Distribution of Bioactive Polyphenolics and Carbohydrate Polymers in a Brown Alga: *Macrocystis pyrifera*

by

Unnati Aum Dave

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Department of Medical Biotechnology
College of medicine and Public Health
Flinders University, South Australia

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Abbreviations

°C	Degree Celsius
ALGF	Alginate Fraction
BP	Bromophenol
CPF	Crude Phenolic Fraction
2-DOG	2-Deoxy Glucose
DPPH	1,1-diphenyl-2-picryl-hydrazyl
DW	Dry weight of seaweed
EtOH	Ethanol
H_2O_2	Hydrogen peroxide
HPLC	High Performance Liquid Chromatography
MeOH	Methanol
MP	*Macrocystis Pyrifera*
M_W	Molecular Weight
PA	Phenolic Acid
PMP	1-phenyl-3-methyl-5-pyrazolone
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
RPM	Rotation Per Minute
RT	Room Temperature
SFP	Sulphated Polysaccharide
UAE	Ultrasound Assisted Extraction
Vc	Vitamin C

WSF	Water Soluble Fraction
WISF	Water Insoluble Fraction

Declaration

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma, and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis or in the notes.

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Abstract

Marine algae have served as an excellent source of bioactive compounds such as phenolics and polysaccharides, which have been utilised in complementary therapies of several diseases. Additionally, algal polysaccharides have also been used for modifying the consistency of food in food industries across the world due to their excellent rheological properties. This study aims to uncover the content of bioactive polyphenolics and polysaccharides in different tissues (leaf, stem, bladder and roots) of *Macrocystis pyrifera*, one of the most abundant brown algae in south-eastern Australia. The results will guide manufacturers to efficiently extract and utilise different bioactive compounds from *M. pyrifera,* which could enhance their applications in food and pharmaceutical industries.

In this study, one crude phenolic fraction (CPF) and three different polysaccharide fractions were extracted and separated by sequential solvent extraction. The total phenolic content of CPF was measured using Folin-Ciocalteu method, while the monosaccharide composition and carbohydrate content of all CPF and polysaccharide fractions was analysed by 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization and high-performance liquid chromatography (HPLC) method. The antioxidant activities of these fractions were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. In different algal tissues, CPF and a water insoluble polysaccharide fraction (WISF) seems to be more abundant with higher yield during extraction compared to the water soluble polysaccharide fraction (WSF) and the alginate fraction (ALGF). Furthermore, the total phenolic and carbohydrate content varies in different algal tissues, although they have very similar monosaccharide composition. Root contains the highest amount of phenolics and total carbohydrates, while bladder shows the lowest amount. This is positively correlated to the results of DPPH assays, revealing highest antioxidant activity in roots followed by leaf, stem and bladder.

In conclusion, this study was successful in fulfilling the goal of estimating the proportion of polyphenolics and polysaccharides in *M. pyrifera*. This species proves to be an excellent source of compounds with potent antioxidant activity having potential applications in pharmaceuticals and cosmetic industries. Furthermore, these properties are beneficial to health industries to develop healthy foods.

Chapter 1: Introduction and Literature Review

1.1 Introduction

Since marine organisms make up around 50% of all species worldwide, the sea provides a huge source of natural bioactive substances and new compounds with novel biological activities. (Li et al., 2011).

Marine algae or seaweeds have been reported to be good sources of secondary metabolites that are highly bioactive and could serve as useful starting points for the creation of novel functional ingredients. They are a huge and diverse collection of unicellular to multicellular, mainly self-sustaining, organisms (Wijesinghe and Jeon, 2012). Marine algae have long been used both as an alternative form of treatment and as food in Asian nations like Korea, Japan, and China. Algae are abundant suppliers of biological substances that support crucial bodily processes: Vitamins A, B, C, K and E, minerals such as magnesium, calcium, copper, iodine, iron, trace elements, carbohydrates, polyphenols, antioxidants etc. The proper functioning of the human body depends on each of these components. It is not surprising that seaweeds have been an important part of Chinese and Japanese diets since 300 BC because they are reduced in calories and fat and abundant in vitamins and minerals.

