioxidative activity was evident from the sensory quality of the product.

In agriculture, macroalgae extracts can be applied to crops and utilized as animal feed. Macroalgae extracts have increased soil fertility and crop production (W. Khan et al., 2009). The use of macroalgae extracts as a feed supplement have shown improved growth, immunity, and fatty acid profile in cattle (Hwang et al., 2014) and higher average weight rain and overall growth in pigs (Draper et al., 2016). Moreover, within aquaculture, the addition of macroalgae extracts can potentially increase the resistance against bacterial and viral diseases. Fucoidan as a dietary feed supplement has demonstrated enhanced survival of fish and crustacean species against bacteria and viruses that are a threat to aquaculture production (Mohan et al., 2019).

Legislative challenges are of importance for potential utilization. With a focus on the European Union regulations, only 22 macroalgae species, including *A. esculenta*, are authorized as food or food supplements by the Novel Food Regulation (Regulation (EC) Ni 259/97). However, as of 2021, only two macroalgae compounds, fucoidan extracted from *Fucus vesiculosus* and *Undaria pinnatifida*, and phlorotannins extracted from *Ecklonia cava*, are authorized as novel food/supplement (Commission Regulation (EU) 2017/2470). Furthermore, food additives are controlled by Regulation (EC) No 1333/2088, which includes eight authorized macroalgae-derived additives, alginate (E401-E405), agar (E406), carrageenan (E407) and euchuema-algae extract (E407a). The use of macroalgae and its derivatives as feed and in packaging materials are further subjected to regulations review by Lähteenmäki-Uutela et al. (2021). The approval of new legislative regulations is a bottleneck for potential applications, as it is a time consuming process and

can take several years.

2.5 Extraction of Bioactive Compounds from Macroalgae

As briefly mentioned, extraction methodology does play a major role in the extraction yield of bioactive compounds. In the case of macroalgae, their rigid and complex structure of the cell wall and the compounds of interest is deeply embedded within this matrix (Ummat et al., 2020), making extraction more challenging.

For these bioactive compounds to be extracted at a commercial scale, high extraction yield, as well as intact biological activities, are required. Conventional extraction methods, such as solid-liquid extraction, soxhlet extraction, maceration, and liquid-liquid extraction (Michalak and Chojnacka, 2014), do not meet these requirements. Current conventional methods for extracting bioactive compounds have several drawbacks, including the need for large solvent volumes, waste generation, loss or contamination of thermally sensitive compounds, and high time and energy consumption (Simoneau et al., 2000). Additionally, resulting in a low yield of bioactive compounds. To address these issues and to facilitate the transition towards more environmentally sustainable extraction technologies, there is a need to develop new safe and effective extraction technologies which give maximum product yield and enable attention of clean label status (Bordoloi and Goosen, 2020). Novel and emerging extraction technologies have been extensively reviewed for its potential application on marine macroalgae by Cikoš et al. (2018) and Sosa-Hernández et al. (2018). Included as a novel and emerging technology is microwave assisted extraction.

2.5.1 Microwave Assisted Extraction

Microwave assisted extraction (MAE), one of the novel and emerging technologies, has received extensive attention for its potential. MAE is based on microwave energy to heat the sample, making an extraction procedure more efficient and selective (Amarante et al., 2020). Microwaves are non-ionizing electromagnetic waves made up of oscillating magnetic and electric fields that are perpendicular to one another, radiating with frequencies ranging from 0.3 to 300 GHz (Seoane et al., 2017). Where the former is responsible for the heating process. Energy is transferred directly to the material during microwave heating. Subsequently, MAE is facilitated by the synergistic combination of mass and heat transfer acting in the same direction, from inside the solid matrix to the outside (Périno-Issartier et al., 2011). The effects favorably affect the structural damage to the cell matrix and enhanced physiochemical and transport properties of the solvent and solutes (Seoane et al., 2017). The principle of microwave assisted extraction (MAE) is illustrated in Figure 2.5. During MAE, the pressure inside the cells build up as a result of the energy absorbed by polar molecules ①, which leads to rapid internal superheating with liquid vaporization ②. Causing the cell wall to rupture ③, enhancing and facilitating the diffusion of solutes that are present in the cell wall or the cytoplasm out of the cell. (Chan et al., 2016; Seoane et al., 2017).

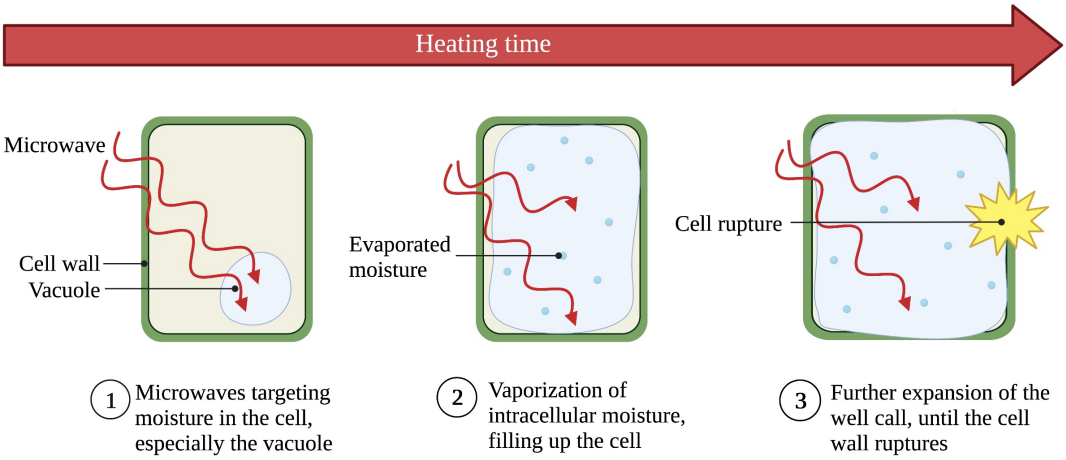

Figure 2.5: Principle of microwave assisted extraction of bioactive compounds, form plant cells. **(1)** Microwave targets polar molecules in the cell, causing the **(2)** moisture in the cell to vaporize, fill up the cell, and pressure build up inside the cell. **(3)** The cell expands until it raptures and released bioactive compounds. Modified from Chan et al. (2016).

The use of MAE also comes with disadvantages. MAE can generate high temperatures and pressures within the sample, which can lead to degradation or modification of the target compounds, especially thermolabile compounds (Ummat et al., 2020. In addition, special equipment is needed. MAE is also dependent on the ability of the microwave energy to generate heat in the sample, as the process will have little efficiency with samples with low thermal conductivity or is highly viscous, which can limit heat transfer (Seoane et al., 2017). Therefore, optimizing the extraction parameters to minimize the risk of sample degradation is essential.

For an efficient, optimized extraction process, several parameters must be considered. For the microwave procedure, microwave power, temperature, and irradiation time play a significant role (Amarante et al., 2020). Pre-treatment, the particle size, solvent, and solvent:solid ratio are important factors for the material to be extracted. Microwave assisted extraction of bioactive compounds has been successfully applied on several different algae species, including the brown algae species; *Fucus vesiculosus* (Amarante et al., 2020; Garcia-Vaquero et al., 2021), *Carpophyllum flexuosum* (Magnusson et al., 2017), and *Ascophyllum nodosum* (Silva et al., 2022), among others. Optimized extraction conditions, temperature, holding time, power, solvent, and solid:solvent ratio have been investigated for the different species. These are summarized in Table 2.4 where different species exhibited various optimal conditions, describing the role of species on extraction conditions. The use of microwaves for extraction has not been to current knowledge performed on the species *A. esculenta*.

Table 2.4: Reported optimum parameters of microwave assisted extraction of several brown algae species.

Species	**Temperature** [°C]	**Hold Time** [min]	**Power** [W]	**Solvent**	**Solid :Solvent Ratio**	**Optimization parameter**	**Reference**
Fucus vesiculosus	75	5	400*	57% Ethanol	N.R.	Phlorotannins	Amarante et al. (2020)
Halomonas elongata	N.R.	16	N.R.	Water	N.R.	TPC	Chamorro et al. (2021)
Sargassum vestitum	30	1.25	960	70% Ethanol	1:50*	TPC	Dang et al. (2018b)
Saccharina japonica	60	25	400	55% Ethanol	1:8	TPC	Z. He et al. (2013)
Carpophyllum flexuosoum	160	3	N.R	Water	1:30	TPC	Magnusson et al. (2017)
Sargassum thunbergi	80	23	574	N.R.	1:27	Polysaccharides	Ren et al. (2017)
Sargassum swartzuii	25*	65	613	52% Ethanol	1:33	TPC	Toan et al. (2021)
Laminaria japonica	60	10	300	Ethanol	1:15	Fucoxanthin	Xiao et al. (2012)

N.R.: Not reported.
TPC: Total polyphenol content
*Constant parameter, not optimized.

Chapter 3

Materials & Methods

A flow chart of the performed extraction methods and analyses of chemical characteristics and bioactivity is illustrated in Figure 3.1. Different parameters of the extraction methods were tested (Figure 3.2) before the total polyphenol content, pH, and fucoxanthin content were determined for the extracts. Further, bioactivity related to the antioxidant properties was assessed using several *in vitro* antioxidant assays; DPPH, ABTS, and FRAP. In addition, the antimicrobial effect of a selection of the extracts was evaluated against the microorganisms *Pseudomonas fluorescence, Listeria innocua* and *Listeria monocytogenes*. Parallels and the number of measurements are elaborated in the respective sections.

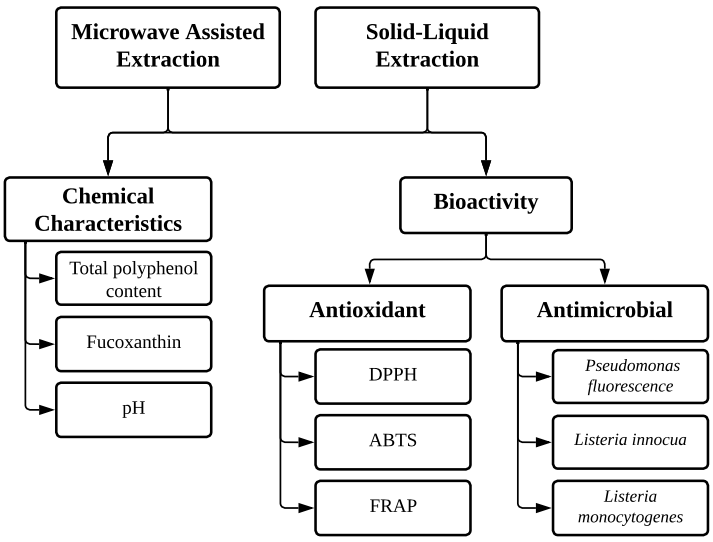

Figure 3.1: Flow chart of the experimental plan, with extraction method, chemical, and bioactivity analyses. Analyses of chemical characteristics included total polyphenol content (TPC), fucoxanthin, and pH measurements. For measuring the bioactivity of the extracts, three *in vitro* antioxidant assays were performed, 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), 2,2´-azin-bis(3-ethylbenithiazoline-6-sulfonic acid) assay (ABTS) and ferric reducing antioxidant power (FRAP), and the antimicrobial effects against the microorganisms *Pseudomonas fluorescence*, *Listeria innocua*, and *Listeria monocytogenes* was investigated. For parallels and the number of measurements, see the respective sections in the present thesis.

3.1 *Alaria esculenta*

Alaria esculenta was obtained in two batches, cultivated and harvested on the 17th of May 2021 (2021 batch) and 19th of May 2022 (2022 batch), from the shores of Frøya, Norway (63°44.89 N 8°53.28 E), by Seaweed Solution AS (SES). After harvest, the raw material was refrigerated in flowing seawater overnight before being frozen and stored (-20°C). The raw material was brought to NTNU in October 2022 from SES. After arriving at NTNU, batches of 500 g frozen *A. esculenta* were freeze-dried (-54°C, <0.133 mbar, 48 h, Labconco FreeZone 12, Kansas, USA) and were manually ground to powder to obtain a homogeneous sample, using a mortar and pestle. The powder was stored at -80°C until further analyses. The 2021 batch was used to perform all experiments (Figure 3.1). The 2022 batch was used to investigate differences in chemical characteristics and antioxidant activity using optimum microwave assisted extraction parameters.

3.2 Extraction Procedures

The extractions were performed using two different extraction methods with different parameters, as presented in Figure 3.2. More exactly, the microwave assisted extraction was performed with the design factors extraction temperature and holding time, and the solid-liquid extraction was performed as a non microwave treated reference method, using two different solvents (water and ethanol:water (30:70)), to investigate the effect of the solvent on the extraction. All extractions was peformed in triplicates (n = 3).

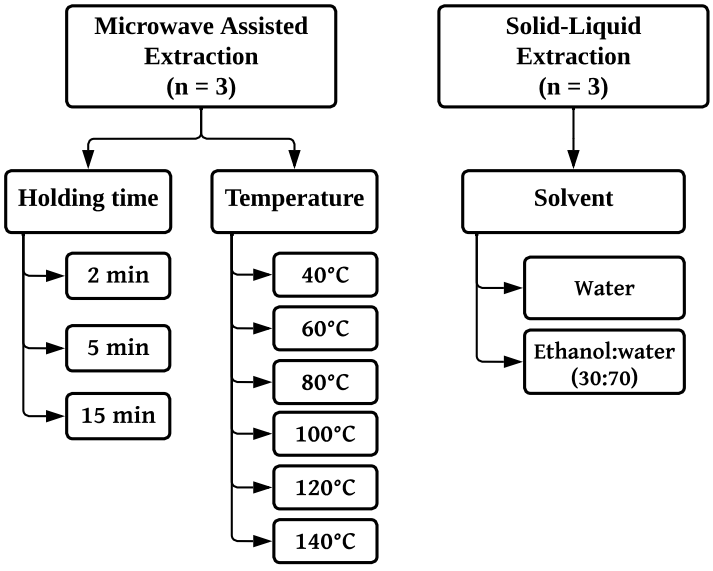

Figure 3.2: Experimental design for the extractions procedures. Different holding times and temperatures were performed for the microwave assisted extraction and solvent types for the solid-liquid extraction. All extracts were produced in triplicates (n = 3).

3.2.1 Microwave Assisted Extraction (MAE)

The microwave assisted extraction was performed on a microwave extraction device (MARS6, CEM Corporation, Matthews, USA) using closed vessels. A total of six different temperatures (40°C, 60°C, 80°C, 100°C, 120°C, and 140°C) and three holding times (2, 5, and 15 minutes) were tested. The extractions had a ramp time of five minutes, power of 350W, stirring on medium speed, organic sample type, and a 10 minutes cooling program was run after the hold time. These settings were equal for all extractions. General observations made during the extractions, and an example of the microwave temperature-power graphs provided by the microwave extraction device for the temperature 120°, are included in Appendix C.

For the extraction, exactly 1.00 g of freeze-dried ground *A. esculenta* and 30.0 mL water was placed in a microwave vessel (MARSXpress^{TM} vessel, CEM Corporation, Matthews, USA) and vortexed for one minute. The vessels were following placed on the microwave turntable and ran on the set program following the experimental plan (Figure 3.1). After cooling, the samples were transferred to centrifugation tubes and centrifuged for 15 minutes at 9000 rpm at 20°C (Kubota, Model 1700, Tokyo, Japan). The extract was obtained by filtrating the supernatant (Sterile syringe filter, 0.2 µm cellulose acetate membrane, VWR International, Radnor, USA). The extracts were stored at -80°C until further analysis, except for the total polyphenol content and pH, which were measured immediately.

3.2.2 Solid-Liquid Extraction (SLE)

Solid-liquid extraction (SLE) was performed using two different solvents, water and ethanol:water (30:70), CAS: 64-17-5, VWR International, Radnor, USA). By homogenizing (Polytron PT 3000, Kinematica AG, Cern, Switzerland), exactly 1.00 g of freeze-dried ground *A. esculenta* and 30.0 mL solvent at 10 000 rpm for 1 minute. The extracts were then centrifuged and filtrated as described for the MAE extracts before the TPC and pH were measured and stored at -80°C until further analysis.

3.3 Total Polyphenol Content

The total phenolic content (TPC) was measured by the Folin-Ciocleout method as described by Singleton et al. (1999), with some modifications. To test tubes, 0.5 mL diluted extract, 0.5 mL Folin-Ciocalteu's reagent (CAS: 12111-13-6, VWR International, Radnor, USA), and 5 mL water were mixed and incubated for three minutes. Following incubation, 1.0 mL 20% sodium carbonate (Na_2CO_3 anhydrous, CAS: 497-19-8, VWR International, Radnor, USA) was added, in addition to 3.0 mL water. The tubes were vortexed and incubated in darkness at room temperature for one hour. After incubation, the absorbance was measured at 725 nm (Shimadzu UV-1800, Shimadzu, Duisburg, Germany), with water as a reference. Blank samples were prepared the same way, with

methanol:water (80:20) (CH_3OH, CAS: 67:66-3, VWR International, Radnor, USA) instead of extract. All extracts were measured in triplicates (n = 3).

A standard curve was prepared using gallic acid (CAS: 5995-86-8, Thermo Fisher Scientific, Waltham, USA), with concentrations ranging from 0-20 mg/L. The standard curve and calculations are included in Appendix B.1. The TPC is expressed as g gallic acid equivalents (GAE)/100g dry weight (dw) biomass of *A. esculenta*.

3.4 pH-measurements

A pH meter was used to measure the pH of each extract (PHM210, MeterLab, Oslo, Norway). The pH meter was calibrated with buffers of pH 4.0 and 7.0 prior to measurement. The pH was measured for each extract (n = 1).

3.5 Detection and Quantification of Fucoxanthin Content

High performance liquid chromatography (HPLC) was used to detect and quantify fucoxanthin in the extract. Quantification of the fucoxanthin content in the 60°C and 80°C MAE extracts was not included due to limited available time. A normal phase HPLC (Agilent 1100 series, Waldbronn, Germany, connected to a diode array detector) was used, with a YMC-Pack 30 column (250 × 4.6mm id, S-5 µm). The extracts were prepared by filtration (PTFE 0.2 µm, Thermo Fisher Scientific, Waltham, USA), and 100 µL of the samples were transferred to HPLC vials. The mobile phase consisted of (A) methanol(CH_3OH, CAS: 67:66-3, VWR International, Radnor, USA):Acetone(CAS: 67-64-1, Honeywell, Charlotte, USA) (60:40), and (B) Acetone (CAS: 67-64-1, Honeywell, Charlotte, USA):water (60:40), with an 80% A, and 20% B gradient. The mobile phase had a flow of 1 mL/min and an analysis time of 20 minutes per sample. The injection volume of each sample was 20 µL, and the column temperature was set to 20°C. Fucoxanthin was detected at 447 nm. A commercial standard was used for the detection and quantification of fucoxanthin (0.093-0.745 mg/mL, DHI, Hørsholm, Denmark). The fucoxanthin was detected for each extract (n = 1).

3.6 Antioxidant assays

The antioxidant effect of the extract was evaluated using three different *in vitro* assays. A combination of assays is desirable because individual assays have limitations according to their specific condition and mechanisms they asses (Gulcin, 2020; Huang et al., 2005). Radical scavenging is one of the mechanisms by which antioxidants inhibit oxidation, whereas the reducing capacity assay provides a measure of the ability of a compound to donate electrons and to reduce the oxidized

intermediates of the peroxidation process (Balboa et al., 2013).

3.6.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay was performed as described by Thiansilakul et al. (2007) and Nenadis et al. (2007), with minor modifications. A 0.15 mM DPPH (CAS: 1898-66-4, Merck, Darmstad, Germany) solution in 96% ethanol (CAS: 64-17-5, VWR International, Radnor, USA) was prepared the night before analysis and was kept overnight at 4°C in darkness.

Before analysis, all sample extracts were diluted with a 1:2 dilution factor with methanol:water (80:20, CH_3OH, CAS: 67:66-3, VWR International, Radnor, USA). Further, 1.5 mL of the diluted extracts were mixed with 1.5 mL of DPPH solution, vortexed, and incubated in the dark for 30 minutes. Blank samples were prepared the same way, with methanol:water (80:20, CH_3OH, CAS: 67:66-3, VWR International, Radnor, USA) replacing the extracts. After incubation, the absorbance was measured at 517 nm (Shimadzu UV-1800, Shimadzu, Duisburg, Germany), with 96% ethanol (CAS: 64-17-5, VWR International, Radnor, USA) as a reference. The DPPH radical scavenging was calculated using equation 3.1. Where A_{Sample} is the absorbance of the sample, and A_{Blank} is the absorbance of the blank. All extracts were measured in triplicates (n = 3).

$$Radical\ scavenging\ activity\,[\%] = \left(1 - \frac{A_{Sample}}{A_{Blank}}\right) * 100\% \tag{3.1}$$

A standard curve was prepared for calibration using different concentrations of Trolox (0-1000 µM, 6-Hydroxy-2,5,7,8-tetrametylkroman 2-carboxylacid, $C_{14}H_{18}O_4$, CAS: 53188-07-1, Thermo Fisher Scientific, Waltham, USA), and are included in Appendix B.2. However, the standard curve was not further used. The results are expressed as radical scavenging activity (RSA) [%].

3.6.2 2,2´-Azin-bis(3-ethylbenzithiazoline-6-sulfonic acid) (ABTS) Assay

The 2,2´-azin-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay was performed as described by Re et al. (1999), Nenadis et al. (2004) and Nenadis et al. (2007) with minor modifications. An $ABTS^{\bullet+}$ solution was prepared by mixing 25 mL ABTS (7 mM, CAS: 30931-67-0 Merck, Darmstad, Germany) with 440 µL potassium persulfate ($K_2S_2O_8$, 140 mM, CAS: 7727-21-1, Merck, Darmstad, Germany). The mixture was covered with foil and left at room temperature overnight (12-16h). The next day, the reaction mixture was diluted with methanol:water (80:20, CH_3OH, CAS: 67:66-3, VWR International, Radnor, USA) until the absorbance was 0.75 ± 0.05 at 734 nm (Shimadzu UV-1800, Shimadzu, Duisburg, Germany), with water as a reference.

All the extract samples were diluted with a 1:3 dilution factor in methanol:water (80:20, CH_3OH, CAS: 67:66-3, VWR International, Radnor, USA), and 200 µL of the diluted sample was added

to 2 mL of $ABTS^{\bullet+}$ in test tubes, vortexed and incubated for six minutes at room temperature. After incubation, the absorbance was read at 734 nm (Shimadzu UV-1800, Shimadzu, Duisburg, Germany), with water as a reference. Blank samples were prepared, replacing the extract with methanol:water (80:20, CH_3OH, CAS: 67:66-3, VWR International, Radnor, USA). All extracts were measured in triplicates (n = 3).

A standard curve for calibration was created using different concentrations of Trolox (0-250µM, 6-Hydroxy-2,5,7,8-tetrametylkroman 2-carboxylacid, $C_{14}H_{18}O_4$, CAS: 53188-07-1, Thermo Fisher Scientific, Waltham, USA), and are included in Appendix B.3. The results were calculated using equation 3.1 and expressed as radical scavenging activity [%].

3.6.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) was performed as described by Benzie and Strain (1996), with minor modifications. The FRAP solution was made by mixing 19 mM Fe(III)-choried-6-hydrate (CAS: 10025-77-1, Merck, Darmstad, Germany), 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ, CAS: 3682-35-7, Merck, Darmstad, Germany) dissolved in 40 mM hydrochloric acid (HCl, CAS: 7647-01-0, Merck, Darmstad, Germany), and acetate buffer (1.505 g sodium acetate(99 % anhydrous, CAS: 127-09-3, Alfa Aesar, Haverhill, USA) + 8.0 ml acetic acid (CAS: 64-19-7, Merck, Darmstad, Germany) added to 500 ml water, pH = 3.6). The solution was incubated at 37°C until use.

In a 96-well plate, 10 µL of diluted extract, 30 µL of water, and 300 µL of FRAP solution was added to each well. The plate was incubated at 37°C for 30 minutes before the absorbance was measured at 593 nm (PowerWaveXS Microplate, BioTek®, in connection with Gen5 2.0 data analysis software). Blanks were prepared the same way, with water replacing the extract volume in the well. All extracts were measured in triplicates (n = 3).

A standard curve was prepared using Trolox (31.35 - 1000 µM, 6-Hydroxy-2,5,7,8-tetrametylkroman 2-carboxylacid, $C_{14}H_{18}O_4$, CAS: 53188-07-1, Thermo Fisher Scientific, Waltham, USA). The standard curve was linear between 31.25-1000 µM Trolox. The standard curve are included in Appendix B.4. Results are expressed as µmol Trolox equivalents (TE)/g dw biomass of *A. esculenta*.

3.7 Antimicrobial Effect

The antimicrobial effect of the extracts was tested against *Pseudomonas fluorescence* (*P. fluorescence*), *Listeria innocua* (*L. innocua*), and *Listeria monocytogenes* (*L. monocytogenes*). Performed based on an inhibitory assay in 96-well plates described by Stupar et al. (2021). For the screening extracts obtained from 40°C 2 minutes holding time (HT2-T40), 120°C 5 minutes holding time (HT5-T120), and 140°C 15 minutes holding time (HT15-T140), from the microwave assisted extraction was chosen. Representing a low, medium, and high factor based on the TPC and antioxidant

activity of the extracts. In addition, two different ratios of extract (2 µL and 100 µL) and two different initial cell concentrations (1x10^4 and 1x10^2 CFU/mL) were tested. The main step of the assay is summarized in figure 3.3.

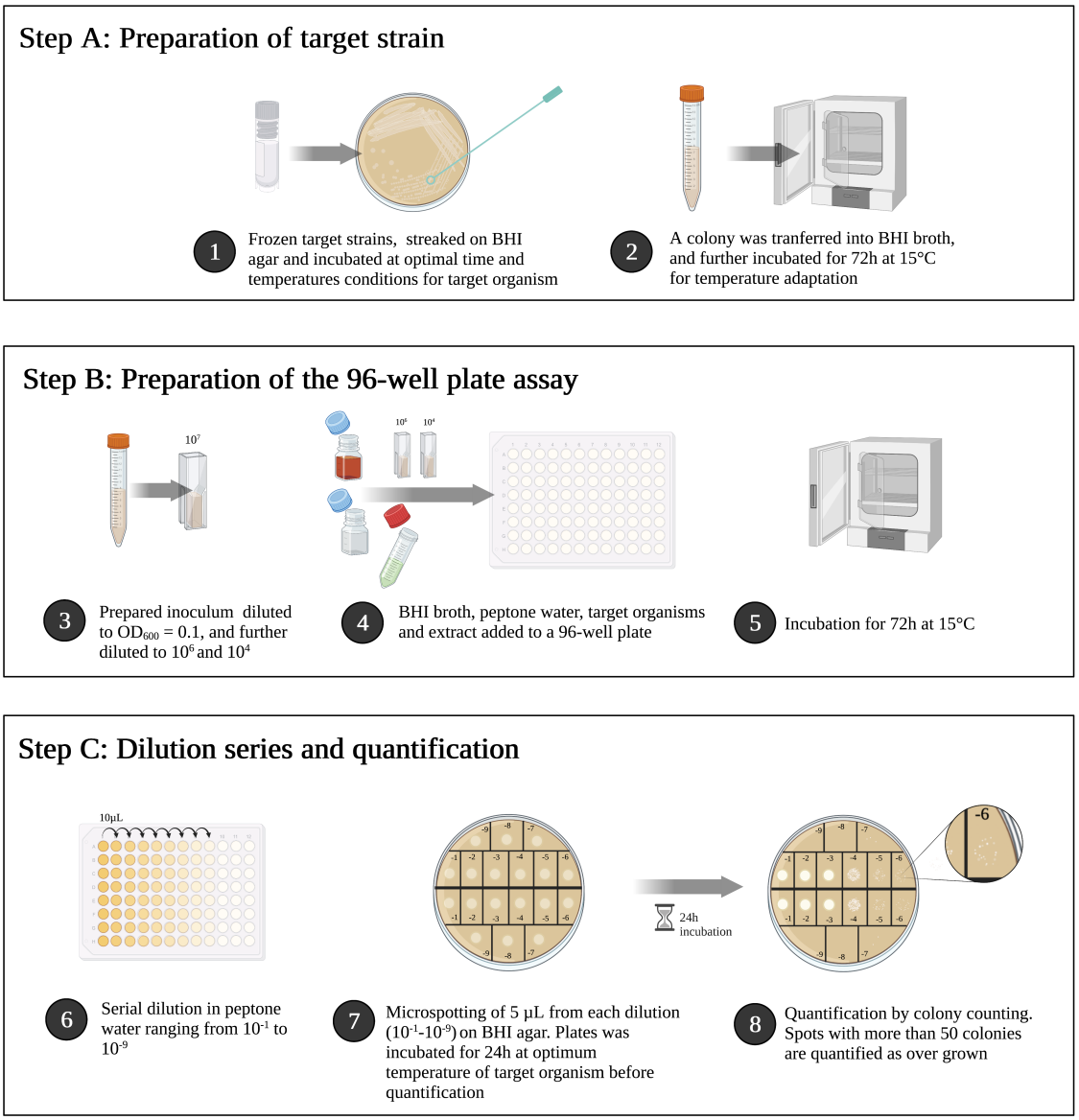

Figure 3.3: The main steps in the 96-well plate inhibition assay. **Step A**: Preparation of target strain. **(1)** Frozen reference strains were streaked on BHI agar and incubated at optimal growth conditions. After incubation, **(2)** a colony was transferred to BHI broth and incubated at 15°C for 72h for temperature adaptation. **Step B:** Preparation of the 96-well plate assay. **(3)** The prepared inoculum was standardized to an OD value of 0.1 and further diluted to obtain the desired initial cell concentrations before **(4)** BHI broth, peptone water, target organisms and extracts were added to a 96-well plate. **(5)** The plate was incubated for 72h at 15°C. **Step C:** Dilution series and quantification. **(6)** Each row from the original 96-well plate was transferred to a new 96-well plate and serial diluted in peptone water ranging from 10^{-1} to 10^{-9}. **(7)** Followed by microspotting of 5 µL from each well on BHI agar and incubated for 24h at optimal temperature conditions for target organism, **(8)** before quantification of each spot by counting colonies, spots with more than 50 colonies are quantified as overgrown.

3.7.1 Preparation of Target Strains

The target organisms *P. fluorescence* (CCUG 1253), *L. innocua* (CCUG 15531), and an environmental strain of *L. monocytogenes* (collected from a salmon deheader, Stupar et al. (2021)) were streaked on brain heart infusion agar (BHIA, Oxoid CM1136, Oslo, Norway) and incubated at optimal temperature and time conditions. For *P. fluorescence* 30°C for 48h, and for *L. innocua* and *L. monocytogenes* 37°C for 24h. After incubation, a colony was transferred to a tube with 10 mL brain heart infusion (BHI) broth (Oxoid CM1135, Oslo, Norway) and incubated for 72h at 15°C, for temperature adaptation. The cultures were further standardized to an optical density (OD) of 0.100 at 600 nm (V-3000PC Spectrophotometer, VWR International, Radnor USA/Shimadzu UV-1700, Shimadzu, Duisburg, Germany) and further diluted with BHI broth to obtain a working cell concentration of $1x10^4$ and $1x10^2$ CFU/mL.

3.7.2 96-Well Plate Inhibition Assay

In a 96-well plate, for the low extract concentration (2 µL), 196 µL of BHI broth, 2 µL extract, and 2 µL of target organism were added to each well. For the high extract concentration (100 µL), the volumes were adjusted, and 98 µL BHI broth, 100 µL extract, and 2 µL of target organisms were added to the wells. Positive controls were prepared with peptone water (1 g/L of peptone (Oxoid, Oslo, Norway) and 8.5 g/L of NaCl (VWR International, Radnor, USA)), replacing the extract volume for both extract volumes and initial cell concentrations. Negative controls were included for the BHI broth alone, the extracts alone, and extracts:BHI broth (1:1). The 96-well plates were incubated for 72h at 15°C.

Each sample, positive and negative controls, was performed in four biological parallels (n = 4). Experiments showing inhibition were repeated in biologically independent replicates, the *L. innocua* screening was repeated twice to validate the method and results.

After incubation, a dilution series in peptone water (1 g/L of peptone (Oxoid, Oslo, Norway) and 8.5 g/L of sodium chloride (NaCl, VWR International, CAS: 497-19-8, Radnor, USA)) from 10^{-1} to 10^{-9} was performed in 96-well plates. Followed by microspotting of 5 µL of each dilution on BHIA. The BHIA plates were incubated for 24h at 37°C for *L. innocua* and *L. monocytogenes*, and 30°C for *P. fluorescence*, before quantification.

After incubation, each spot was quantified by colony counting. Spots with more than 50 colonies were quantified as overgrown. The number of colonies was further transformed to colony forming units per mL [CFU/mL] and log-transformed. The determination of growth inhibition (log reduction CFU/mL), equation 3.2 was used. Where $\bar{x}$ is the average of the control samples (n = 4), and x is the average of sample with extract and target organism (n = 4).

$$log reduction \frac{CFU}{mL} = \bar{x} log \frac{CFU}{mL} target\ organism - x\, log \frac{CFU}{mL} target\ organism\ with\ extract \quad (3.2)$$

3.8 Data Analysis and Statistics

A General Linear Model (GLM), univariate, full factorial, with temperature and holding time as fixed factors, were used to evaluate the microwave extraction. Furthermore, one-way analysis of variance (ANOVA), followed by Tukeys honest significant difference (Tukeys HSD) pairwise comparison post hoc test, was used to investigate significant differences within each temperature and holding time and to compare the different treatment combinations of the microwave extraction with the solid-liquid extractions. Statistical analyses were performed on log-transformed data for the antimicrobial assay. A GLM univariate, was used with the three fixed factors: extract, extract volume, and initial cell concentration, for a main effect analysis. Investigation of differences between the 2021 and 2022 batches was evaluated using an independent samples T-test. The statistical analysis was performed using IBM Statistical Package for the Social Sciences statistics software (release 29, IBM Corporation, USA). The alpha level was set to 5% ($p<0.05$), and the results are presented as the mean ± standard deviation (SD) for each measurement unless otherwise stated.

Pearson's correlation, bivariate, was used to investigate the relationship between chemical characteristics and antioxidant activity. The correlation was performed and visualized in R version 4.0.2 (R Core Team et al., 2020) and RStudio version 1.4.1717 (RStudio Team, 2020), using the corrplot package (T. Wei and Simko, 2021). Furthermore, the multivariate analysis principal component analysis (PCA) was used to investigate the relationship between the variables and extract. Prior to the PCA, the data was standardized, as the variables have different units. The PCA was visualized using the factoextra package (Kassambara and Mundt, 2017).

3.9 Preliminary Trails

A preliminary trial of the MAE was performed to find sufficient extraction power, temperature, and holding times, in addition to getting familiar with the equipment. Initial testing using the temperatures 30°C, 60°C, and 90°C, and the holding times of 15 and 30 minutes were performed. Two different samples types on the microwave device were tested, "Organic" and "Environmental (Extraction)", if the mixing pre-treatment between the MAE and SLE did affect the results, different biomass:solvent ratio (1:10, 1:20, 1:30, and 1:40), and two different methods to obtain *A. esculenta* powder from the freeze-dried material was investigated (mortar and pestle, and coffee bean grinder). All pre-tests were evaluated based on the TPC content of the extracts.

A preliminary trial was also performed for the antimicrobial assay to fit the methodology to the extracts. The assay was performed using *L. innocua*, with the MAE extract obtain at 120°C 5 minutes holding time. Three different extract volumes were tested (2, 20, and 100 µL), two cell concentrations ($1x10^2$ and $1x10^4$ CFU/mL), and two different general growth medias BHIA and plate count agar. The final setup of the assay was based on the results obtained in this trial. However, it was later discovered an error in the experimental work, so the results were discarded.

Chapter 4

Results

The present thesis included an investigation of the different design factors for microwave assisted extraction, temperature and holding time, and different solvents, water and ethanol:water (30:70), for solid-liquid extraction, affected the chemical characteristics and bioactive properties of *A. esculenta* extracts. The extraction procedures were studied in detail and compared with each other. The main essence was to feature the performance of the different extraction methods parameters by investigating the chemical characteristics of the extracts and their relation to antioxidant properties. Moreover, to investigate the antimicrobial activity of *A. esculenta* extracts, and compare two batches of the raw material harvested in two different years. The results from the preliminary trial are presented in Appendix C.

4.1 Total Polyphenol Content

The total polyphenol content in the extracts was determined using the Folin-Ciocalteu method. The extraction of TPC was affected by the experimental design (GLM: F = 176.52, $p < 0.001$), showing that the temperature was the strongest design factor contributing to variations between the groups (GLM: F = 533.30, $p < 0.001$). Showing an increase in TPC with increasing temperature up to 120°C before the TPC declined at the extraction temperature of 140°C (Table 4.1). The fixed factor holding time was also identified as significant (GLM: F = 132.51, $p < 0.001$), the holding time of 5 minutes providing a significantly higher TPC than the holding time of 15 minutes within all temperature groups (Table 4.1). However, the holding times of 2 and 15 minutes were not significantly different ($p = 0.084$), independent of temperature. The highest detected TPC, 1.22 ± 0.06 g GAE/100 g dw, was achieved with an extraction temperature of 120°C and a holding time of 5 minutes. Moreover, a significant interaction was observed between the two factors (GLM: F = 8.86, $p < 0.001$).

Table 4.1: Total polyphenol content, given as g gallic acid equivalents/100 g dry weight biomass (n = 9), in *Alaria esculenta* microwave extracts, using different microwave extraction temperatures (40°C, 60°C, 80°C, 100°C, 120°C, and 140°C) and holding times (2, 5, and 15 minutes).

Holding time[A]	**2 min**	**5 min**	**15 min**	**p-value**[*]
Temperature[A]				
40°C	$0.62 \pm 0.01^{d, x}$	$0.64 \pm 0.03^{e, x}$	$0.57 \pm 0.02^{e, y}$	< 0.001
60°C	$0.63 \pm 0.02^{d, x}$	$0.64 \pm 0.03^{e, x}$	$0.59 \pm 0.03^{e, y}$	< 0.001
80°C	$0.65 \pm 0.04^{d, y}$	$0.76 \pm 0.05^{d, x}$	$0.64 \pm 0.03^{d, y}$	< 0.001
100°C	$0.70 \pm 0.05^{c, y}$	$0.92 \pm 0.06^{c, x}$	$0.75 \pm 0.02^{c, y}$	< 0.001
120°C	$1.06 \pm 0.06^{a, y}$	$1.22 \pm 0.06^{a, x}$	$1.00 \pm 0.06^{a, y}$	< 0.001
140°C	$0.92 \pm 0.06^{b, y}$	$1.00 \pm 0.08^{b, x}$	$0.88 \pm 0.05^{b, y}$	$= 0.002$
p-value[*]	< 0.001	< 0.001	< 0.001	

[A]General Linear Model (GLM) full factorial design: GLM_{Model}: F = 176.52, $p < 0.001$, $GLM_{Temperature}$: F = 533.30, $p < 0.001$, $GLM_{Holding\ time}$: F = 132.51, $p < 0.001$, $GLM_{Interaction}$: F = 8.86, $p < 0.001$.
[*]Different superscripts within each row (holding time) (^{xy}) and column (temperature) (^{abcde}) indicate significant differences ($p < 0.05$) between groups by one-way ANOVA and Tukey HSD pairwise comparison test.