Seaweeds are typically found in the benthic littoral zone. Generally, there are three subgroups of seaweeds based on their pigments: red (Rhodophyta), brown (Phaeophyta), and green (Chlorophyta) (Otero et al., 2021). Seaweed polyphenols have drawn particular interest due to their wide range of health benefits and high concentration in different parts of the world. These compounds are among the most abundant and diverse classes of seaweed phytochemicals, which are essential to facilitate seaweeds to survive in harsh ocean environment (Otera *et al*., 2021). The other crucial bioactives in seaweeds are polysaccharides. Their importance is because majority of them are the sulphated polysaccharides (SFP), which have number of health advantages, including prebiotic effects and cholesterol metabolism (Otera *et al*., 2021).

Different brown seaweeds, including *Himanthalia elongata*, *Stypocaulon scoparium, Ascophyllum nodosum, Ecklonia stolonifera, and Fucus vesiculosus*, have been used to extract bioactive compounds (Leyton et al., 2016).

The major components of the brown seaweeds are phlorotannins, pigments, lipids, low molecular weight organic compounds, proteins, and some inorganic compunds like (mannitol, fucoidan, alginate, and laminaran are a few examples of these). Many studies concentrate on the

discovery of novel chemical entities from natural resources. Seaweeds are a viable source of materials to find new physiologically active compounds, according to recent developments in active compound investigation from natural sources. Additionally, a number of studies deal with bioactive chemicals derived from seaweeds, which has been a particularly busy research area recently (Wijesinghe and Jeon, 2012).

Alginate is usually extracted from brown seaweed as a hydrocolloid for a variety of applications, most notably as gelling agent in the cosmetics, pharmaceutical, and food processing industries. However, due to potential medicinal uses of brown seaweed, other substances like phlorotannins and fucoidan (sulfated polysaccharides) are gaining more interest. Additionally, research has been done on the possibility of fermenting mannitol and laminarin into ethanol or butanol biofuels (Zang et al., 2020).

Brown seaweeds have been investigated and have been reported to possess qualities of fertilizer, water retention property and biodegradability and could serve in horticulture as an substitute to plastic pots and artificial fertilizer (Chbani, Mawlawi and Zaouk, 2013). Industrial manufacturers employ *M. pyrifera* as one of these. Brown seaweed *M. pyrifera* grows quickly, and its high productivity helps meet the rising demand for basic materials (Zou et al., 2021). It is commonly known as giant kelp or bladder kelp and is largest of all the algae (Guiry et al., 2021).

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Figure 1.2 Diagram representing different parts (tissues) of *M. pyrifera* (Baldasarre-Keenan/slide serve/presentation).

On the extraction of bioactives from *M. pyrifera*, there is, however, little information. This brown seaweed, *M. pyrifera*, is widely distributed throughout the Pacific Ocean and is particularly prevalent in Chile's coastal regions. *M. pyrifera* is a fast-growing alga that is employed in the food and cosmetics sectors as a feed source and thickening agent. As a result, creating extraction conditions that enable the successful isolation of bioactives from *M. pyrifera* may prove to be of economic importance (Leyton et al., 2016).

Therefore, this article will mainly reveal the quantity of different polysaccharides and polyphenolic compounds in various parts of brown algae that can prove to be a good source of secondary metabolites that are highly bioactive and could serve as useful starting points for the creation of novel functional ingredients that would have applications in cosmetic, pharmaceutical and food industries.

Table 1.1. Different seaweed species and their total phenolic content (TPC) and their unit of expression (PGE: phloroglucinol equivalents) (Ford et al., 2019)

Seaweed species	Collection date and location	Extraction method	TPC and units expressed
Ascophyllum nodosum	March 2007, southwest Iceland.	7:3 (v/v) acetone/water extraction.	15.9 g PGE/100 g extract
Ascophyllum nodosum	Supplied by Portomuinos company, collected in August (no year given).	Dried in oven at 40°C, water extraction for analysis	0.96 g PGE/100 g extract
Ascophyllum nodosum	Nova Scotia, Canada between 2000 and 2003.	Methanol/water extract (1:1).	5.26% phenolic content
Fucus serratus	March 2007, southwest Iceland.	7:3 (v/v) acetone/water extraction.	24.0 g PGE/100 g extract
Fucus vesiculosus	March 2007, southwest Iceland.	7:3 (v/v) acetone/water extraction	24.2 g PGE/100 g extract
Fucus vesiculosus	Supplied by Portomuinos company, collected in August (no year given).	Dried in oven at 40°C, water extraction for analysis	1.15 g PGE/100 g extract
Fucus vesiculosus	Nova Scotia, Canada between 2000 and 2003.	Methanol/water extract (1:1).	23.21% phenolic content