For the SLE, the TPC was significantly affected by the solvent used, 0.70 ± 0.05 and 1.20 ± 0.07 g GAE/100g, for water and ethanol:water (30:70), respectively ($p < 0.001$). Furthermore, an investigation of the TPC of the MAE extracts in comparison to the SLE extracts was performed (One-way ANOVA: $p < 0.001$), as presented in Figure 4.1. In general, the use of MAE temperatures of 120°C and 140°C resulted in significantly higher TPC than SLE using water as the solvent. The highest detected TPC using MAE matched the TPC for the SLE using ethanol:water (30:70) as the solvent, 1.22 ± 0.06 and 1.16 ± 0.06 g GAE/100 g dw, respectively (p = 0.717). These were significantly higher than all other extracts (Figure 4.1).

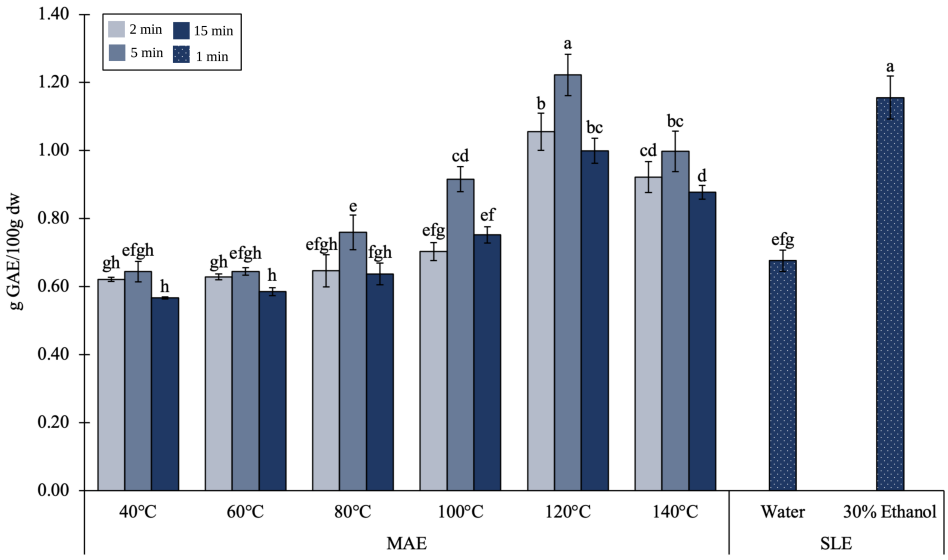

Figure 4.1: Comparison of the total polyphenol content in *Alaria esculenta* extracts, given as g gallic acid equivalents (GAE)/100 g dry weight (dw) biomass (n = 9), between the different extraction methods, microwave assisted extraction (MAE) and the solid-liquid extraction (SLE). One-way ANOVA: $p < 0.001$, different letters (abcdefgh) represent significant differences from Tukey HSD pairwise comparison test.

4.2 pH Measurements

The pH of each extract was measured. The pH of the MAE extracts (Table 4.2) was affected by the experimental design (GLM: F = 63.65, $p < 0.001$). The fixed factor temperature showed to be the biggest cause of variation between the groups (GLM: F = 200.42, $p < 0.001$), and the fixed factor holding time was also found to be significant (GLM: F = 13.58, $p < 0.001$). In addition, a significant interaction between the two factors was found (GLM: F = 5.47, $p < 0.001$). The extracts pH was in the range of 6.17-6.50, where the pH decreased with increasing extraction temperature and holding time (Table 4.2).

Table 4.2: Extract pH (n = 3), in *Alaria esculenta* microwave extracts, obtained at different microwave extraction temperatures (40°C, 60°C, 80°C, 100°C, 120°C, and 140°C) and holding times (2, 5, and 15 minutes).

Holding time[A]	**2 min**	**5 min**	**15 min**	**p-value**[*]
Temperature[A]				
40°C	6.50 ± 0.46^{a}	6.52 ± 0.03^{a}	6.54 ± 0.01^{a}	= 0.301
60°C	6.53 ± 0.01^{a}	6.52 ± 0.015^{a}	6.51 ± 0.02^{a}	= 0.302
80°C	6.48 ± 0.02^{a}	6.46 ± 0.05^{a}	6.42 ± 0.03^{b}	= 0.168
100°C	6.35 ± 0.03^{b}	6.37 ± 0.01^{b}	6.33 ± 0.01^{c}	= 0.058
120°C	$6.35 \pm 0.02^{x, b}$	$6.31 \pm 0.03^{y, c}$	$6.30 \pm 0.02^{y, c}$	= 0.034
140°C	$6.29 \pm 0.01^{x, b}$	$6.20 \pm 0.01^{x, c}$	$6.17 \pm 0.03^{y, d}$	< 0.001
p-value[*]	< 0.001	< 0.001	< 0.001	

[A]General Linear Model (GLM) full factorial design: GLM_{Model}: F = 63.65, $p < 0.001$, $GLM_{Temperature}$: F = 200.40, $p < 0.001$, $GLM_{Holding\ time}$: F = 13.58, $p < 0.001$, $GLM_{Interaction}$: F = 5.47, $p < 0.001$.
[*]Different superscripts within each row (holding time) (^{xy}) and column (temperature) (^{abcd}) indicate significant differences ($p < 0.05$) between groups by one-way ANOVA and Tukey HSD pairwise comparison test.

The use of different solvents affected the pH of the SLE extracts, 6.53 and 6.63, for the solvents water and ethanol:water (30:70), respectively (p = 0.008). The MAE extracts were compared with the SLE extracts (One-way ANOVA: $p < 0.001$), as presented in Figure 4.2. Generally, the use of water as the solvent resulted in a lower pH of the extracts compared with ethanol:water (30:70) as the solvent, regardless of the extraction method (MAE and SLE) (Figure 4.2). In contrast, the pH in the SLE water extract did not significantly differ from the MAE until the MAE temperature exceeded 80°C, except for a 15 minute holding time at 80°C.

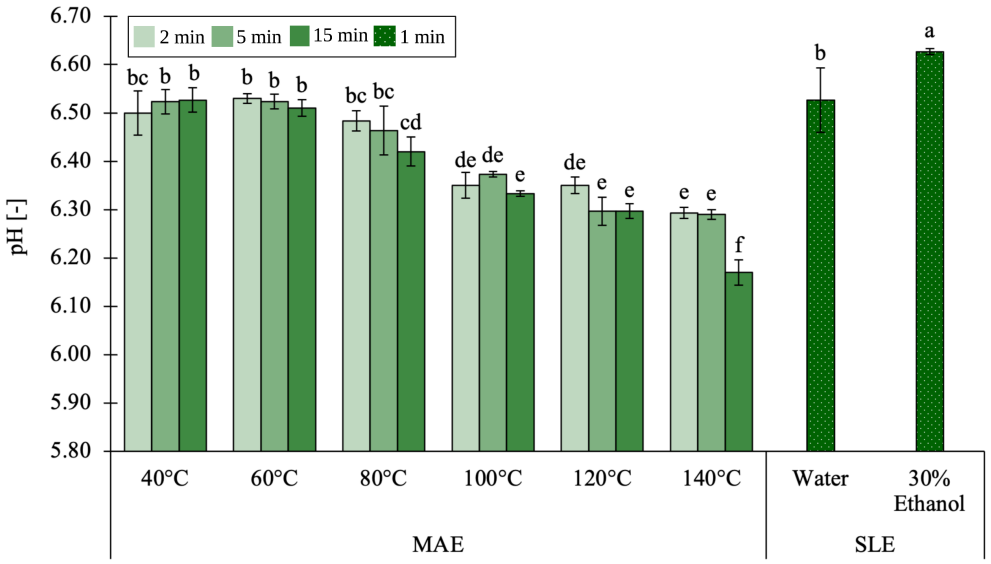

Figure 4.2: Comparison of extract pH in *Alaria esculenta* extracts (n = 3) between the different extraction methods, microwave assisted extraction (MAE) and solid-liquid extraction (SLE). One-way ANOVA: $p < 0.001$, different letters (abcdef) represent significant differences from Tukey HSD pairwise comparison test.

4.3 Fucoxanthin Quantification

Quantification of the pigment fucoxanthin was calculated based on an HPLC analysis. Extraction of fucoxanthin (Table 4.3) was affected by the experimental design (GLM: $F = 98.17$, $p < 0.001$). The temperature was identified as the main design factor affecting the variations between the groups (GLM: $F = 322.64$, $p < 0.001$), where increasing temperature resulted in enhanced extraction of fucoxanthin. The fixed factor holding time was also a significant factor (GLM: $F = 26.36$, $p < 0.001$). However, significant differences in holding time were only found within the extraction temperature 140°C, where the holding time of 15 minutes provided an outstandingly high yield of 84.63 ± 8.79 mg/100 g dw (Table 4.3). Moreover, the factors were found to significantly interact with each other (GLM: $F = 9.87$, $p < 0.001$).

Table 4.3: Fucoxanthin content, given in mg/100 g dry weight of biomass (n = 3), in *Alaria esculenta* microwave extracts, using different microwave extraction temperatures (40°C, 60°C, 80°C, 100°C, 120°C, and 140°C) and holding times (2, 5, and 15 minutes).

Holding time[A] **Temperature**[A]	**2 min**	**5 min**	**15 min**	**p-value**[*]
40°C	2.07 ± 1.63^{c}	4.07 ± 1.33^{c}	3.65 ± 0.77^{d}	= 0.171
100°C	22.11 ± 3.55^{b}	23.89 ± 3.31^{b}	26.09 ± 0.95^{c}	= 0.305
120°C	41.91 ± 6.13^{a}	45.15 ± 5.80^{a}	52.70 ± 3.37^{b}	= 0.106
140°C	$50.40 \pm 4.70^{y, a}$	$57.03 \pm 9.17^{y, a}$	$84.63 \pm 8.79^{x, a}$	< 0.001
p-value[*]	< 0.001	< 0.001	< 0.001	

[A]General Linear Model (GLM) full factorial design: GLM_{Model}: F = 98.17, $p < 0.001$, $GLM_{Temperature}$: F = 322.64, $p < 0.001$, $GLM_{Holding\ time}$: F = 26.36, $p < 0.001$, $GLM_{Interaction}$: F = 9.87, $p < 0.001$.
[*]Different superscripts within each row (holding time) (^{xy}) and column (temperature) (^{abcd}) indicate significant differences ($p < 0.05$) between groups by one-way ANOVA and Tukey HSD pairwise comparison test.

For the SLE, no significant difference was observed between water and ethanol:water (30:70) as the solvents, 5.19 ± 0.38 and 9.94 ± 4.10 mg/100 g dw, respectively (p = 0.116). Moreover, the MAE extracts were examined in comparison to the SLE extracts (One-way ANOVA: $p < 0.001$) and are presented in Figure 4.3. The MAE extraction temperature of 40°C and both SLE solvents performed poorly compared to the higher MAE temperatures for the extraction of fucoxanthin (Figure 4.3).

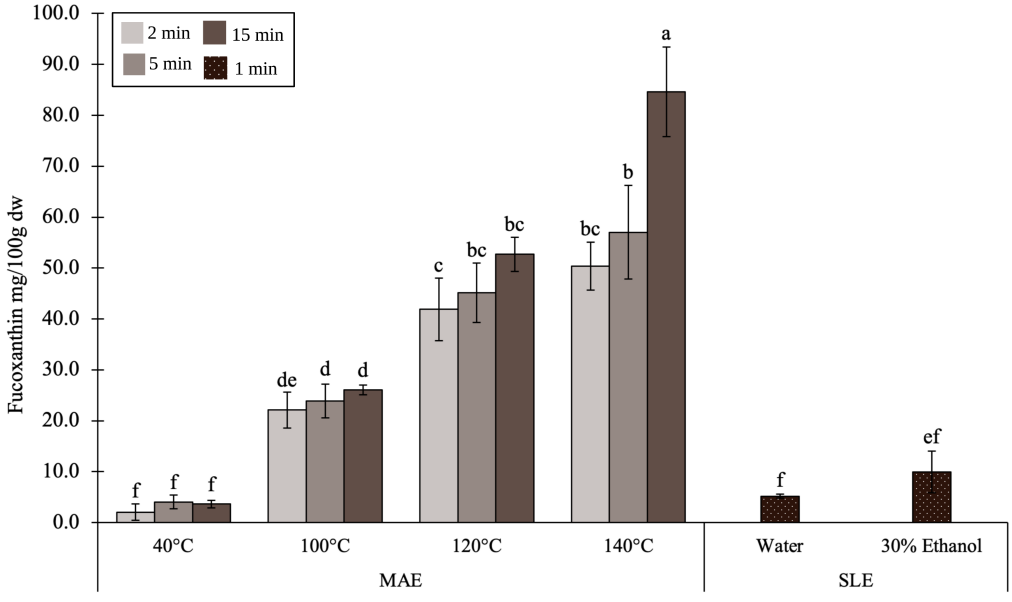

Figure 4.3: Comparison of the fucoxanthin content in *Alaria esculenta* extracts, given in mg/100g dry weight (dw) biomass (n = 3), between the different extraction methods, microwave assisted extraction (MAE) and the solid-liquid extraction (SLE). One-way ANOVA: $p < 0.001$, different letters (abcdef) represent significant differences from Tukey HSD pairwise comparison test.

4.4 Antioxidant Assays

Three *in vitro* antioxidant assays were used to evaluate the antioxidant activity of the extracts: DPPH, ABTS, and FRAP, which measure the ability to neutralize ROS via electron transfer (DPPH and ABTS) and to reduce Fe^{3+} (FRAP). The results from the antioxidant assays as affected by the design factors for the MAE have been investigated and are presented in Table 4.4. The inclusion of several assays is preferred to gain a better understanding of the antioxidant properties of the extracts.

Table 4.4: Antioxidant activity measured by the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), 2,2´-azin-bis(3-ethylbenithiazoline-6-sulfonic acid) assay (ABTS) and ferric reducing antioxidant power (FRAP) in *Alaria esculenta* extracts obtained by microwave assisted extractions using different microwave extraction temperatures (40°C, 60°C, 80°C, 100°C, 120°C and 140°C), and holding times, (2, 5 and 15 minutes) as parameters. The results from the DPPH and ABTS assay are presented as radical scavenging activity [%] (n = 9), and FRAP is presented as µmol Trolox equivalents/g dry weight biomass (n = 9).

	Holding time	**2 min**	**5 min**	**15 min**	**p-value***
Assay	**Temperature**				
DPPH[A]	**40°C**	46.45 ± 0.21^{d}	47.17 ± 1.54^{e}	48.09 ± 0.39^{d}	= 0.177
	60°C	44.79 ± 1.43^{d}	45.54 ± 0.81^{e}	41.43 ± 1.91^{d}	= 0.702
	80°C	55.96 ± 1.22^{c}	56.50 ± 1.21^{d}	59.46 ± 1.34^{c}	= 0.193
	100°C	65.76 ± 1.22^{b}	67.20 ± 1.62^{c}	67.73 ± 0.65^{b}	= 0.208
	120°C	69.61 ± 0.65^{ab}	70.32 ± 0.18^{b}	69.97 ± 1.08^{b}	= 0.532
	140°C	$73.37 \pm 0.74^{a, y}$	$75.27 \pm 0.17^{a, y}$	$80.07 \pm 2.26^{a, x}$	= 0.003
	p-value*	< 0.001	< 0.001	< 0.001	
ABTS[B]	**40°C**	50.74 ± 0.33^{d}	52.27 ± 3.16^{d}	53.87 ± 1.96^{d}	= 0.282
	60°C	$58.61 \pm 2.39^{x, c}$	$52.69 \pm 0.83^{y, d}$	$54.43 \pm 2.99^{xy, d}$	= 0.045
	80°C	62.33 ± 5.87^{bc}	62.41 ± 3.49^{c}	61.90 ± 1.00^{c}	= 0.987
	100°C	$69.49 \pm 2.55^{y, b}$	$77.37 \pm 0.85^{x, b}$	$77.76 \pm 0.60^{x, b}$	= 0.001
	120°C	79.50 ± 0.13^{a}	$82.72 \pm 1.09^{y, ab}$	81.41 ± 2.11^{b}	= 0.072
	140°C	$82.06 \pm 0.96^{y, a}$	$84.54 \pm 3.64^{y, a}$	$91.49 \pm 0.98^{x, a}$	= 0.005
	p-value*	< 0.001	< 0.001	< 0.001	
FRAP[C]	**40°C**	5.20 ± 0.28^{c}	5.87 ± 0.78^{b}	6.05 ± 0.52^{d}	= 0.227
	60°C	$6.25 \pm 0.54^{bc, y}$	$6.34 \pm 0.38^{b, y}$	$7.79 \pm 0.62^{cd, x}$	= 0.023
	80°C	6.81 ± 0.54^{b}	7.19 ± 0.13^{d}	7.55 ± 0.46^{cd}	= 0.174
	100°C	7.38 ± 0.41^{b}	7.39 ± 0.47^{b}	7.66 ± 0.93^{c}	= 0.839
	120°C	10.17 ± 0.15^{a}	10.39 ± 1.24^{a}	10.02 ± 0.36^{b}	= 0.835
	140°C	$10.74 \pm 0.55^{a, y}$	$11.84 \pm 0.74^{a, y}$	$13.91 \pm 0.56^{a, x}$	= 0.002
	p-value*	< 0.001	< 0.001	< 0.001	

[A]General Linear Model (GLM) full factorial design: GLM_{Model}: F = 233.63, $p < 0.001$, $GLM_{Temperature}$: F = 782.96, $p < 0.001$, $GLM_{Holding\ time}$: F = 13.76, $p < 0.001$, $GLM_{Interaction}$: F = 2.93, p = 0.009.

[B]General Linear Model (GLM) full factorial design: GLM_{Model}: F = 92.00, $p < 0.001$, $GLM_{Temperature}$: F = 303.86, $p < 0.001$, $GLM_{Holding\ time}$: F = 7.03, p = 0.003, $GLM_{Interaction}$: F = 4.76, $p < 0.001$.

[C]General Linear Model (GLM) full factorial design: GLM_{Model}: F = 47.08, $p < 0.001$, $GLM_{Temperature}$: F = 147.60, $p < 0.001$, $GLM_{Holding\ time}$: F = 14.63, p = 0.003, $GLM_{Interaction}$: F = 3.30, p = 0.004.

*Different superscripts within each row (holding time) (^{xy}) and column (temperature) (^{abcde}) indicate significant differences ($p < 0.05$) between groups by one-way ANOVA and Tukey HSD pairwise comparison test.

By investigating the design factors for the MAE, the same trend was observed in all performed assays. The antioxidant activity of the extracts was affected by the experimental design (Table 4.4), showing the fixed factor temperature to be the main discriminant (Table 4.4), where the antioxidant activity increased with increasing extraction temperature. Additionally, the fixed factor holding time and the interaction between the two factors were significant (Table 4.4). Moreover, the extract obtained at an extraction temperature of 140°C with a 15 minute holding time was found to have the significantly highest antioxidant activity in all assays.

For the radical scavenging assays, SLE with ethanol:water (30:70) as the solvent gave a significantly higher RSA compared to SLE using water as the solvent, 82.62 ± 0.28% and 59.28 ± 0.38% ($p < 0.001$), for the DPPH assay, and 92.05 ± 2.21% and 63.46 ± 2.66% ($p < 0.001$) for the ABTS assay. Moreover, a comparison between the RSA in the extracts from the MAE and the SLE extraction methods was performed, as presented in Figure 4.4 and Figure 4.5, for the DPPH (One-way ANOVA: $p < 0.001$) and ABTS assay (One-way ANOVA: $p < 0.001$), respectively. The same was observed in both assays, SLE using water as the solvent was equal to MAE extracts, using an extraction temperature of 80°C. Further SLE using ethanol:water (30:70) as the solvent had an equally high RSA as the MAE extract obtained at extraction temperature of 140°C with a 15 minute holding time (Figure 4.4 & Figure 4.5).