1.2 Bioactive compounds from seaweeds

1.2.1 Ulvan

With repeating sequences of disaccharides like sulfated rhamnose, uronic acids (glucuronic or iduronic acids), and xylose, Ulvan has a distinctive heterogeneous makeup (Otero et al., 2021). According to Pangestuti and Kurnianto (2017), ulvan is generally made up of approximately 65% ulvanobiuronic acid and 30% uronic acids, with trace amounts of galactose, mannose, and arabinose. The two crucial repeating disaccharides are aldobiuronic acids presented as in Figure1.2.1: type A, ulvanobiuronic acid 3-sulfate (A_{3s}) and type B, ulvanobiuronic acid 3-sulfate (B_{3s}). Partially sulfated xylose residues at $O\text{-}_2$ can also occur in place of uronic acids. The sulphate percentage can vary from 3 to 8% of the dry weight, and its increase is associated with increased anticoagulant activity (Robic et al., 2009).

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Figure 1.2.1: Structure of the major repeating disaccharides in Ulva ulvan: ulvanobiuronic acids A3s and B3s and ulvanobioses U_{3s} and $U_{2's,3s}$ (Robic et al., 2009)

1.2.2 Fucoidan

Fucoidan is a kind of fucan that is found in brown macroalgae and is distinguished by having L-fucose (fucopyranose) as its primary monosaccharide component. The amount of fucose in fucoidan is typically 40% of all monosaccharides, but in certain species, it can reach 80% (Bittkau, Neupane, and Alban 2020). Depending on the type of algae, fucoidan typically has a highly diverse structure (many branches). However, the Fucales can be largely distinguished depending on their structural similarities (Otero et al., 2021). Additionally, *Sargassum stenophyllum*-produced fucoidan had a backbone made of -(1,6)-D-galactosyl and -(1,2)-D-mannosyl residues, suggesting that sulfated galactose may be another important fucoidan sugar in some species (Duarte et al. 2001).

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Figure 1.2.2: L-fucose units that are 1,3 and 1,4 linked and forming Fucoidan (Lin, Jiao and Pour, 2022)

1.2.3 Alginate

Nearly most of the brown seaweeds contain alginate as the primary component of the cell wall and cell matrix. Its nature makes it an acidic molecule. Alginate is basically made up of two linearly linked monomers: -L-guluronate (G-blocks) linked through -(1,4) linkage to -D-mannuronate (M-blocks). These monomers can be sequential (G-G-G, M-M-M) or insinuated (M-G-M) (Cardoso, Costa, and Mano, 2016). Alginates from *A. nodosum*, *M. pyrifera*, and species of the genus Laminaria have higher M-block indexes (yielding weaker gels), whereas alginates from cortex or more mature blades from *L. hyperborea* were responsible for significantly higher G-block composition (Draget 2009).

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Figure 1.2.3: molecular structure of sodium Alginate consisting of mannuronic acid and guluronic acid linked through – (1,4) linkage (Salisu et al., 2015)

1.2.4 Laminarin

Laminarins are low M_W chain-linked β-(1,3)-D-glucan storage polymers with 6-O-branching in the primary chain (Otera et al., 2021). Laminarin differs from other SFP in that it is smaller (on average) and has a polymerisation degree of 20–25 glucose moieties. Laminarin is mainly a homopolymer with two different forms of laminarin chains which can be differentiated based on the polyols present in their reducing end: M chains and G chains. While G chains have glucose as the reducing end, M chains terminate with a β-(1,3)-D mannosyl (Otera et al., 2021). Numerous techniques, such as oxidation, reduction, sulfation, and radiation, have been employed over time to alter the laminarin backbone and enhance its mechanical and physicochemical characteristics. For instance, a study reported that under the identical circumstances, sulfated laminarin had greater anticancer activity compared to plain laminarin in colorectal adenocarcinoma cells in human (Ji et al., 2013).

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Figure 1.2.4: Structure of laminarin consisting of G chain and M chain

1.2.5 Phlorotannins

Within cells, physodes—vesicles that are found both on the periplasmic membrane and in the perinuclear regions of the cell—are responsible for the production of phlorotannins. These are primary and secondary metabolites that are exclusively present in brown seaweeds and are oligomers of phloroglucinol. (Cotas et al., 2020). It is believed that the Golgi apparatus produces phloroglucinol, the monomeric unit of phlorotannins, through the acetate-malonate polyketide route (Santos et al., 2019). Six categories of phenolotannins are distinguished by the type of structural relationship they share as shown Figure 1.2.5: (1) phloretols (aryl-ether bonds), (2) fucols (aryl-aryl bonds), (3) fucophloretols (ether or phenyl linage), (4) eckols (dibenzo-1,4-dioxin linkages), (5) fuhalols (ortho/para ether bridges with an extra hydroxyl group on one unit), and (6) carmalols (dibenzodioxin moiety) (Cotas et al., 2020).

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Figure 1.2.5: Chemical structures of phlorotannins: (A) Phloroglucinol; (B) Tetrafucol A; (C) Tetraphlorethol B; (D) Fucodiphlorethol A; (E) Tetrafuhalol A; and (F) Phlorofucofuroeckol (Cotas et al., 2020)

1.2.6 Bromophenols

Bromophenols (BP) are secondary metabolites that have multiple roles such as chemical defence and prevention, and investigations have revealed a wide range of positive ecological actions. BP are found in every major algal groups; they initially evolved from the red algae *Neorhodomela larix* (previously identified as *Rhodomela larix*), and have since been observed and extracted from all taxonomic categories of marine macroalgae, including red, green, and brown algae (Cotas et al., 2020). Much less has been learned about bromophenols than phlorotannins owing to the small amount of these substances in seaweeds, resulting in lesser separation and bioactive characterisation. More research is needed to identify and characterise the category of compounds. Nonetheless, there have been some reports that link the separated molecule to bioactivities like antioxidant, anticancer, antimicrobial, and revealing their function as binding with estrogen receptor and behaving as an endocrine disruptor (Liu et al., 2011).

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Figure 1.2.6: Chemical structures of bromophenols: (A) 2,4-bromophenol; (B) 2,6-bromophenol; (C) 2,4,6-tribromophenol

1.2.7 Phenolics acids

Bioactive compounds known as phenolic acids (PAs) are involved in many processes in the body, including photosynthesis, allelopathy, enzyme function, and nutritional uptake. These are often linked to other molecules, such as simple and/or complex carbohydrates, organic acids, and and other bioactive substances like terpenoids or flavonoids (Pietta et al., 2003). These PAs consist of a solitary phenol ring and atleast one functional carboxylic acid group. Their classification is usually determined by the number of carbons in the chain that is joined to the phenolic ring. (Cotas et al., 2020). Several investigations have demonstrated the PAs' presence in seaweeds. These investigations, however, are few and focus mostly on chemical structures only. (Luna-Guevara et al., 2018).

1.2.6 Flavonoids

These are the phenolic molecules with heterocyclic oxygen bound to two aromatic rings, and their structural properties can change depending on the degree of hydrogenation (Hussain et al., 2019). More than 2000 chemicals have been identified in terrestrial vegetation, and these have been grouped into broad categories such flavones, flavanones, flavonols, anthocyanins, and isoflavones (Cotas et al., 2020). According to several studies, seaweed is a great source of catechins and other flavonoids. Numerous species of Chlorophyta, Rhodophyta, and Phaeophyceae were found to contain flavonoids, including rutin, quercetin, and hesperidin (Santos et al., 2019). The brown macroalgae *Durvillae antarctica*, *Lessonia spicata*, and *Macrocystis pyrifera* (formerly known as *Macrocystis integrifolia*) have been reported to have a significant amount of flavonoid glycosides (Santos et al., 2019). Flavonoids are also reported to play several biological functions in human body like inducing apoptosis, scavenging of free radicals, UV protection, resistance to metal toxicity, fight against microorganisms, improve glucose transport etc.