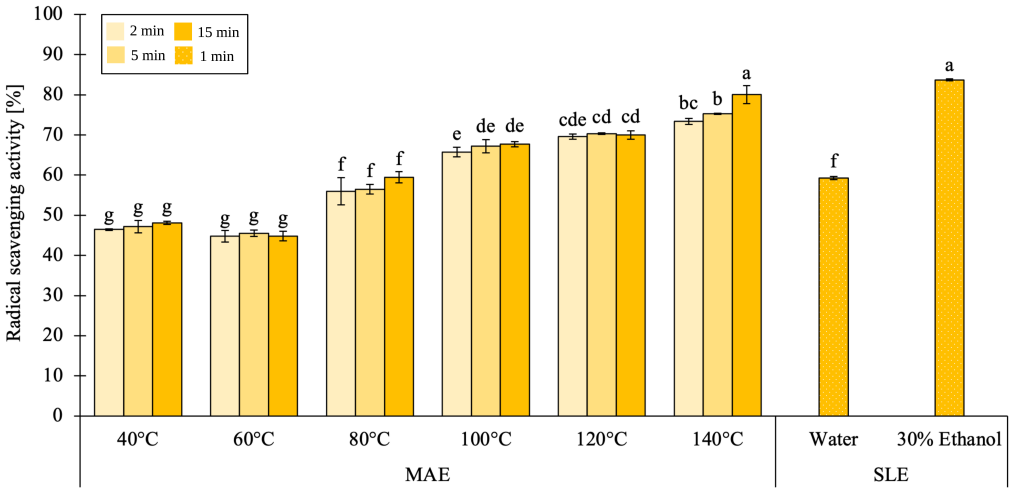

Figure 4.4: Comparison of the radical scavenging activity (RSA) given in percent, in *Alaria esculenta* extracts, measured by the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay, between the different extraction methods, microwave assisted extraction (MAE), and the solid-liquid extraction (SLE). One-way ANOVA: $p < 0.001$. Different letters (abcdefg) represent significant differences from Tukey HSD pairwise comparison test.

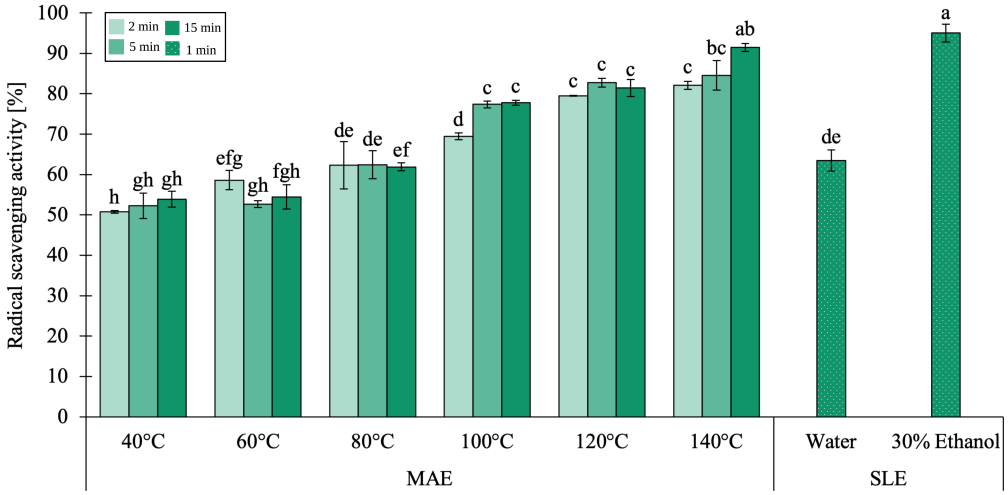

Figure 4.5: Comparison of the radical scavenging activity (RSA) given in percent, in *Alaria esculenta* extracts, measured by the2,2´-azin-bis(3-ethylbenithiazoline-6 sulfonic acid) assay, between the different extraction methods, microwave assisted extraction (MAE), and the solid-liquid extraction (SLE). One-way ANOVA: $p < 0.001$. Different letters (abcdefgh) represent significant differences from Tukey HSD pairwise comparison test.

In the FRAP assay, the use of different solvents in the SLE affected the antioxidant activity in the extracts, 6.23 ± 0.58 and 8.31 ± 0.61 µmol TE/g dw biomass, for water and ethanol:water (30:70), respectively ($p = 0.013$). Furthermore, the MAE extracts were compared to the SLE extracts (One-way ANOVA: $p < 0.001$) and are presented in Figure 4.6. The extracts from the MAE temperatures of 120°C and 140°C exhibited significantly greater antioxidant activity than all the other extracts, including both SLE extracts (Figure 4.6). Only minor differences were found between the other extracts.

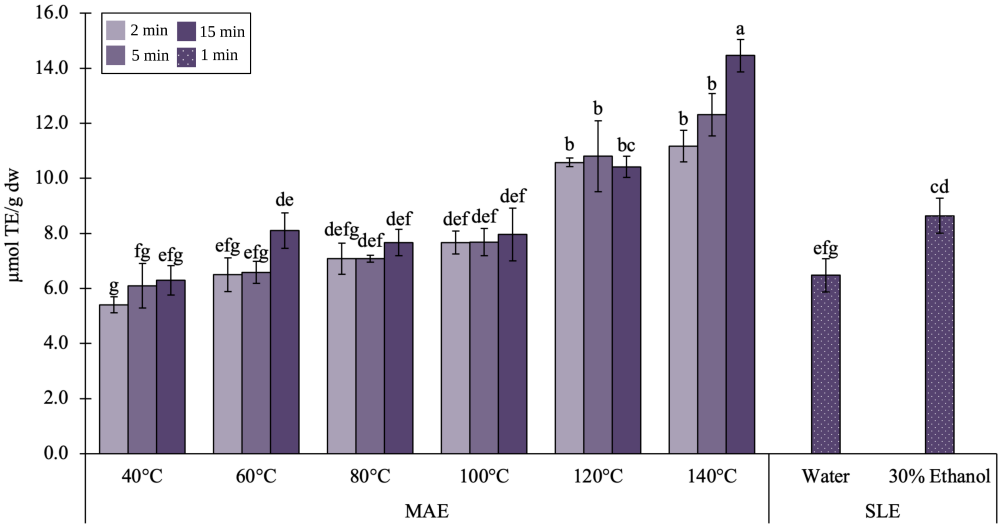

Figure 4.6: Comparison of the ferric reducing antioxidant power, given in µmol Trolox equivalents (TE)/g dry weight (dw) biomass, in *Alaria esculenta* extracts, measured by ferric reducing antioxidant power assay, between the different extraction methods, microwave assisted extraction (MAE), and the solid-liquid extraction (SLE). One-way ANOVA: $p < 0.001$. Different letters (abcdefg) represent significant differences from Tukey HSD pairwise comparison test.

4.5 Correlation Between Chemical Characteristics and Antioxidant Activity

For the purpose of establishing a connection between the measured chemical characteristics (TPC, fucoxanthin, and pH) and the antioxidant activity of the MAE and the SLE extracts, Pearson's correlation analysis was used. The correlation coefficients (r) are presented in Figure 4.7.

For the MAE extracts, all correlations were found to be significant, as shown in Figure 4.7 [**A**]. However, the TPC was found to have a weaker relationship between the antioxidant assays as opposed to the very strong positive correlation for the fucoxanthin and a very strong negative correlation with the pH. In contrast, the SLE extracts demonstrated a very strong positive significant correlation between the TPC and the antioxidant assays but no significant correlation between the fucoxanthin and the antioxidant assays. Furthermore, the pH was only found to be significantly positively correlated with the ABTS assay, and the correlation between the chemical content and the FRAP assay was, in general, weaker than the other assays (Figure 4.7 [**B**]).

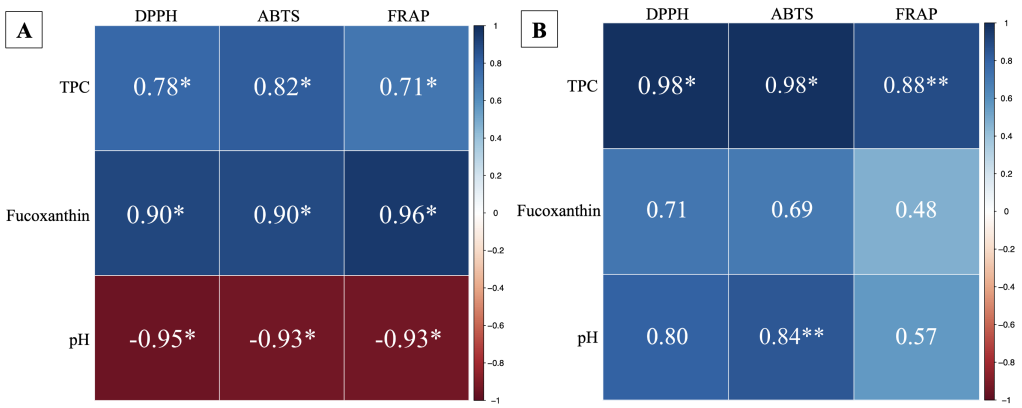

Figure 4.7: Correlation matrices showing correlation (r) between the chemical characteristics (total polyphenol content (TPC), fucoxanthin content, and measured pH) and the antioxidative activity measured by the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), 2,2´-azin-bis(3-ethylbenithiazoline-6-sulfonic acid) assay (ABTS) and ferric reducing antioxidant power (FRAP) in *Alaria esculenta* extracts, for **[A]** the microwave assisted extraction (N = 36), and **[B]** the solid-liquid extraction (N = 6). *Correlation is significant at the 0.01 level (2-tailed). **Correlation is significant at the 0.05 level (2-tailed)

4.6 Principal Component Analysis

Principal component analysis was performed to gain an overview of the similarities and differences between the chemical characteristics and the antioxidant activity of the extracts obtained using different extraction parameters. The PCA was performed twice, solely for the MAE extracts and with all extracts included (MAE and SLE), presented as PCA biplots in Figure 4.8 and Figure 4.9, respectively. The complementary loading plots for the variables and scree plots for the PCA are

shown in Appendix D.

For the MAE extracts, the first two principal components (PC), PC1 (Dim 1, Figure 4.8) and PC2 (Dim 2, Figure 4.8), explained 89.8% and 6.6% of the variation in the data, respectively. All variables, except TPC, were found to be mostly explained by PC1 and were highly correlated with each other. However, the TPC was identified as the variable contributing the most to the variation in the data (Figure D.1 [**A**], Appendix D).

From the biplot, the temperature group 40°C is located close to each other, and further separation between the temperature groups was observed as the temperature increased in PC1. In the PC2 dimension, the temperature groups were separated after the holding time, and the biggest difference was found between the extract obtained with the MAE temperature of 120°C with a holding time of 5 minutes (MAE-HT5-T120, Figure 4.8) and the MAE temperature of 140°C with a holding times of 15 minutes (MAE-HT15-T140, Figure 4.8), located on opposite sides of the PC2.

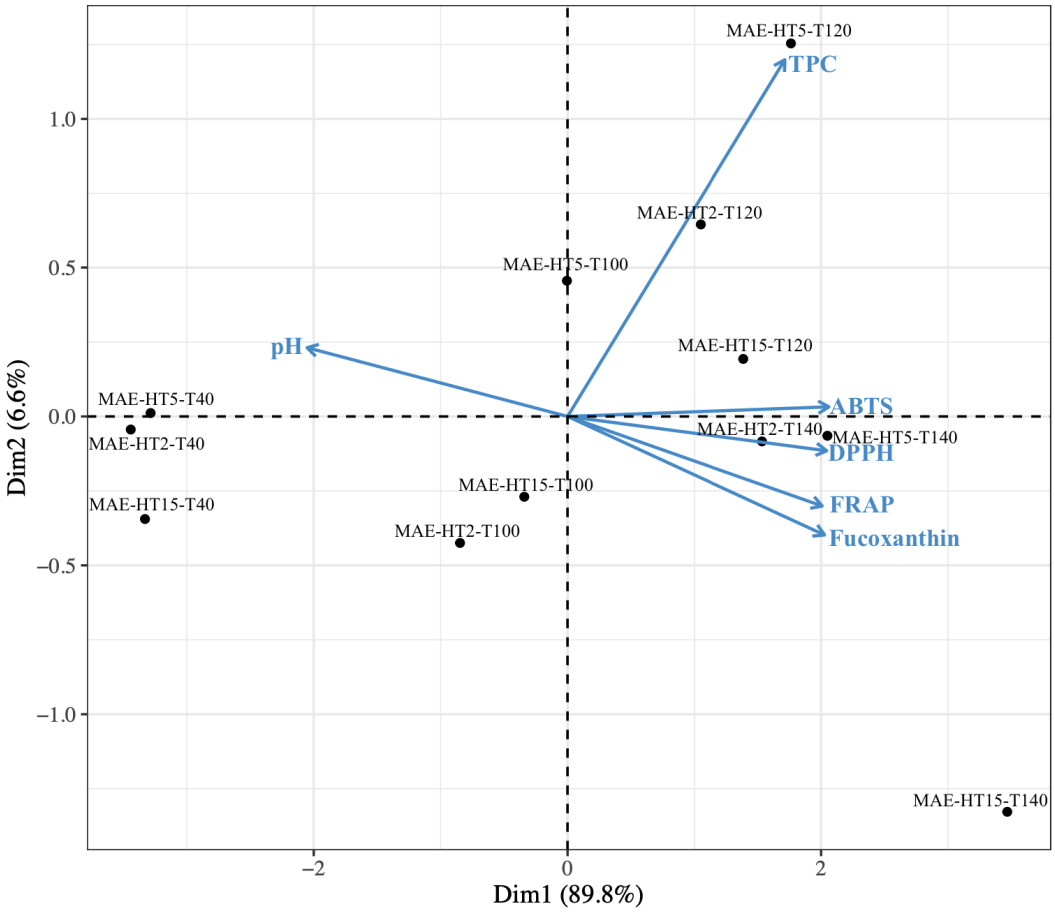

Figure 4.8: Principal component analysis (PCA) of the extracts. Variables are total polyphenol content (TPC), pH, fucoxanthin, and the antioxidative assays the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), 2,2´-azin-bis(3-ethylbenithiazoline-6-sulfonic acid) assay (ABTS) and ferric reducing antioxidant power (FRAP). The microwave assisted extraction (MAE) is defined after holding time (HT) and temperature (T).

For the PCA including all extracts, the first two PC, PC1 (Dim 1, Figure 4.9) and PC2 (Dim 2, Figure 4.9), explained 77.6% and 16.5% of the variation in the data, respectively. All variables were discovered to be mostly explained by PC1. However, the variables for the chemical composition (TPC, pH, and fucoxanthin) were marginally separated by the PC2 dimension. The TPC was found to have a higher correlation with the radical scavenging assays, the DPPH and ABTS, located adjacent to each other, and the fucoxanthin content was found to correlate with the FRAP assay. The pH, with its negative correlation, was also correlated more with the fucoxanthin and the FRAP assay. In this case, the fucoxanthin was found to contribute the most to the variation in the data, and TPC contributed the least (Figure D.1 [**B**], Appendix D).

The MAE extracts were mostly separated horizontally. Thus, PC1 appeared to explain most of the variation between the MAE groups. In contrast, the SLE extracts were mostly separated by the PC2 dimension, where the SLE ethanol:water (30:70) extract (SLE-30%EtOH, Figure 4.9) can be found solitary in the upper part of the biplot.

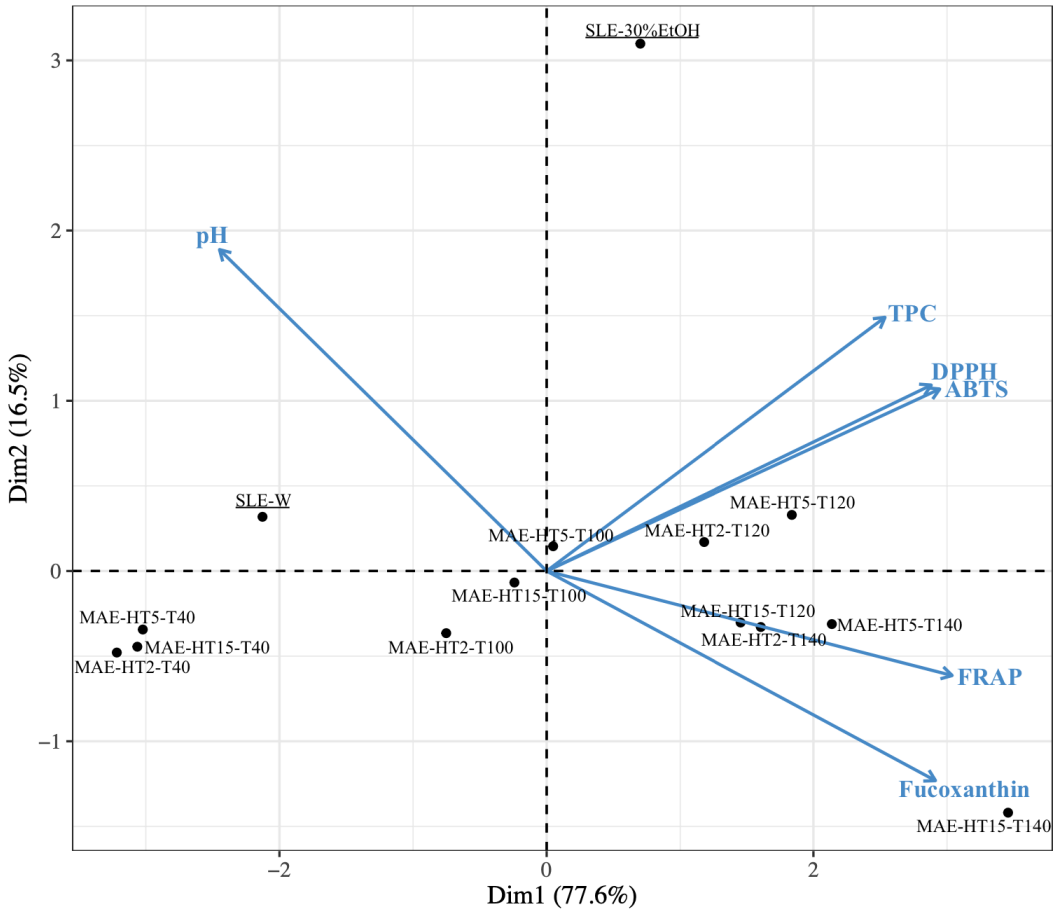

Figure 4.9: Principal component analysis (PCA) of all extracts. Variables are total polyphenol content (TPC), pH, fucoxanthin, and the antioxidative assays the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), 2,2´-azin-bis(3-ethylbenithiazoline-6-sulfonic acid) assay (ABTS) and ferric reducing antioxidant power (FRAP). The microwave assisted extraction (MAE) is defined after holding time (HT) and temperature (T) in the plot. The solid-liquid extraction (SLE) extrscts are underlined, and indicated by solvent water (SLE-water) and ethanol:water (30:70) (SLE-30%EtOH).

4.7 Antimicrobial Effect of Microwave Extracts

The antimicrobial effect of the tested *A. esculenta* MAE extracts (HT2-T40, HT5-120, and HT15-140) were investigated using a 96-well plate inhibition assay against the microorganisms *P. fluorescence*, *L. innocua* and *L. monocytogenes*. The extracts are considered as extracts with different chemical compositions and not as affected by MAE. The extracts were chosen based on their chemical characteristics, representing low, medium, and high factors of TPC, fucoxanthin, and pH.

The inhibitory effects are presented in Table 4.5, given as log reduction CFU/mL, the difference between the average cell concentration (CFU/mL) of the target organism (positive control) and the cell concentration (CFU/mL) of the target organisms obtained with the extract. The calculation was based on the quantified cell concentrations, which are included in Appendix E. Negative controls confirmed no contamination and the absence of microorganisms in the extracts (Appendix E). Table 4.5 shows that both *Listeria* strains were inhibited by *A. esculenta* extracts, no inhibitory effect was found for *P. fluorescence*.