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Figure 1.2.8: Main classes of flavonoids found in algae: (A) Flavones; (B) Flavonols; (C) Flavanones; (D) Flavan-3-ol (Cotas et al., 2020)

1.3 Extraction Methodologies

There are numerous approaches that could be utilized to investigate seaweed bioactive compounds, from pre-treatment to their characterization. To determine the techniques for carrying out the extraction, isolation, and analysis of the bioactive compound bioavailability and their bioactivities, it is first essential to choose the desired seaweed species. After that, it is crucial to discover and determine the substances to be extracted—whether they are intracellular or extracellular (Cotas et al., 2020).

In a nutshell, the first step is to prepare the macroalgae, which may require washing to remove salt and contaminants, freeze-drying, or grinding to create a uniform powder. The extracts could next undergo a pretreatment to remove substances like lipids or pigments that might obstruct the active compounds extraction (Otero et al., 2021).

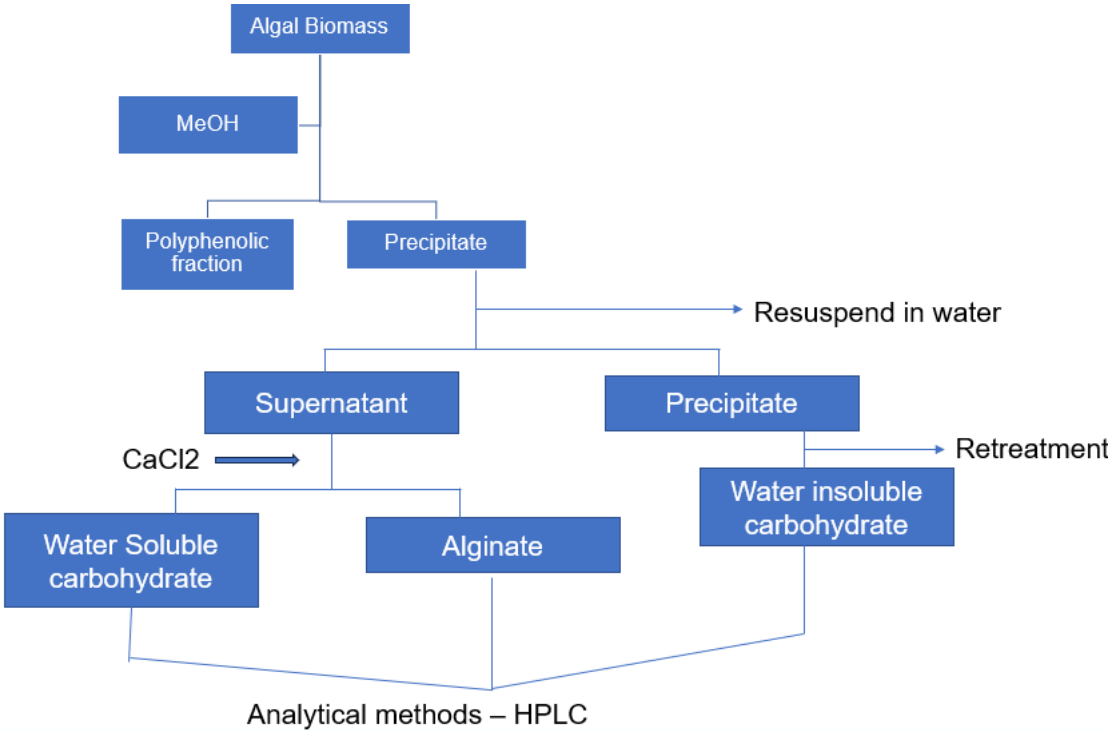

Figure 1.3: Flow chart representing the general process of extraction and quantification of seaweeds bioactives

1.3.1 Traditional Techniques

Both studies and businesses frequently employ conventional methods like heat-assisted extraction (HAE) and Soxhlet extraction. HAE is a method for extracting liquids from solids that frequently uses heat and/or agitation to increase the solubility of the desired chemicals in the solvent being used (Vaquero et al., 2020). However, Soxhlet extraction is distinguished from HAE by the continuous passage of solvent through the sample, which increases the amount extracted (Vaquero et al., 2020).

1.3.2 Innovative Methods

Microwave-aided extraction (MAE), ultrasound-assisted extraction (UAE), pressurised liquid extraction (PLE), enzyme-assisted extraction (EAE), and pulse-electric field extraction (PEF) are the most widely used approaches for SFP extraction at the moment (Otero 2021). **Microwave assisted extraction:** This method is currently regarded as one of the most effective emerging technologies. The samples are heated using microwaves, which causes the intracellular fluids to expand that cause increase in the volume and ultimately in pressure. This results in cell break down and the subsequent release of intracellular chemicals into the solvent. By using MAE instead of conventional methods, less solvent is required, and the process is more effective (Dobrinčić et al. 2020). **Ultra-sound assisted extraction:** The UAE, which relies on the

application of ultra-sound waves over 20kHz to generate bubbles and pressure gradients, is another example of cutting-edge methods for extracting SP. These bubbles expand and contract, breaking down cell walls and particles in the process, favouring contact between the sample and the solvent. Due to its ease of use, affordable machinery, and speed, UAE is seen to be a technology that is ideal for businesses of all kinds. Additionally, because it functions at low temperatures, thermo-labile chemicals can be preserved, and it can be used in conjunction with other extraction techniques, including MAE (Ciko et al. 2018; Y. Wang et al. 2021). **Enzymatic assisted extraction:** This method involves using digestive enzymes to break down the intricate cell walls of seaweeds and liberate the cytoplasmic material, increasing the pace of extraction. Proteases, such as Alcalase or Flavourzyme, and carbohydrases, such as Viscozyme or Celluclast, constitute a few of the enzymes used for SP extraction (Dobrinčić et al., 2020). Temperature, pH, solvent (water or buffer), and the ratio of enzymes/sample are crucial elements in EAE that have a significant impact on its effectiveness and must be thoroughly taken into account for an optimised extraction (Khalil et al., 2018). **Pulse-electrified field:** This extraction technique uses strong electric pulses to break the bonds between the SFP molecules that make up the cell wall, disrupting the structure of the cell. The spacing between the electrodes and intensity are the primary variables. Since there are typically no harmful solvents needed, it is regarded as a green extraction technique (Polikovsky et al., 2016).

1.4 Purification, Quantification and Characterization

After the extraction process, isolation and quantification of the target bioactives are done. Depending on the type of compound that needs to be separated, a variety of techniques could be used (Cotas et al., 2020). The origin, the extraction as well as purification methods used, the sample particle size, the conditions of preservation, as well as the existence of undesirable components in extracts such fatty acids or pigments all have an impact on the detection of bioactive compounds (Shahidi and Naczk, 2003).

The separation, purification, and characterization of a variety of bioactive compounds are all made possible by HPLC when used in conjunction with the proper detectors (Zuet al., 2019). It offers a number of benefits since it is a quick approach, necessitates a small amount of extract sample, and identifies a specific target bioactive compound (Otero at al., 2020).

Retention in reversed-phase liquid chromatography (RP-HPLC), which requires a non-polar stationary phase and a polar hydro-organic mobile phase for analysis, is influenced by the solutes' hydrophobicity, the stationary phase's hydrophobicity, and the mobile phase's polarity.

(Zuvela et al., 2019). As a result, the partitioning procedure and the compounds' adsorption are used to achieve the separation (Rafferty et al., 2007). However, RP-HPLC is typically used to identify and measure phlorotannins, with detection in the UV range of the spectrum and solvent combinations of methanol/acetonitrile and water.

Mass spectrometry and liquid or gas chromatography combined allowed for the characterisation of phenolic substances (Cotas et al., 2020). Since the 1990s, one of the most popular techniques, liquid chromatography-tandem mass spectrometry (LC-MS/MS), has significantly advanced the field of quantitative analysis because of its built-in specificity, sensitivity, and time consumption. It is now commonly known that the ideal technique for measuring compounds, small molecule medications, and other foreign substances in biological matrices (plasma, blood, serum, urine, and tissue) (Xu et al., 2007). For evaluating the several physical and chemical properties of novel chemical entities (NCEs), researchers have been using HPLC-MS for performing tests like solubility, permeability, and chemical stability. In addition, numerous drug metabolism and pharmacokinetics (DMPK) investigations are carried out as part of the process of finding novel medicines (Korfmacher, 2005).

It is possible to employ a variety of ionisation sources as the interface between the mass spectrometer and the HPLC eluant. The two most popular sources are atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI). Both of these source types are currently included as standard equipment on mass spectrometers used for LC-MS applications (Korfmacher, 2005).