Table 4.5: Inhibitory effect of the different *Alaria esculenta* extract, given as log reduction CFU/mL, the difference between the average cell concentration (CFU/mL) of the target organism (positive control) and the cell concentration (CFU/mL) of the target organisms obtained with extract, on the target organisms; *Pseudomonas fluorescence* (*P. fluorescence*), *Listeria innocua* (*L. innocua*), and *Listeria monocytogenes* (*L. monocytogenes*), for the different initial cell concentrations (of the target organisms) ($1x10^2$ and $1x10^4$), and applied extract volumes (2 and 100 µL).

	Extract volume	**2 µL**		**100 µL**	
	Cell concentration	**10^2**	**10^4**	**10^2**	**10^4**
Target organism	**Extract**				
P. fluoresence	**HT2-T40**	0.19 ± 0.13	-0.11 ± 0.07^{b}	-0.11 ± 0.10^{b}	0.09 ± 0.08
(n = 4)	**HT5-T120**	0.06 ± 0.14	0.10 ± 0.12^{ab}	-0.12 ± 0.12^{ab}	0.06 ± 0.02
	HT15-T140	-0.07 ± 0.17	0.01 ± 0.11^{a}	0.02 ± 0.15^{a}	0.03 ± 0.09
	p-value*	= 0.324	= 0.037	= 0.040	= 0.81
L. innocua	**HT2-T40**	-0.06 ± 0.25	0.23 ± 0.21	0.13 ± 0.18^{b}	0.06 ± 0.24^{b}
(n = 12)	**HT5-T120**	0.06 ± 0.20	0.14 ± 0.12	1.22 ± 0.12^{a}	1.22 ± 0.11^{a}
	HT15-T140	0.09 ± 0.16	0.17 ± 0.30	1.25 ± 0.11^{a}	1.36 ± 0.14^{a}
	p-value*	= 0.189	= 0.820	< 0.001	< 0.001
L. monocytogenes	**HT2-T40**	0.11 ± 0.16	0.20 ± 0.20	0.04 ± 0.09^{c}	0.06 ± 0.06^{c}
(n = 8)	**HT5-T120**	0.28 ± 0.22	0.03 ± 0.10	1.25 ± 0.05^{b}	1.37 ± 0.05^{b}
	HT15-T140	0.14 ± 0.20	0.13 ± 0.15	2.03.± 0.08^{a}	2.05.± 0.06^{a}
	p-value*	= 0.110	= 0.110	< 0.001	< 0.001

*Different superscripts within each column (extract) (^{abc}) indicate significant differences ($p < 0.05$) between groups by one-way ANOVA and Tukey HSD pairwise comparison test.

The fixed factors, extract (HT2-T40, HT5-120, and HT15-140), extract volume (2 and 100 µL), and initial cell concentration ($1x10^2$ and $1x10^4$ CFU/mL), were investigated for a main effect analysis, presented in Table E1, Appendix F. From the main effect analysis, the antimicrobial effect of the extracts was not significantly affected by the initial cell concentration (GLM: $p > 0.05$) for any of the three target organisms (Table E1, Appendix F). Hence, the factor of initial cell concentration was combined for further analysis of the effect of type of extract and extract volume for the target organisms *L. innocua* and *L. monocytogenes*, presented in Figure 4.10 and Figure 4.11, respectively.

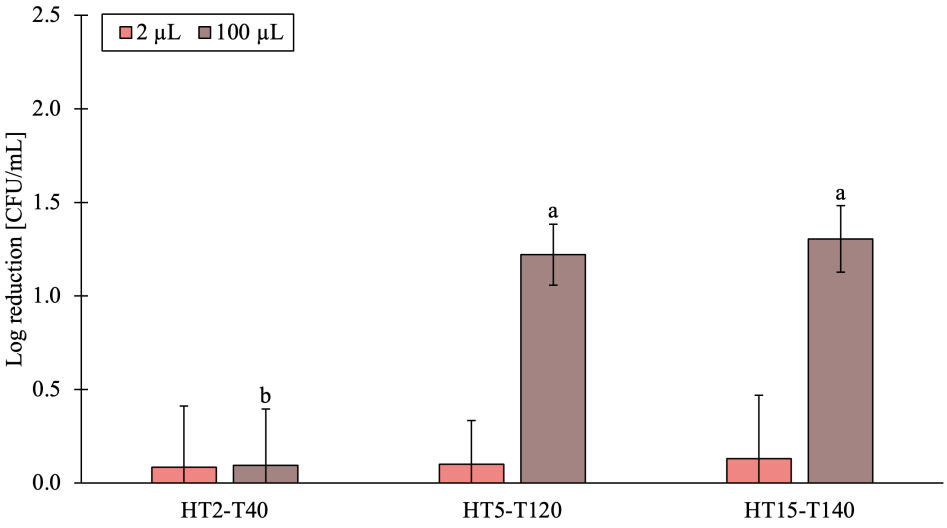

Figure 4.10: Main effects of extract (HT2-T40, HT5-T120, and HT15-T140) and extract volume (2 and 100 µL) on inhibitory effect, given as log reduction CFU/mL, the difference between the average cell concentration (CFU/mL) of *Listeria innocua* (positive control) and the cell concentration (CFU/mL) of *Listeria innocua* obtained with extract. Different letters (ab) represent significant differences between the extracts for the extract volume from Tukey HSD pairwise comparison test.

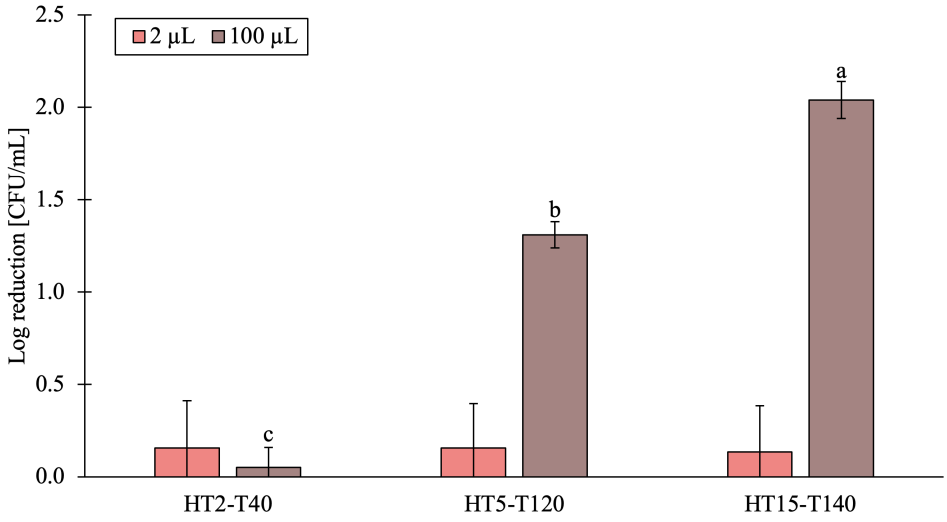

Figure 4.11: Main effects of extract (HT2-T40, HT5-T120, and HT15-T140) and extract volume (2 and 100 µL) on inhibitory effect, given as log reduction CFU/mL, the difference between the average cell concentration (CFU/mL) of *Listeria monocytogenes* (positive control) and the cell concentration (CFU/mL) of *Listeria monocytogenes* obtained with extract. Different letters (abc) represent significant differences between the extracts for the extract volume from Tukey HSD pairwise comparison test.

The extracts showed significantly different inhibitory effects on both *L. innocua* (GLM: F = 50.97, $p < 0.001$) and *L. monocytogenes* (GLM: F = 39.48, $p < 0.001$). The HT2-T40 extract did not demonstrate any inhibitory effect on neither of the strains (Figure 4.10 & Figure 4.11). For *L. innocua*, there was no significant difference between the HT5-T120 and HT15-T140 extracts (Figure 4.10), but for *L. monocytogenes*, HT15-140 had a significantly stronger inhibitory effect compared to HT5-120 (Figure 4.11). Furthermore, the extract volume was significant for both *L. innocua* (GLM: F = 188.76, $p < 0.001$) and *L. monocytogenes* (GLM: F = 114.70, $p < 0.001$). All detected inhibitory effects were obtained using the high extract volume (100 µL), and no inhibition was observed with the low extract volume.

4.8 Effect of Harvest Year in Chemical Characteristics and Antioxidant Activity of *Alaria esculenta*

Differences in chemical characteristics and antioxidant activity were investigated in *A. esculenta* cultivated at the same site in two different years, May 2022 and May 2022, respectively. The optimized MAE parameters for TPC (120°C, 5 minutes hold time) were used for the extraction, and the results for the chemical characteristics are presented in Table 4.6. The total polyphenol content in the 2021 batch was found to be higher than the 2022 batch, with yields of 1.22±0.05 g GAE/100 g and 1.07±0.03 g GAE/100g, respectively (T-test, p = 0.042). However, no differences were detected in the fucoxanthin content (T-test, p = 0.342) or the measured extract pH (T-test, p = 0.530) of the extracts.

Table 4.6: Comparison between *Alaria esculenta* harvested in 2021 and 2022, at the same location, of total polyphenol content (TPC), fucoxanthin content, and measured pH in extracts obtained from microwave assisted extraction with a temperature of 120°C and hold time of 5 minutes.

	2021	**2022**	**p-value**
TPC [g GAE/100g dw]	1.22 ± 0.05	1.07 ± 0.03	= 0.042
Fucoxanthin [mg/100g dw]	45.15 ± 5.80	51.16 ± 9.17	= 0.342
pH [−]	6.30 ± 0.03	6.31 ± 0.02	= 0.530

Furthermore, the difference in harvest year on the *in vitro* antioxidant activity was investigated. No significant difference was found for neither of the radical scavenging assays, DPPH (T-test, p = 0.788) and ABTS (T-test, p = 0.177), as seen in Figure 4.12, nor the reducing activity assay, FRAP (T-test, p = 0.162), shown in Figure 4.13.

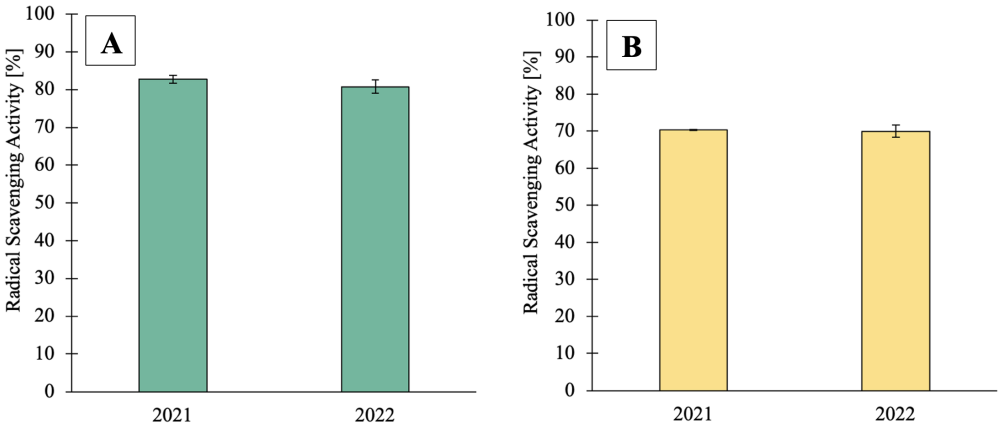

Figure 4.12: Antioxidant activity, from the [**A**] 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH) assay, and [**B**] 2,2´-azin-bis(3-ethylbenithiazoline-6-sulfonic acid) (ABTS) assay, given in radical scavenging activity [%], for *Alaria esculenta* harvested in two different years (2021 & 2022) at the same season and location.

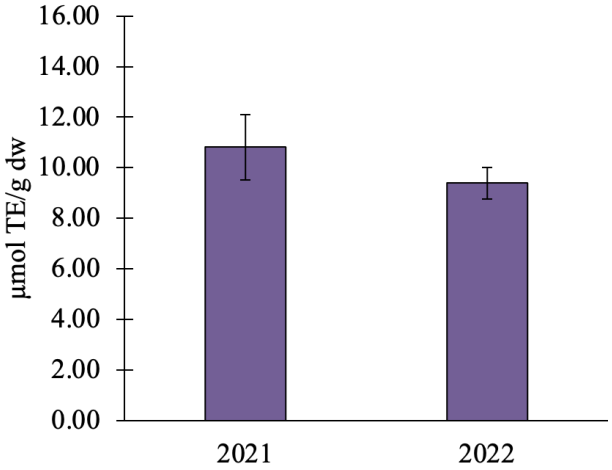

Figure 4.13: Ferric reducing antioxidant power (FRAP) assay, given as µmol Trolox equivalents (TE)/g dry weight (dw) biomass, from *Alaria esculenta* harvested in two different years (2021 & 2022) at the same season and location.

Chapter 5

Discussion

The present thesis features the application of microwave assisted extraction of bioactive compounds from the brown algae *A. esculenta* and its antioxidant and antimicrobial properties. The results show that the MAE temperature and holding time influence the chemical composition and antioxidant activity of the extracts. Moreover, the solvents effect of extraction was displayed. Additionally, the antimicrobial properties of the extracts were detected. Lastly, the effect of harvest year was evaluated. The results of the present thesis demonstrates the potential of microwave assisted extraction and contributes to the increased knowledge of the bioactivity of *A. esculenta*.

5.1 Chemical Characterization of *Alaria esculenta* Extracts

The chemical content, as affected by the different extraction protocols of the *A. esculenta* extracts were determined for the total polyphenol content, fucoxanthin, and pH. Variations were found within all characteristics for the different applied protocols for microwave assisted extraction and solid-liquid extraction.

Distinctive optimum parameters were identified for the TPC and fucoxanthin content. For the TPC, a holding time of 5 minutes was the most efficient (Table 4.1). In this case, a holding time of 2 minutes is inadequate for polyphenol extraction, and a holding time of 15 minutes is excessive, causing the heat-sensitive polyphenols to degrade (Antony and Farid, 2022). On the other hand, for fucoxanthin extraction, the holding time of 15 minutes was the most effective. However, solely for the extraction temperature of 140°C, no difference in holding times was observed for the other temperature groups (Table 4.3). Furthermore, the MAE temperature of 120°C provided the highest TPC, while the extraction temperature of 140°C provided the highest fucoxanthin yield, at the extraction temperature of 140°C the TPC decreased due to heat degradation. Overall, these findings suggest that the optimal extraction parameters for MAE vary depending on the specific chemical component being extracted.

The optimum MAE parameters for TPC in the present thesis, which used water as the solvent, differ from the findings reported by Magnusson et al. (2017). Magnusson et al. (2017) reported an optimum MAE temperature of 160°C with a holding time of 3 minutes for TPC from the brown alga *Carpophyllum flexsoum*. The difference in species can account for the difference in optimum parameters, as different species have reported to have different optimum extraction parameters (as mentioned in section 2.5.1). Furthermore, the findings in the present thesis show that the highest fucoxanthin content was obtained at the highest investigated MAE parameters (Table 4.3). Hence, higher MAE temperatures and holding times, using water as the solvent, should be applied to properly determine the optimal parameters for fucoxanthin extraction. In a study by Xiao et al. (2012), the optimum MAE parameters for fucoxanthin extraction were reported to be an extraction temperature of 60°C with a 10 minute holding time, using ethanol as the solvent. The difference in extraction temperature can be attributed to the different properties of the solvents.

The solvent is a fundamental component of an extraction procedure. For MAE, the physical properties of the solvent are of high importance because the solvents capacity to absorb microwave energy is essential (Seoane et al., 2017). Water has a higher ability to absorb microwave radiation than ethanol due to its larger dielectric constant and dielectric loss (Veggi et al., 2012). These properties cause water exposed to microwave radiation to generate heat rapidly. The present thesis showed ethanol:water (30:70) to significantly increase the extraction of TPC, compared to water (Figure 4.1). The difference was expected since ethanol had been reported to increase the solubility of phenolic compounds, as demonstrated by Afonso et al. (2021). Afonso et al. (2021) compared the TPC between aqueous and ethanolic extracts of *A. esculenta* and reported values of 44.1 ± 4.0 and 95.1 ± 15.2 mg GAE/100 g dw, respectively. The difference between the two solvents reported by Afonso et al. (2021) is consistent with the findings of the present thesis. Conversely, there was no difference in fucoxanthin extraction between the two solvents used in the present thesis (Figure 4.3). Based on previous studies of fucoxanthin extraction from *A. esculenta* using ethanolic solvents, the applied extraction time of 1 minute was insufficient. For instance, Stévant et al. (2017) reported a fucoxanthin content of 87.1 mg/100 g dw, extracted using SLE with ethanol:water (60:40) for 2 hours, and Afonso et al. (2021) used SLE ethanolic extraction for 5 minutes and reported a fucoxanthin content of 66.1 mg/100 g dw. These studies indicate that higher fucoxanthin content can be achieved with longer extraction times. Thus, longer extraction times should have been applied.

Studies of MAE on different brown algae species have reported aqueous ethanolic solutions, in combination with lower extraction temperatures, to be the optimized extraction solvent for TPC (Amarante et al., 2020; Dang et al., 2018b; Z. He et al., 2013) and, as mentioned fucoxanthin (Xiao et al., 2012). In contrast, the present thesis and that of Magnusson et al. (2017), which reported higher optimum extraction temperatures using water as the solvent for these compounds. Considering the solvent properties, the difference in optimum extraction temperature could be related to the solvent used, explaining the lower optimized extraction temperatures reported for fucoxanthin by Xiao et al. (2012), compared to the present thesis. Hence, the optimum MAE parameters found for water as the solvent are not applicable to other types of solvents.

The use of water as the solvent allows the extraction of other water-soluble compounds, such as polysaccharides, proteins, and organic acids (Charoensiddhi et al., 2016). The pH of the extracts is an indicator of the potential presence of other compounds in the extracts. The pH was observed to decrease as affected by the extraction parameters (Table 4.2). This decrease in pH can be caused by the presence of organic acids, which can feature antioxidant and antimicrobial effects (Q. Liu et al., 2019). Moreover, creating a more acidic environment in the extract could be favorable for the extraction of other non-detected compounds. Charoensiddhi et al. (2016) tested several pH-adjusted waters in the range of pH = 4.5-8.0 for extraction of polysaccharides from the brown alga *Escklonia radiata*. The study showed an increase in the polysaccharide extraction with decreasing pH, expressing the facilitation of polysaccharides in a more acidic environment. A divergence was also observed for the pH values of the two tested solvents (Figure 4.2). The ethanol:water (30:70) extract exhibited a significantly higher pH than the water extract. However, since ethanol has a higher kPa constant than water (Deleebeeck et al., 2021, the solution could initially have had higher pH. The pH was not measured prior to extraction and should have been performed to investigate how much the pH was affected by the extraction.

Similarities were also observed between the characteristics as affected by the MAE. To achieve a considerable change in both TPC and extract pH, temperatures exceeding 100°C were required (Table 4.1 & Table 4.2). This can be linked to the observation of initially the freeze-dried ground *A. esculenta* material appeared at the bottom of the microwave vessel before extraction (Table A1, Appendix A). After extraction, for temperatures below 100°C the material was maintained at the bottom of the vessel, and for temperatures above 100°C the material was floating on top of the solvent in the vessel (Table A1, Appendix A). Indicating a decrease in the density of the material caused by cell rupture (Thompson and Islam, 2021), which further releases bioactive compounds and enhances the extraction. Additionally, this explains the low variation between the temperature groups 40-80°C (Table 4.1 & Table 4.2). Moreover, as these groups were equal to the SLE water extraction, these MAE extraction temperatures appear to be insufficient. A compatible observation could not be found for the fucoxanthin content, as the 60°C and 80°C temperature groups were not quantified. However, the fucoxanthin content of these extracts is expected to be between 2.07 - 26.09 mg/100 g dw, the range between the 40°C and 100°C extracts (Table 4.3).