Quantitative nuclear magnet resonance (qNMR) has recently demonstrated its effectiveness in identifying and quantification of metabolites (Nerantzaki et al., 2011). In general, the measurement of Fourier transformed signals and their conversion to radio-frequency impulses yield the NMR spectrum. Thus, NMR has a lower mass sensitivity than other spectroscopy techniques (Bluminch et al., 2018).

Following the application of this technique and optimised rapid solvent extraction, the phenolic profile of *Ulva intestinalis* (Chlorophyta) could be seen (Cotas et al., 2020).

Season, water temperature, light intensity, and nutrient availability all affect the concentration of polyphenols found in algae. In the temperate and tropical Atlantic regions, their content can reach a maximum of up to 20% in algal dry weight, while they contains the least levels in the tropical Pacific region (Aminina et al., 2020).

Traditional colorimetric procedures, such as the Folin-Ciocalteu, Folin-Denis, or Prussian blue assays, are used to measure phenolic content (Mekini´c et al., 2019). Folin-Ciocalteu is the test that is more frequently used to measure phenolic compounds; through a redox reaction with the reagent, phenolic compounds can be measured spectrophotometrically and quantified. However, this method's drawback is the interference of non-phenolic reducing agents (Bravo, 2009). Today, preparative chromatography methods such as column chromatography, high-pressure liquid chromatography (HPLC), or thin-layer chromatography (TLC) are used to isolate phenolic chemicals. However, these chromatographic techniques have advanced to be utilized for the isolation, purification, quantitation, separation, and identification of many phenolic substances (Mekini´c et al., 2019).

Carotenoids, polysaccharides, vitamins, and their precursors, as well as polyphenols, which help to prevent oxidation processes, are responsible for the antioxidant activity of seaweeds (Kumar, Tarafdar and Badgujar 2021). Because of their potential to prevent several diseases linked to oxidative stress, which happens when there is imbalance between antioxidants and reactive oxygen species (ROS) due to either depletion of antioxidants or accumulation of ROS, antioxidants are appealing as supplements (Corsetto et al., 2020).

Antioxidants are thought to be chemicals that, at relatively low concentrations, have the ability to postpone or stop the oxidation of a specific substrate. They function as "free radical scavengers" by preventing and repairing oxidative stress-related damage. While the advantages of antioxidants from terrestrial plants are widely acknowledged, the advantages of antioxidants from seaweed have received less attention. Seaweed cultivation, which has been practised for decades and is expanding quickly worldwide, especially in China, has shown effective pollutant remediation and water quality improvement (Liu and Sun 2020). The antioxidant activity present in natural sources is assessed using a variety of in-vitro techniques. The radical scavenging ability of antioxidants is assessed by scavenging assays using 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid radical. People frequently utilise the thiobarbituric acid reactive substances tests and the -carotene-linoleate model systems to evaluate the antioxidant property of food subtances and organic molecules containing lipids. The ferric antioxidant power reduction and cupric ion reducing antioxidant capacity assays are used to measure the reducing ability of antioxidants. These tests are frequently used to find potential sources of antioxidants (Khan et al., 2020)

1.5 Potential applications of extracted bioactives

Since bioactive compounds in seaweeds are underexplored compared to that of terrestrial plants, additional investigation is needed to expand the biological library and increase the likelihood of finding novel substances with potential applications in various fields or businesses. As a result, this field is still developing from isolation to application (Cotas et al., 2020).

1.5.1 Food Industry

As a safe and organic food stabiliser and preservative, these chemicals' antioxidant abilities may be of interest to the food industry. But it is important to remember that oxidised phenolic compounds can combine with amino acids to generate complexes that are insoluble which might cause blockage of proteolytic enzymes and alleviate the nutritional value of a food item (Wong and Cheung, 2001). However, the limitations on the use of artificial substances in the food business may mark a turning point for the utilization of marine bioactives as secure substitutes (Freile-Pelegrín and Robledo, 2013). Their antioxidant properties are helpful in the dietary supplement industry, not only as nutraceutical molecules for functional food items, in which they are highly advantageous in enhancing health (as food supplements), but also for boosting its lifespan period when utilised in food processing (functional foods) (Wang et al., 2010).