Direct comparison with other findings of chemical characteristics is challenging due to differences caused by seasonal variation, geographical location, extraction medium, and variations in protocols. For instance, the TPC values reported by Afonso et al. (2021) are considerably lower than those reported in the present thesis. Afonso et al. (2021) used *A. esculenta* harvested in March 2017, while the raw material in the present thesis was harvested in May 2021. The difference in harvest season could explain the discrepancy in the TPC values. The effect of seasons has been demonstrated by Schiener et al. (2015). Schiener et al. (2015) examined *A. esculenta* harvested in March and May 2011 and reported a TPC of 0.31% and 1.40% of total biomass, respectively, showing the effect of seasons on the TPC. Furthermore, the TPC was comparable to literature values obtained using other novel and emerging extraction technologies. A TPC of 1.04 g GAE/100 g dw was reported by Einarsdóttir et al. (2021a) using pulsed electric field supported extraction, and

a TPC of 1.12 g GAE/100g dw was demonstrated by Sapatinha et al. (2022) using enzyme assisted extraction. In the case of fucoxanthin, the highest yield obtained from the MAE, 84.63 ± 8.79 mg/100 g dw, is consistent with the values reported in the literature. In addition to the mentioned quantification by Stévant et al. (2017), Shannon and Abu-Ghannam (2017) extracted fucoxanthin, using 62.2% acetone and reported a fucoxanthin content of approximately 86.0 mg/100 g dw. Intriguingly, none of these were acquired using water as the extraction medium nor with a thermal extraction procedure, the results provide a new insight into the potential of fucoxanthin extraction using the time-efficient MAE, using water as the solvent.

Moreover, additional factors affect the effectiveness of an extraction protocol, which is specific to each extraction method. The effect of temperature, holding time, and solvent were demonstrated, as well as the role of solid:solvent ratio was demonstrated (Figure C.2, Appendix C). A ratio of 1:30 (v/W) was found to be the most effective, similar to the optimized ratios reported by Magnusson et al. (2017), Toan et al. (2021), and Ren et al. (2017). The effect of pre-treatment on the raw material is also an essential factor in an extraction process. In the present thesis, the raw material was freeze-dried before extracted. The conservation of bioactive compounds is affected by pre-treatment. Sappati et al. (2019) and Badmus et al. (2019) investigated the effect of various drying methods and discovered freeze-drying to outperform oven-drying in the case of the yield of bioactive compounds. However, freeze drying was employed in the present thesis. It is a time-consuming and expensive procedure that might not be economically feasible for industrial purposes (Petrova et al., 2018). Nevertheless, this technique effectively preserves bioactive compounds. It is also crucial to obtain a homogeneous sample from the raw material before extraction because numerous studies have demonstrated intrathallus differences of the compounds of interest (Shannon and Abu-Ghannam, 2017, T. Wang et al., 2009). Furthermore, other pre-treatments using novel and emerging technologies, such as pulsed electric field and ultrasonic assisted extraction, to enhance extraction are of further interest to be investigated. Considering both ultrasonic assisted extraction (Ummat et al., 2020) and pulsed electric field assisted extraction (Einarsdóttir et al., 2021a) have been proven as efficient extraction methods on their own.

The limitations of the TPC assay must be considered. The measured TPC may not be completely accurate. The Folin-Ciocalteu assay lacks sensitivity and accuracy and is based on the reducing capacity of bioactive compounds (Huang et al., 2005). Other compounds present in the extracts, such as sugars, proteins, and thiols, may interact and confer false-positive results (Ford et al., 2019). The TPC content is not regarded as false-positive in the present thesis because macroalgae, particularly *A. esculenta*, are known to be rich in polyphenols. However, the results obtained could have been overestimated. To accurately determine the polyphenol concentration, further analyses of specific polyphenols must be performed. Therefore, the TPC content was regarded as an estimation. However, the Folin-Ciocalteu assay, which is frequently used for the determination of the TPC (Gulcin, 2020), demonstrated that the TPC of the extracts differed between the different protocols used.

5.2 Antioxidant Properties of *Alaria esculenta* Extracts

The antioxidant properties of the extracts were assessed using three *in vitro* assays DPPH, ABTS, and FRAP. The individual methods analyze a specific mechanism under specific conditions (Gulcin, 2020; Huang et al., 2005). Comparison between studies complicates further for antioxidant properties because, in addition to the mentioned factors for the chemical characteristics, the presentation of antioxidants is given in different values and divergence in applied protocols. However, in the present thesis, the antioxidant assays provide information about the performance of MAE and SLE and their ability to yield extracts with different antioxidant activities as affected by the design factors.

For MAE, the extracts exhibited similar trends in all antioxidant assays (Table 4.4). The antioxidant activity of the extracts increased with increasing extraction temperature. However, there were only minor differences between the holding times within the temperature groups. The major exception was within the 140°C temperature group, where the holding time of 15 minutes provided the extract with the significantly highest antioxidant activity (Table 4.4). The small divergence in the antioxidant activity between the holding times is intriguing, as the TPC significantly differs between the holding times (Table 4.1). Thus, the results indicate that polyphenols play a less prominent role in the antioxidant activity of *A. esculenta* extracts, supported by the correlation between the chemical characteristics and antioxidant activity (Figure 4.7).

In general, brown algae have been reported to have a very strong correlation between the TPC and antioxidant activity as reviewed by Balboa et al. (2013). Contrary to the current findings, where the TPC had a moderate correlation with the DPPH and FRAP assay and a strong correlation with the ABTS assay (Figure 4.7). The moderate correlation build on the existing knowledge of polyphenols having a less prominent role in antioxidant activity of *A. esculenta*. For instance, Q. Liu et al. (2019) investigated the correlation between TPC and DPPH in *A. esculenta* extracts and reported a correlation of r = 0.78, identical to the correlation coefficient for the DPPH assay in the present thesis, and Einarsdóttir et al. (2021b) reported a correlation between the TPC, and DPPH and FRAP assays, r = 0.76 and r = 0.77, respectively. Additionally, Afonso et al. (2021) reported a correlation between TPC and the ABTS assay of r = 0.73. These results further indicate that polyphenols are not the main component responsible for the antioxidant activity of *A. esculenta* extracts. The presence of other detected compounds (fucoxanthin) and non-detected water-soluble antioxidant compounds, such as polysaccharides and organic acids (as indicated by the pH), in the extracts accounts for this observation, further supported by the strong relationship between the fucoxanthin, extract pH, and the antioxidant assays (Figure 4.7).

In fact, the fucoxanthin content had a very strong correlation with all antioxidant assays (Figure 4.7), proposing that fucoxanthin could be a stronger contributor to the antioxidant activity of the *A. esculenta* extracts. A similar observation was reported by Foo et al. (2017), reporting fucoxanthin to contribute more towards the antioxidant activity in the case of the iron chelating antioxidant properties. Fucoxanthin, as well as polyphenols, have the ability to scavenge free

radicals, as demonstrated for fucoxanthin by Sachindra et al. (2007). Sachindra et al. (2007) investigated fucoxanthin compared to α-Tocopherol (E307) and reported fucoxanthin to be similar to α-Tocopherol for the radical scavenging activity, demonstrating its strong potential as a radical scavenger. Further, this could explain why the antioxidant activity was not affected by the MAE holding time for the majority of the MAE temperature groups (Table 4.4). The MAE extract with the highest antioxidant activity was, in addition, the extract with an outstandingly high fucoxanthin content. However, the strong correlation between the antioxidant assays and the fucoxanthin is contradicting from research by Dang et al. (2018a). Dang et al. (2018a) reported polyphenols as the main contributor towards the antioxidant activity and fucoxanthin as a moderate contributor for brown algae. As the antioxidant assays also had a strong negative correlation with the pH (Figure 4.7), it could be indicative of other non-detected compounds in the extracts contributing to the antioxidant activity, such as polysaccharides or organic acids.

The PCA MAE biplot in Figure 4.8 indicates that the use of higher extraction temperatures enhances the extraction of a wider diversity of bioactive compounds and antioxidant properties of the extracts. In the biplot, most of the variance was explained in PC1 (89.8%), and low variance was explained in PC2 (6.6%). The temperature groups were located adjacent to each other and had similar chemical characteristics in terms of pH, fucoxanthin, and antioxidant activity, as these variables were associated with PC1. Furthermore, the MAE temperature groups are separated after the holding time, accounting for the variation in TPC. The PCA MAE biplot is consistent with the observed results, as TPC is the variable with the most variation between the holding times of the variables. However, the variance explained in PC2 is low, meaning that the separation of the extract within the holding times is minimal. The observation is similar to Santos et al. (2016), who applied PCA to examine the effect of extraction temperature and extraction time on the extraction of phenolic compounds from beet rot. Santos et al. (2016) also observed that the extract grouped after temperature with increasing antioxidant activity. Moreover, the three antioxidant assays have a strong relationship with each other (Figure 4.8), related to their similar chemical behaviors (Huang et al., 2005).

Farvin and Jacobsen (2013) explored the polyphenolic profile of several brown algae aqueous and ethanolic extracts and found their properties regarding specific polyphenols and antioxidant activity to be distinctive. Hence, the chemical composition and the antioxidant activity of the different extract types are different depending on the solvent. The difference was evident by the inclusion of both extraction methods in the PCA (Figure 4.9). The SLE water extract is similar to the MAE 40°C extracts, as opposed to the SLE ethanol:water (30:70) extract, which is distinguishable from all extracts, caused by the difference in the solvent as different solvent properties results in the extraction of different compounds. The DPPH and ABTS assays are now located close to each other, showing a strong correlation between these variables due to chemical mechanism, as both are radical scavenging assays (Gulcin, 2020). However, in this case, the TPC was located close to the RSA assays, indicating that RSA is more related to TPC. Following, the reducing capacity (FRAP) is more correlated with fucoxanthin and negatively correlated with pH. From the biplot (Figure 4.9), the insufficiency of the MAE temperature of 40°C can be seen, as the SLE water

extract do appear to have higher antioxidant activity than the MAE temperature 40°C extracts.

Compared with its high pH and low fucoxanthin content, the SLE ethanol:water (30:70) extract exhibited a high RSA in the DPPH and ABTS assays (Figure 4.4 & 4.5). Considering the above mentioned difference in the polyphenolic profile and related antioxidant properties. The ethanolic solvent, being a non-polar solution, allowing the extraction of lipophilic antioxidant compounds that were not present in the aqueous extracts. The difference is also evident by that the MAE extract with similar TPC to the SLE ethanol:water (30:70) extracts (Figure 4.1), do not exhibit the same antioxidant activity in any of the assays (Figure 4.4, 4.5, and 4.6). However, the high antioxidant activity, as measured by the DPPH and ABTS assays, was not consistent in the FRAP assay. The FRAP assay is performed under acidic conditions (pH = 3.6), which can suppress the antioxidant activity of some antioxidant compounds (Huang et al., 2005), explaining why the SLE ethanol:water (30:70) extract performed poorly in the FRAP assay. In contrast to the MAE extracts, the correlation between the TPC and the antioxidant assays was found to be significant very strong in these extracts (Figure 4.7). However, apart from the correlation between the pH and the ABTS assay, no significant correlation was observed for the fucoxanthin and pH and the antioxidant assays. However, the small number of measurements must be considered. The correlation was also performed by combining the SLE results, and the mentioned difference between aqueous and ethanolic extracts must be considered, as they could influence the results.

All performed assays are based on the same antioxidant mechanism (Gulcin, 2020). Other assays, such as oxygen radical absorbance capacity and metal-chelating properties, investigate other antioxidant mechanisms and could have been performed to gain a deeper understanding of the antioxidant capacity of the *A. esculenta* extracts. Nevertheless, these are *in vitro* assays that are useful for assessing the potential of the extracts. However, they do not represent complex systems, such as food. Further investigations on the antioxidant activity of the extracts in food systems are of interest to evaluate the performance, especially for possible applications within the food industry.

5.3 Antimicrobial Properties of *Alaria esculenta* Extracts

The antimicrobial effect of the *A. esculenta* microwave extracts were investigated for three microorganisms *P. fluorescence*, *L. innocua*, and *L. monocytogenes* (Table E1). The extracts had an inhibitory effect of 1-2 log reduction CFU/mL for both *Listeria* strains, whereas no inhibitory effect of *P. fluoresence* was observed (Table E1). To the best of the authors knowledge, this is the first study to demonstrate the antimicrobial effect of *A. esculenta* towards *L. monocytogenes*.

Based on *P. fluorescence* being a gram-negative bacteria and *Listeria* is a gram-positive bacteria, cell morphology could play a role in the antimicrobial properties of the extracts. Karpiński and Adamczak (2019) investigated the antimicrobial effect of isolated fucoxanthin on six gram-positive and seven gram-negative bacteria and discovered a greater inhibitory impact on the gram-

positive bacteria. *Pseudomonas aeringnosa* among the investigated gram-negative bacteria was discovered to have the lowest inhibition zone (agar disc-diffusion method) and the highest minimal inhibitory concentration. The same was reported by Z. Liu et al. (2019). However, no inhibitory effect was detected in the present thesis, for *P. fluorescence*, it could be presumed that *Pseudomonas* are not very susceptible to fucoxanthin nor in general algae extracts. However, isolated fucoxanthin is used in the above mentioned studies. The extract used in the present thesis is crude extracts, which include multiple bioactive compounds.

A variety of crude algae methanolic extracts were tested for their antimicrobial effect against two gram-positive and gram-negative bacteria in a study conducted by Cox et al. (2010). In the study, few differences were found between the gram-positive and gram-negative bacteria in terms of their inhibitory effects. The same was reported in a study performed by Lerfall et al. (2018), in which *A. esculenta* ethanolic extracts were tested against numerous gram-positive and gram-negative bacteria, with two of the gram-positive and two of the gram-negative bacteria inhibited by the *A. esculenta* extract. Indicating that polyphenols and other compounds are more effective towards gram-negative bacteria than fucoxanthin is. Considering this, it should be noted that the results obtained in the present thesis indicate gram-positive bacteria to be more susceptible towards *A. esculenta* extracts, than gram-negative bacteria. Only one gram-negative bacteria was investigated. Moreover, intraspecific differences exist and must be considered (Stupar et al., 2021). The addition of several gram-negative and gram-positive bacteria, with the inclusion of several strains of the same species, should be performed before determining if the effect of cell morphology influences the antimicrobial effect of the extracts.

In general, the extracts exhibited a stronger inhibitory effect on *L. monocytogenes* than on *L. innocua*. The *Listera* strains used in the present thesis were also used in a study by Stupar et al. (2021). Stupar et al. (2021) investigated the potential of bioconservation using lactic acid bacteria isolated from seafood and also reported a higher inhibitory effect towards the *L. monocytogenes* strain, compared to *L. innocua*. Which implies that the *L. monocytogenes* strain used is generally more susceptible than the *L. innocua* strain. The observed difference in the *Listeria* strains raises the question of *L. innocua* as a substitute for *L. monocytogenes*, as addressed by Stupar et al. (2021).

No difference in log reduction was found between the HT5-T120 and HT15-T140 extracts for *L. innocua*, with log reductions of 1.22 ± 0.16 and 1.31 ± 0.18, respectively (Figure 4.10). The extracts used are different from each other in terms of chemical composition, the HT5-T120 extracts have a higher TPC content (1.22 g GAE/100 g dw) than the HT15-T140 extract (0.88 g GAE/100 g dw), and fucoxanthin content of the HT5-T120 (45.15 mg/100 g dw) extracts was almost half of the content in the HT15-T140 extract (84.63 mg/100 g dw). Suggesting that both polyphenols and fucoxanthin contribute to the observed antimicrobial activity. However, this was not the case for *L. monocytogenes*. For *L. monocytogenes*, the log reduction was greater for the HT15-T140 extract (log reduction CFU/mL = 2.04 ± 0.10), compared to the HT5-T120 extract (log reduction CFU/mL = 1.31 ± 0.07), as shown in Figure 4.11. As mentioned, the HT15-T140 extract has

a higher fucoxanthin content and lower TPC, which could indicate that fucoxanthin contributes more toward antimicrobial activity than polyphenols. However, other non-detected, such as polysaccharides and organic acids, can also have antimicrobial properties. Furthermore, the pH of the HT14-T140 extract (pH = 6.31) was lower than of the HT5-T120 extract (pH = 6.17). Both are within the pH range in which *Listeria* can grow (George et al., 1988). The slightly lower pH in the HT15-T140 extract could have contributed to the higher inhibitory effect of this extract.

The extracts exhibited inhibitory effects. However, the inhibitory effect is considered to be moderate (log reduction = 1 - 2). The extracts used were crude extracts obtained using MAE, with water as the solvent. Lipophilic compounds have also been reported to have higher antimicrobial activity compared to hydrophilic compounds (Shannon and Abu-Ghannam, 2016). In the present thesis, crude aqueous extracts were used, and the presence of lipophilic compounds was considered low to non-present. It could be that *A. esculenta* extracts have a higher antimicrobial activity if lipophilic substances are to be included. In addition, the extracts were used directly. An up-concentration of the extract could be favorable for a stronger antimicrobial effect. As indicated by the results, where a low volume of extracts had little to no inhibitory effect, and a high volume of extract showed an inhibitory effect. Supported by the results reported by Cox et al. (2010), who investigated the inhibitory effect of different concentrations of extract and found an increased inhibitory effect with increasing extract concentration.

The results can have significant implications within the food industry, as the present thesis proposes *A. esculenta* extracts as a potential alternative to synthetic preservatives, as both antioxidant and antimicrobial properties were demonstrated, extracted using a more sustainable method. Potentially, the use of *A. esculenta* as a natural preservative can help to prolong the shelf life of food products, thus reducing food waste and improving food safety. The extracts inhibitory effect against *L. monocytogenes* is of particular importance for food safety, as *L. monocytogenes* is a ubiquitous pathogenic bacteria frequently found in food products (Farber and Peterkin, 1991). However, a barrier for potential applications is legislative regulations. Further research is needed for future possible applications of *A. esculenta* extracts.

5.4 Effect of Harvest Year in Chemical Characteristics and Antioxidant Activity of *Alaria esculenta*

The difference in chemical characteristics and *in vitro* antioxidant activity between two batches of *A. esculenta* was investigated. The raw material was grown for the same time period (Jan-May) in the respective years at the same site.

The effect of the year did show a significant difference in the TPC, whereas the fucoxanthin content and the pH of the extract were found to be similar. Roleda et al. (2019) also investigated the effect of harvest year and found no significant difference in TPC. However, Roleda et al. (2019) combined *A. esculenta* harvested from different geographical areas (France, Trondheim and Bodø, Norway). The combining of these locations could influence the results in regards to harvest year. Gager et al. (2020) also reported a significant difference in TPC in *A. esculenta* harvested in 2015 and 2016 at the same time (autumn) and location (France). However, the ocean climate is fluctuating and is different at different geographical locations, which can contribute to different growth conditions in different years. In particular abiotic factors, such as weather and nutrient availability, do greatly vary, which influence the chemical composition of the alga (Kraan, 2020).

For the antioxidant activity, no difference between the two batched was observed for any of the antioxidant assays. Gager et al. (2020) reported a significant difference in reducing power, as measured by the FRAP assay, in the case of the batch with the highest TPC has the highest antioxidant activity. No significant difference was found between the two batches in the FRAP assay in the present thesis, the TPC was barely different from each other, the difference in the TPC reported by Gager et al. (2020) was greater, and could explain the significant difference found. However, the FRAP was found to be numerically lower in the 2022 batch, possibly attributed to the lower TPC content. Additionally, Gager et al. (2020) reported no difference for the DPPH assay, as consistent with the findings of the present thesis.

The results provide a foundation that the chemical composition and the antioxidant activity of *A. esculenta* as harvested from the same location at the same time is stable. However, only two different harvest years were included using a small sample size, further investigations on bigger data sets are needed to validate the effect of harvest year.

Chapter 6

Conclusion

MAE was performed to extract bioactive compounds from the brown algae *A. esculenta*, using different extraction temperatures and holding times, and its bioactivity has been investigated. The chemical characteristics of the extracts were determined for the TPC, fucoxanthin content, and pH. The *in vitro* antioxidant activity of the extracts and their relationship with the chemical characteristics were evaluated. Moreover, the extracts antimicrobial effect on the microorganisms *P. fluorescence*, *L. innocua*, and *L. monocytogenes* were examined. In addition, the difference in the chemical characterization and antioxidant activity of *A. esculenta* harvested in May 2021 and May 2022 were evaluated.

The potential of MAE using water as the extraction medium was demonstrated, and optimization of MAE parameters was found to be compound depended. The optimization parameters of the TPC were determined to an extraction temperature of 120°C with a holding time of 5 minutes, and for fucoxanthin, an extraction temperature of 140°C with a holding time of 15 minutes. Considering that the optimized fucoxanthin parameters were obtained from the highest tested extraction temperature and holding time, the optimization is inconclusive. Additionally, a change in the pH was found for increased extraction temperatures and holding times.

The antioxidant activity increased with higher MAE temperatures and holding times. The relationship between the *in vitro* antioxidant activity and chemical characteristics found fucoxanthin to be a greater contributor than polyphenols towards the antioxidant activity of the extract. Moreover, the two solvents affected the extraction yield and antioxidant activities. The antimicrobial properties of *A. esculenta* extracts on *L. innocua* and *L. monocytogenes* was demonstrated, whereas *P. fluorescence* showed resistance against the extracts. Lastly, the effect of harvest year was only found to be different for the TPC, where the 2021 batch had a higher TPC than the 2022 batch.

In conclusion, MAE is a promising extraction method to extract high yields of bioactive compounds from *A. esculenta*, with antioxidant and antimicrobial properties. Proposing bioactive compounds from *A. esculenta* as potential candidates for possible applications within the food industry.

Chapter 7

Further Work and General Remarks

The present thesis provided a strong demonstration of the promise of microwave assisted extraction as a novel and green extraction technology, using water as the solvent. The optimum parameters for the fucoxanthin could not be properly determined. Therefore, investigation of higher MAE temperatures and holding times should be studied. However, for a proper assessment of the performance of the MAE, the method should be tested against more traditional extraction methods than what was performed in the present thesis.

Moreover, the inclusion of different pre-treatment methods to improve the extraction. Especially, with the use of other novel green technologies, such as pulsed electric field (PEF) and ultrasonication, as pre-treatments. The use of PEF original to be included in the present thesis. Due to equipment delivery delays, it was not possible to include. The PEF was to be applied as a pre-treatment, in combination with MAE, and as its own.

Considering the limitations of the Folin-Ciocalteu method, it is of interest to identify the concrete polyphenolic profile of the extracts and evaluate if there is a difference in how the polyphenolic compounds contribute towards the bioactivity of the extract. Further, the antioxidant and antimicrobial activity was only assessed *in vitro*, and it will be important to see the efficacy of these extracts in complex food systems while multiple oxidation-reduction reactions occur simultaneously. It is important to acknowledge that the obtained *in vitro* results cannot be extrapolated to the stability and availability of food products. Investigation of the stability of these compounds should also be applied to further enhance their potential to be applied in the food industry. Further inclusion of the extracts in packaging material, especially edible coating and films (nanocellulose, chitosan), is a potential use for the extracts. The present thesis showed *A. esculenta* to have antioxidant and antimicrobial properties, further studies should take place.

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Appendix A

Microwave Assisted Extraction Observations

During the extraction process of microwave-assisted extractions, visual observations were conducted to observe its behavior. Table A1 summarizes key observations, such as the sinking and floating of the sample, the dropping of microwave power, and exceeding temperature levels set for the extraction process. Figure A.1 provides an example of temperature and power behavior throughout the extraction with varying hold times at 120°C.

Table A1: Key observations made from the microwave assisted extractions process. For the extraction process, and visually observations from the sample.

Key Finding	**Description**
Sink and Float	Initially, the sample sank to the bottom of the microwave vessel and was found at the bottom of the vessel after extraction, for temperatures 40-80°C. But, for the temperatures 100-140°C the sample was found to be floating on top of the solvent in the vessel.
Power Drop	The microwave power dropped drastically once the temperature reached the set temperature level for the given extraction program.
Temperature Exceeding the set Level	The temperature exceeded the set temperature level at the end of the ramp time for almost all performed extractions before stabilizing at the set temperature.

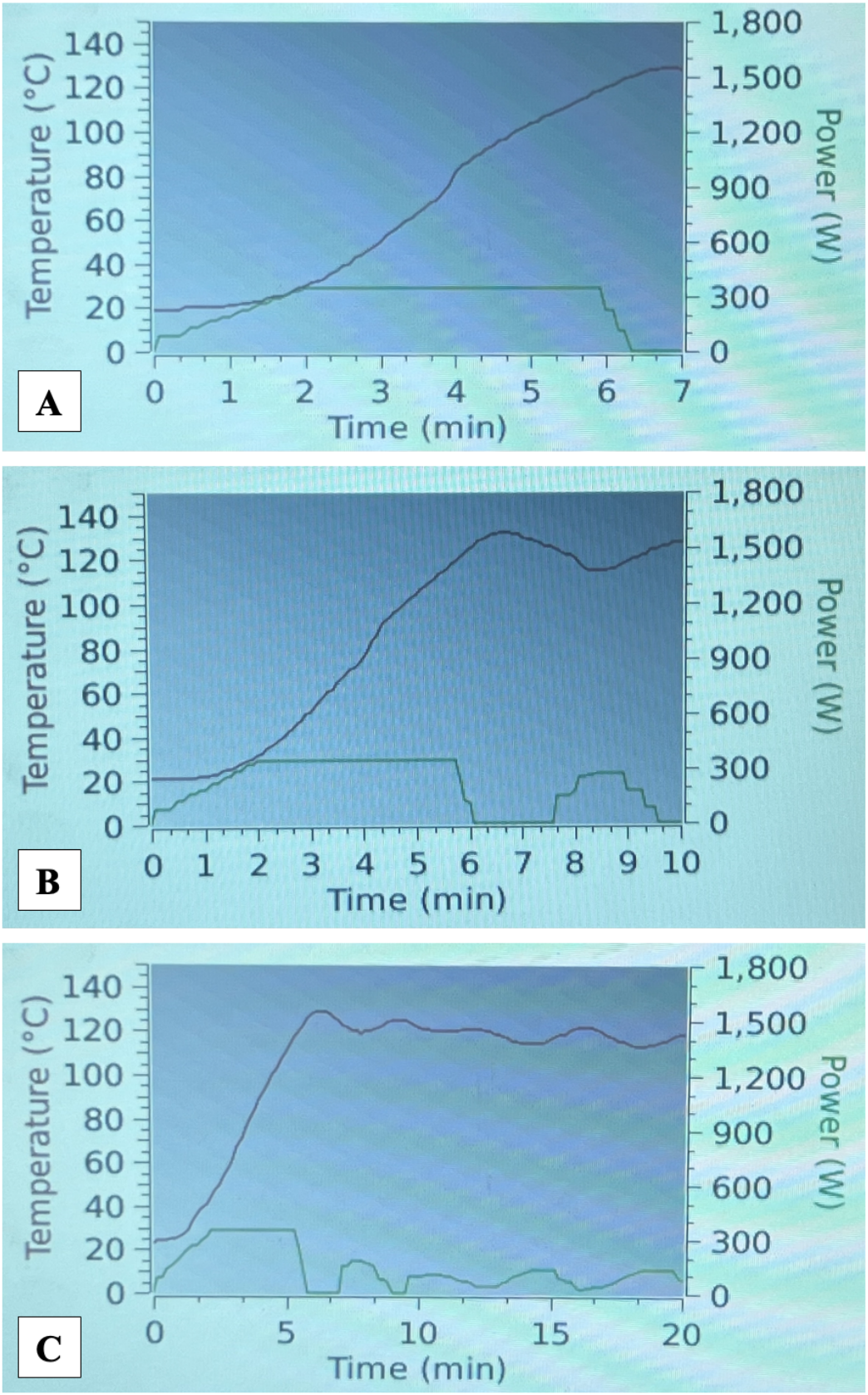

Figure A.1: Temperature-power graphs for the microwave assisted extractions, for the temperature 120°C, and [A] 2 minutes hold time, [B] 5 minute hold time, and [C] 15 minute hold time. The first 5 minutes are ramp time.

Appendix B

Standard Curves & Calculations

B.1 Total Polyphenol Content

The total polyphenol content (TPC) was determined using the Folin-Ciocalceu method, a standard curve was obtain using gallic acid (CAS: 5995-86-8, Thermo Fisher Scientific, Waltham, USA), see Figure B.1 and used for determination of the TPC of the extracts, using the regression equation.

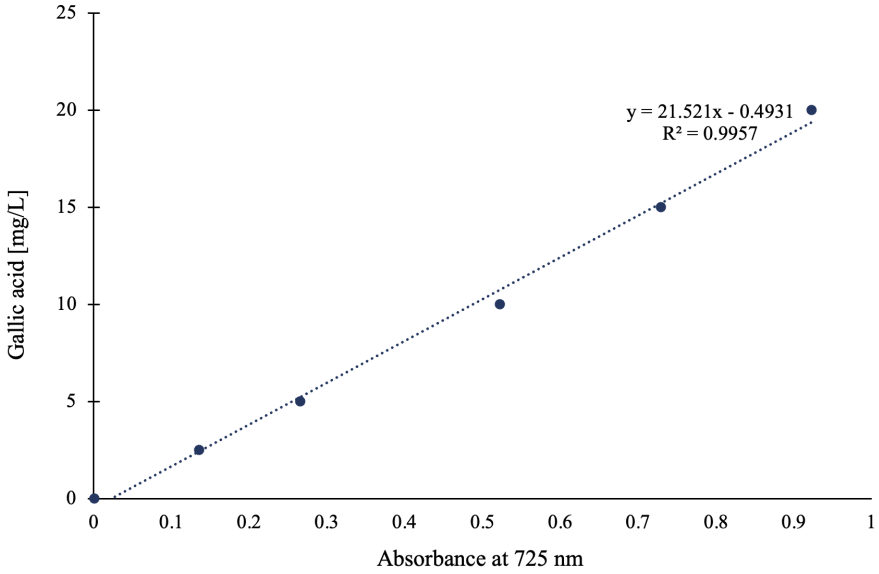

Figure B.1: Standard curve for total polyphenol content with different concentrations of gallic acid (0-20 mg/L), and absorbance measured at 725 nm. Equation: y = 21.521x - 0.49

The TPC was calculated based on the equation obtained from the standard curve (Figure B.1), using the equations; B.1, and B.2. Where *Abs* is the measured absorbance of the sample, and *c* is the concentration of the extract (g/L).

$$Gallic\,acid\,equivalents\,(GAE) = Abs \times 21.521 \tag{B.1}$$

$$\frac{g\,GAE}{100\,g\,dry\,weight\,biomass} = \frac{GAE \times dilution\,factor}{c \times 10g} \tag{B.2}$$

B.2 DPPH Assay

A standard curve was prepared with different concentrations of Trolox. The absorbance at 517 nm were plotted against the concentrations (µM) of Trolox to obtain the standard curve. Further, curve was evaluated by linear regression (Figure B.2). The curve was only used to verify the method, and was not further used.

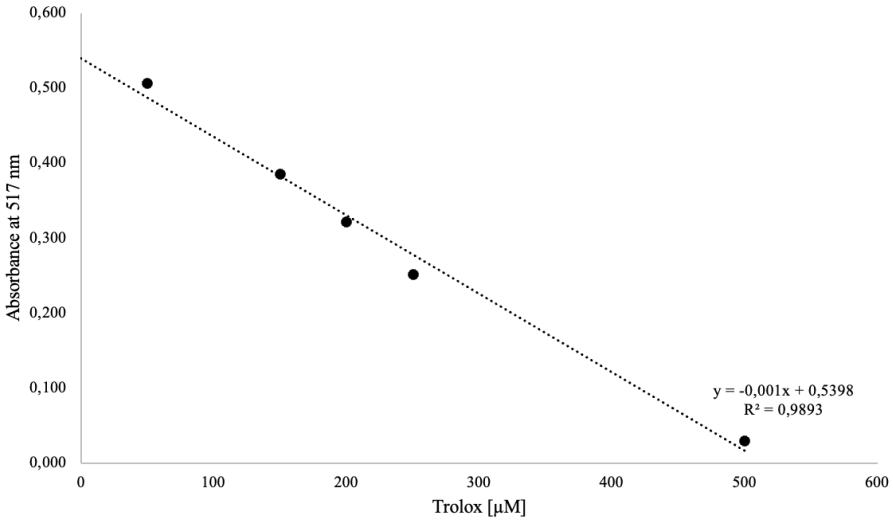

Figure B.2: Standard curve for DHHP assay with different concentrations of trolox (0-500 µM), and absorbance measured at 517 nm

B.3 ABTS Assay

A standard curve was prepared with different concentrations of Trolox. The absorbance at 734 nm were plotted against the concentrations (µM) of Trolox to obtain the standard curve. Further, curve was evaluated by linear regression (Figure B.3). The curve was only used to verify the method, and was not further used.

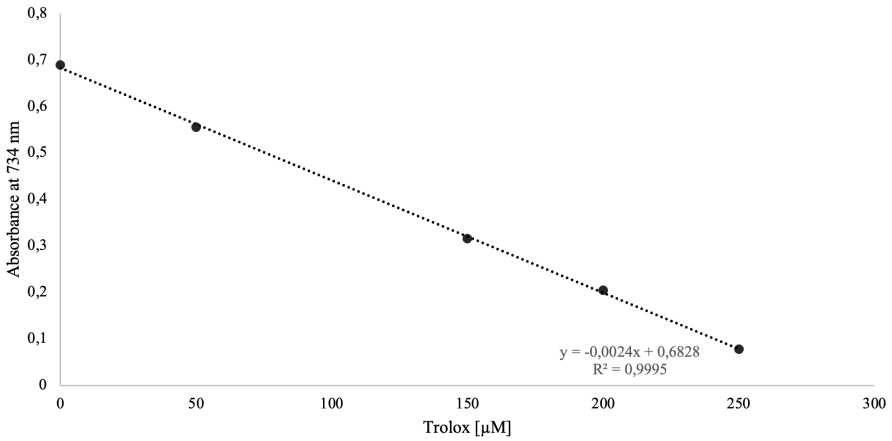

Figure B.3: Standardcurve for ABTS assay with different concentrations of trolox (0-250 µM), and absorbance measured at 734 nm

B.4 FRAP Assay

To assess the antioxidant activity of the extracts using the FRAP assay, a standard curve was established by preparing a series of Trolox standard solutions and measuring their absorbance at 593 nm. The resulting data was then plotted with Trolox concentration (µM) on the x-axis and absorbance on the y-axis, and a linear regression was performed to derive the equation for the curve, shown in Figure B.4.

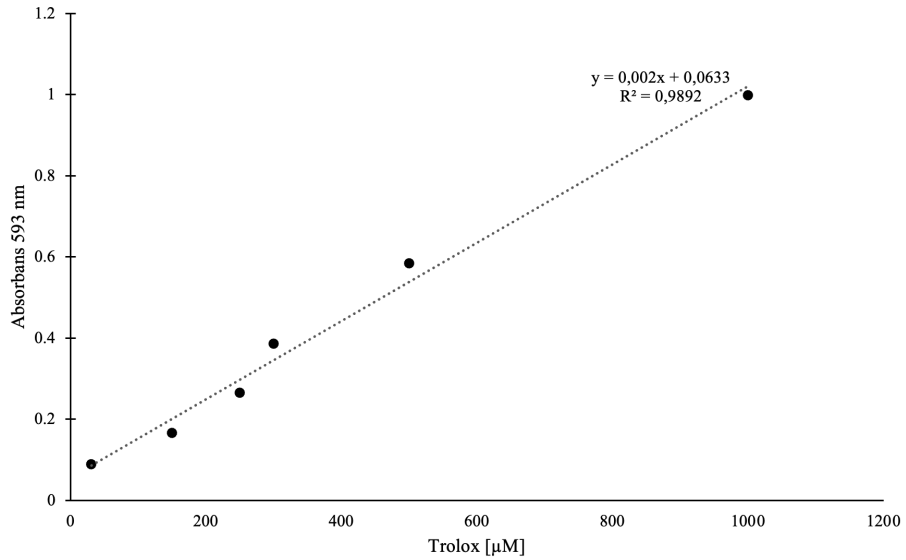

Figure B.4: Standard curve for FRAP assay with different concentrations of Trolox (31.25-1000 µM) and absorbance measured at 593 nm.

Appendix C

Results from Preliminary Trail

Initial microwave assisted extraction testing, using the temperatures 30°C, 60°C and 90°C was used, in combination with a 15 and 30 minute holding time, using a 1:10 (biomass:water) ratio. The initial testing showed an increasing TPC content with increasing temperature (GLM, $F = 32.78$, $p < 0.001$) and a decreasing TPC with an increased holding time (GLM, $F = 153.41$, $p < 0.001$). No interaction was observed between temperature and holding time (GLM, $F = 0.04$, $p = 0.965$). Thus, higher temperatures are increase extraction yield in terms of the TPC, and lower yields was obtained at long holding times. Implying shorter hold times to be more effective.

Table C1: Total polyphenol content given in g gallic acid equivalents (GAE)/100 g dry weight of biomass, for microwave assisted extraction using the temperatures 30°C, 60°C, and 90°C, and holding time of 15 and 30 minutes (n = 3). GLM: $P_{\text{Temperature}} < 0.001$, $P_{\text{Holding Time}} = 0.083$, $P_{\text{Interaction}} = 0.965$.

	15 minutes	**30 minutes**
30°C	0.334 ± 0.021	0.258 ± 0.003
60°C	0.377 ± 0.008	0.303 ± 0.011
90°C	0.393 ± 0.014	0.316 ± 0.014

The microwave device also had several settings for sample types. Including ”Organic” and ”Environmental (Extraction)”. The effect of these sample type settings was tested in regards to the TPC, see Figure C.1. The extraction was performed with a 60°C temperature and 15 minute holding time. No difference was observed between the two sample types (T-test, $p = 0.776$), ”Organic” and ”Environmental (Extraction)” which gave a TPC of 0.369 ± 0.012 and 0.367 ± 0.010 g GAE/100g dw, respectively. Hence, the ”Organic” sample type was used for the extractions.

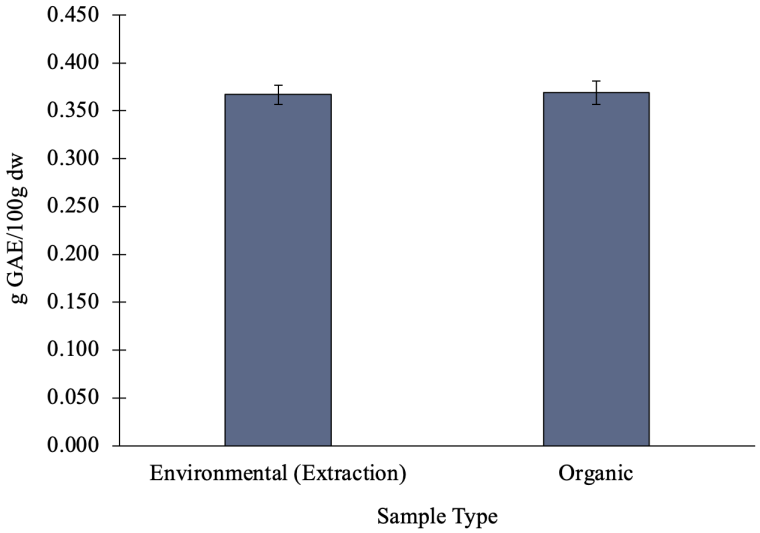

Figure C.1: Total Polyphenol Content given in g gallic acid equivalents (GAE)/100g dry weight (dw) of biomass, for the two different sample types in the microwave device setting. Independent-Samples T-Test, p = 0.776.

The influence of pre-treatment on the samples prior to MAE was examined to determine whether differences in pre-treatment between microwave-assisted extractions and solid-liquid extractions affected the results. A ratio of 1:10 biomass to water was used, and two different MAE temperatures (30°C and 60°C) were employed, with a 15-minute hold time. The TPC was not affected by homogenizing the samples prior to MAE ($p > 0.05$), as indicated in Table C2.

Since no difference was found between the different mixing procedures, vortexing of the microwave vessel was favored to avoid possible loss of samples during transfer between the homogenization tube and microwave vessel. Homogenization within the microwave vessels was not possible.

Table C2: The effect of pre-treatment on the total polyphenol content was investigated by comparing homogenization (10.000 rpm, 1 minute) and vortexing (1 minute) of the samples prior to microwave-assisted extraction. The extractions were carried out at temperatures of 30°C and 60°C, with a 15 minute hold time. The results are expressed in g gallic acid equivalents/100g dry weight biomass, and differences in pre-treatments were evaluated using an independent samples T-test.

	Homogenized	**Vortexed**	**p-value**
30°C	0.351 ± 0.017	0.334 ± 0.021	= 0.327
60°C	0.369 ± 0.011	0.376 ± 0.008	= 0.439

To investigate the effect of ratio on extraction yield, four different ratios (1:10, 1:20, 1:30, and 1:40) were tested. The 1:30 and 1:40 ratios were found to be the most effective (one-way ANOVA, $p < 0.001$), as shown in Figure C.2. Since the 1:30 ratio had a numerically higher value than the 1:40 ratio, it was selected for the extractions.

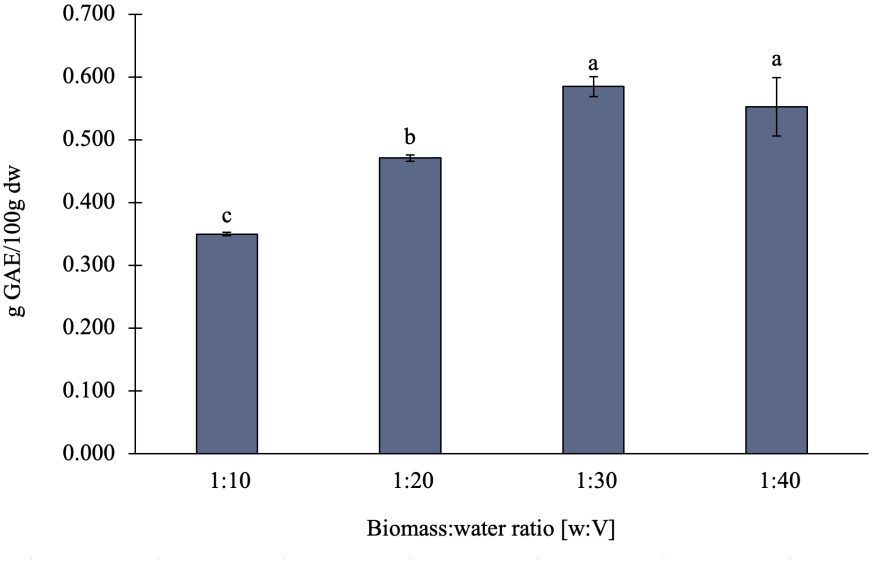

Figure C.2: The total polyphenol content was measured in g gallic acid equivalents (GAE)/100g dry weight (dw) of biomass for four different ratios (1:10, 1:20, 1:30, and 1:40) extracted using microwave-assisted extraction at 60°C for 15 minutes. A one-way ANOVA showed a significant difference (p ¡ 0.001) in the extraction yields among the different ratios. One-way ANOVA: $p < 0.001$. Homogeneous groups are shown by the same letter ANOVA followed by Tukey's HSD.

Two different methods were tested for obtaining powder: manually grinding using a mortar and pestle and using a coffee bean grinder. The coffee bean grinder was able to produce smaller particles than the mortar and pestle, as shown in Figure C.3. However, the use of the coffee bean grinder generated a lot of heat, which could have led to compound degradation. The total polyphenol content (TPC) was 0.358 ± 0.020 and 0.644 ± 0.011 g GAE/100g dw for the coffee bean grinder and mortar and pestle, respectively (t-test, p ¡ 0.001). In addition, visual observation suggested that fucoxanthin may have been degraded during the grinding process, as the powder obtained using the coffee bean grinder appeared greener than the powder obtained using the mortar and pestle.

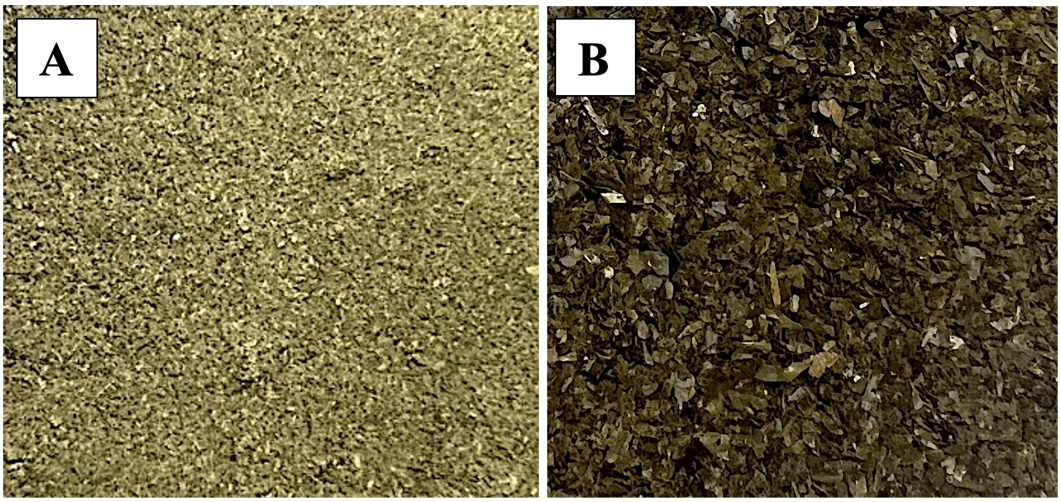

Figure C.3: Visually observation of the raw material of the two different crushing methods tested, [**A**] using a coffee bean grinder and [**B**] using a mortar and pestle.

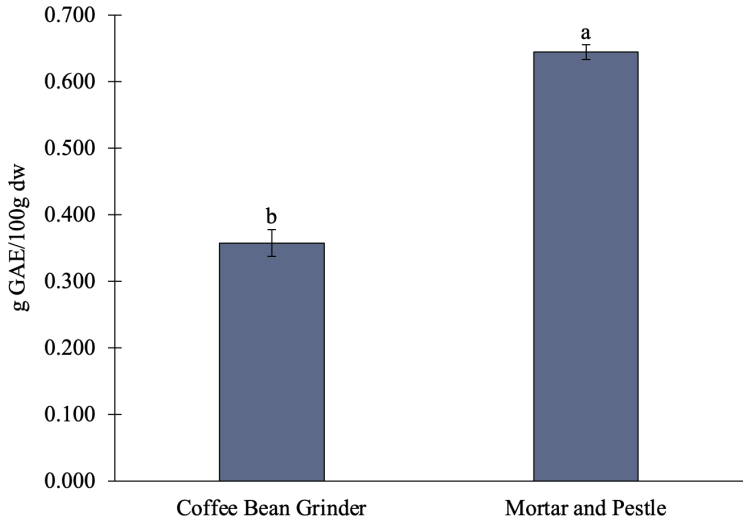

Figure C.4: Total Polyphenol Content given in g gallic acid equivalents (GAE)/100g dry weight (dw) of biomass, for the two different methods used to obtain ground the freeze-dried *Alaria esculenta*. Extracted using microwave assisted extraction, with a temperature of 60°C and 5 minute hold time. Independent-Samples T-test, $p < 0.001$.

Appendix D

Principal Component Analysis

Loading plot for the variables for the MAE extracts, and all extracts are given in Figure D.1, with the associated screeplot are presented in Figure D.2.

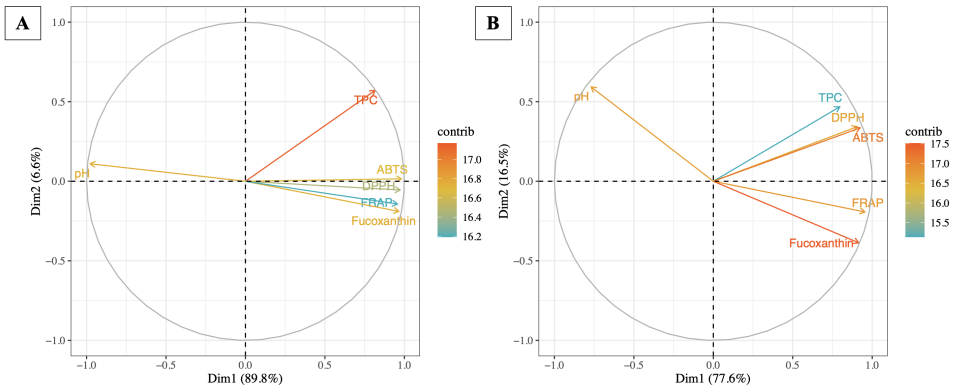

Figure D.1: Loading plot for the variables for the principal component analysis for [**A**] the microwave assisted extraction extracts, and [**B**] for all extracts (microwave assisted extraction and solid-liquid extraction.) The ledgend show how much each variable contributes (contrib) to the variation in the data, given in percent.

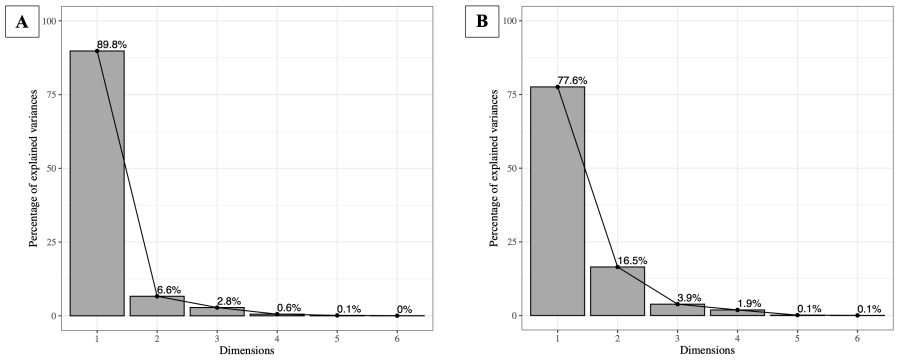

Figure D.2: Screeplot for the principal component analysis for [**A**] the microwave assisted extraction extracts, and [**B**] for all extracts (microwave assisted extraction and solid-liquid extraction).

Appendix E

Quantified Cell Concentrations from the Antimicrobial Inhibitory Assay

The calculated cell concentrations from the antimicrobial assay, including positive and negative controls are shown for *Pseudomonas fluorescence* in Table E1, the three replicates of *Listeria innocua* in Table E2-E4, and the two replicates of *Listeria monocytogenes* in Table E5 and Table E6.

Table E1: Log CFU/mL of *Pseudomonad fluorescence*, for the positive control and negative controls. No detected (ND) growth was found for the negative controls.

Extract volume	**2 µL**		**100 µL**	
Cell concentration	**10^2**	**10^4**	**10^2**	**10^4**
Controls				
Positive	8.79 ± 0.13	8.82 ± 0.30	8.49 ± 0.13	8.78 ± 0.23
Negative BHI	ND	ND	ND	ND
Negative HT2-T40	ND	ND	ND	ND
Negative HT5-T120	ND	ND	ND	ND
Negative HT15-T140	ND	ND	ND	ND
Negative BHI + HT2-T40	ND	ND	ND	ND
Negative BHI + HT5-T120	ND	ND	ND	ND
Negative BHI + HT15-T140	ND	ND	ND	ND
Extract				
HT2-T40	8.60 ± 0.34	8.75 ± 0.14	8.60 ± 0.32	8.69 ± 0.08
HT5-T120	8.73 ± 0.43	8.72 ± 0.41	8.61 ± 0.53	8.68 ± 0.47
HT15-T140	8.82 ± 0.62	8.75 ± 0.39	8.47 ± 0.32	8.81 ± 0.61

Table E2: Log CFU/mL of *Listeria innocua* replication 1, for the positive control and negative controls. No detected (ND) growth was found for the negative controls.

Extract volume	**2 µL**		**100 µL**	
Cell concentration	**10^2**	**10^4**	**10^2**	**10^4**
Controls				
Positive	8.29 ± 0.12	8.38 ± 0.41	7.97 ± 0.57	7.94 ± 0.67
Negative BHI	ND	ND	ND	ND
Negative HT2-T40	ND	ND	ND	ND
Negative HT5-T120	ND	ND	ND	ND
Negative HT15-T140	ND	ND	ND	ND
Negative BHI + HT2-T40	ND	ND	ND	ND
Negative BHI + HT5-T120	ND	ND	ND	ND
Negative BHI + HT15-T140	ND	ND	ND	ND
Extract				
HT2-T40	8.08 ± 0.51	8.03 ± 0.34	8.01 ± 0.42	8.19 ± 0.17
HT5-T120	8.13 ± 0.33	8.21 ± 0.29	6.61 ± 0.45	6.70 ± 0.45
HT15-T140	8.11 ± 0.29	7.90 ± 0.27	6.58 ± 0.57	6.45 ± 0.68

Table E3: Log CFU/mL of *Listeria innocua* replication 2, for the positive control and negative controls. No detected (ND) growth was found for the negative controls.

Extract volume	**2 µL**		**100 µL**	
Cell concentration	**10^2**	**10^4**	**10^2**	**10^4**
Controls				
Positive	7.80 ± 0.17	7.91 ± 0.30	7.88 ± 0.51	7.93 ± 0.46
Negative BHI	ND	ND	ND	ND
Negative HT2-T40	ND	ND	ND	ND
Negative HT5-T120	ND	ND	ND	ND
Negative HT15-T140	ND	ND	ND	ND
Negative BHI + HT2-T40	ND	ND	ND	ND
Negative BHI + HT5-T120	ND	ND	ND	ND
Negative BHI + HT15-T140	ND	ND	ND	ND
Extract				
HT2-T40	7.86 ± 0.40	7.81 ± 0.52	7.61 ± 0.24	7.67 ± 0.42
HT5-T120	7.79 ± 0.12	7.89 ± 0.44	6.75 ± 0.26	6.70 ± 0.16
HT15-T140	7.73 ± 0.25	8.05 ± 0.17	6.71 ± 0.38	6.60 ± 0.42

Table E4: Log CFU/mL of *Listeria innocua* replication 3, for the positive control and negative controls. No detected (ND) growth was found for the negative controls.

Extract volume	**2 µL**		**100 µL**	
Cell concentration	**10^2**	**10^4**	**10^2**	**10^4**
Controls				
Positive	8.05 ± 0.16	8.31 ± 0.21	8.07 ± 0.27	8.00 ± 0.21
Negative BHI	ND	ND	ND	ND
Negative HT2-T40	ND	ND	ND	ND
Negative HT5-T120	ND	ND	ND	ND
Negative HT15-T140	ND	ND	ND	ND
Negative BHI + HT2-T40	ND	ND	ND	ND
Negative BHI + HT5-T120	ND	ND	ND	ND
Negative BHI + HT15-T140	ND	ND	ND	ND
Extract				
HT2-T40	8.39 ± 0.48	8.07 ± 0.12	7.91 ± 0.17	7.83 ± 0.53
HT5-T120	8.05 ± 0.34	8.08 ± 0.51	6.91 ± 0.45	6.80 ± 0.12
HT15-T140	8.02 ± 0.22	8.14 ± 0.51	6.87 ± 0.33	6.78 ± 0.40

Table E5: Log CFU/mL of *Listeria monocytogenes* replication 1, for the positive control and negative controls. No detected (ND) growth was found for the negative controls.

Extract volume	**2 µL**		**100 µL**	
Cell concentration	**10^2**	**10^4**	**10^2**	**10^4**
Controls				
Positive	7.72 ± 0.19	7.73 ± 0.53	7.77 ± 0.27	7.75 ± 0.37
Negative BHI	ND	ND	ND	ND
Negative HT2-T40	ND	ND	ND	ND
Negative HT5-T120	ND	ND	ND	ND
Negative HT15-T140	ND	ND	ND	ND
Negative BHI + HT2-T40	ND	ND	ND	ND
Negative BHI + HT5-T120	ND	ND	ND	ND
Negative BHI + HT15-T140	ND	ND	ND	ND
Extract				
HT2-T40	7.56 ± 0.57	7.64 ± 0.21	7.66 ± 0.51	7.78 ± 0.54
HT5-T120	7.69 ± 0.10	7.46 ± 0.24	6.37 ± 0.18	6.50 ± 0.27
HT15-T140	7.50 ± 0.15	7.64 ± 0.57	5.74 ± 0.14	5.66 ± 0.42

Table E6: Log CFU/mL of *Listeria monocytogenes* replication 2, for the positive control and negative controls. No detected (ND) growth was found for the negative controls.

Extract volume	**2 µL**		**100 µL**	
Cell concentration	**10^2**	**10^4**	**10^2**	**10^4**
Controls				
Positive	7.71 ± 0.19	7.74 ± 0.53	7.71 ± 0.28	7.72 ± 0.78
Negative BHI	ND	ND	ND	ND
Negative HT2-T40	ND	ND	ND	ND
Negative HT5-T120	ND	ND	ND	ND
Negative HT15-T140	ND	ND	ND	ND
Negative BHI + HT2-T40	ND	ND	ND	ND
Negative BHI + HT5-T120	ND	ND	ND	ND
Negative BHI + HT15-T140	ND	ND	ND	ND
Extract				
HT2-T40	7.04 ± 0.73	7.50 ± 0.12	7.76 ± 0.27	7.62 ± 0.54
HT5-T120	7.69 ± 0.10	7.46 ± 0.24	6.37 ± 0.17	6.48 ± 0.33
HT15-T140	7.67 ± 0.16	7.54 ± 0.14	5.66 ± 0.58	5.76 ± 0.43

Appendix F

Main Effects Analysis from the Antimicrobial Inhibitory Assay

The fixed factors cell concentration ($1x10^2$ and $1x10^4$), extract volume (2 and 100 µL), and extract (HT2-T40, HT5-T120, and HT15-T140) used in the antimicrobial assay, was asses in a main effect analysis. The results from the analysis is shown in Table E1.

Table E1: Effects of the fixed factors initial cell concentration, extract volume, and extract used antimicrobial inhibition assay, given as log reduction [CFU/mL] for the three target organisms; *Pseudomonas fluoreescence* (*P. fluorescence*) (n = 4), *Listeria innocua* (*L. innocua*) (n = 12), and *Listeria monocytogenes* (*L. monocytogenes*) (n = 8).

Factor	***P. fluorescence***	***L. innocua***	***L. monocytogenes***
Cell Concentrations			
10^2 CFU/mL	-0.01 ± 0.09	0.44 ± 0.59	0.64 ± 0.80
10^4 CFU/mL	0.02 ± 0.12	0.53 ± 0.57	0.66 ± 0.76
P_C-value*	= 0.198	= 0.147	= 0.978
Extract Volume			
2 µL	0.00 ± 0.11	0.10 ± 0.23^b	0.15 ± 0.18^b
100 µL	0.01 ± 0.11	0.87 ± 0.57^a	1.13 ± 0.84^a
P_V-value*	= 0.732	< 0.001	< 0.001
Extract			
HT2-T40	-0.04 ± 0.10	0.09 ± 0.24^b	0.10 ± 0.15^c
HT5-T120	0.05 ± 0.12	0.71 ± 0.59^a	0.73 ± 0.61^b
HT15-T140	0.00 ± 0.09	0.67 ± 0.61^a	1.09 ± 0.98^c
P_E-value*	= 0.244	< 0.001	< 0.001

*General Linear Model (GLM) analyses of variance of main effects, where P_C, P_V, and P_E are the significance levels of the fixed factors Cell concentration (10^2 and 10^4 CFU/mL), Extract volume (2 and 10 µL), and Extract (HT2-T40, HT5-T120, and HT15-140). Different superscripts (^{abc}) within each column indicate significant variation ($p < 0.05$) between variables by Tukey's comparison test.

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