In terms of cancer, diabetes, arthritis, neurological diseases, obesity, osteoporosis, liver disease, and cardiovascular disease, brown seaweeds have been the subject of the most studies. There haven't been many clinical trials, but one was done to see how osteoarthritis patients responded to a seaweed extract formulation made from *Fucus vesiculosis, Macrocystis pyrifera,* and *Laminaria japonica*. The mixture, according to the stud lessen osteoarthritis symptoms in a dose-dependent manner (Lomartire and Goncalves, 2022).

1.5.2 Cosmetic Industry

Certain seaweed extracts which include corresponding phenolic substances, particularly phlorotannins, are currently used in cosmetics like skin-care and anti-aging products. Seaweeds have long been employed for this goal; for example, an extract from the seaweed *Saccharina japonica* (formerly known as *Laminaria japonica*) is employed in cosmetics and has anti-inflammatory, antioxidant, and anti-microbial properties (Cotas et al., 2020). The company Natural Solution creates two licenced seaweed extracts with phenolic compounds as their main active components.

Recent studies strongly support the inclusion of polysaccharide as active components in cosmetic compositions. Polysaccharides serve a variety of cosmetic products purposes, including

thickening, moisturising, emulsifying, and conditioning hair. Ulvan from green seaweeds, fucoidan, alginate, and laminarin from brown seaweeds, agar, and carrageenan from red seaweeds are significant polysaccharides. Agar and alginate are commonly utilized as thickening and gelling agent in the food industires (Jesumani et al., 2019).

1.5.3 Pharmaceutical applications

In traditional healthcare, seaweeds have been utilised for generations as a typical treatment of variety of illnesses (Jerald, Joshi and Jain, 2008). The Celtic, British, and Roman people who lived close to the ocean employed them for wound healing, as vermifuges, or as anthelmintics for a thousand years, so the contemporary hunt in pharmaceutical and biomedical fields is growing and in constant progression (Smit, 2004). Phenolic chemicals are being studied for their potential use in improving or curing some of the most prevalent illnesses in the world today, including cardiovascular, diabetic, neurodegenerative, and mental disorders (Audibert et el., 2010).

Numerous studies have demonstrated that fucoidan suppresses the invasion, metastasis, and angiogenesis of cancer cells as well as inducing death in a variety of cancer cell types. According to reports, kelp polysaccharides have regulatory effects that include reducing systemic inflammation by reducing the recruitment of inflammatory cells to the liver and the expression of inflammatory mediators. Purified kelp polysaccharides exhibit considerable antioxidant, 1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging, and antibacterial action (Zhong et al., 2020).

1.5.4 Animal Healthcare

It has been proven that the polyphenolic chemicals in seaweed are accessible to animals through the colon (Keyrouz et al., 2011). The phenolic substances can be ingested either in their original form directly in the upper digestive tract or following being modified by microbes in the digestive tracts in the lower intestine (Chojnacka et al., 2011 and Galleano et al., 2010). Phlorotannins may be present in the diets of pigs and chickens only at tiny amounts, typically up to 5-6% and not exceeding 10 % in developing livestock in order to avoid over dose which may lead to adverse effects in animal health. Phlorotannins act as prebiotics in both grazing and monogastric breeds and the recommended amount is administered at a level under 5% in the animal diet (Cotas et al. 2020).

Beyond providing essential nutrients, in vitro research and some animal research have demonstrated the health benefits of seaweed; however, several of these investigations used

incorrect biomarkers to validate their conclusions and did not conduct well-designed experiments to evaluate efficiency. Some seaweed components are attractive as functional food ingredients; however, additional evidence from animal nutritional studies, including mechanistic evidence, is needed to evaluate the nutritional benefit, the efficacy of claimed bioactivities, and any potential drawbacks (Morais et al., 2020).

1.5.5 Agriculture

Due to its anti-microbial action, the seaweed phenolic compounds found in some commercialised seaweed extracts may provide defence against plant illnesses (Wijesinghe et al., 2012). Due to the understanding of phlorotannins, this topic is now being developed, and brown seaweeds are typically used as the primary research material. A registered invention currently exists for the commercialisation of a solution containing phenolic chemicals as a stimulating agent for mycorrhizal and rhizobial symbiosis, which can occur through fertilisation or by treatment which is based on the Fucus and Ascophyllum genera (Cotas et al., 2020).

The seaweeds polysaccharides are frequently referred to as moisture-holding hydrogels in agriculture for improving soil water retention, which is an essential soil characteristic. In agricultural areas, super-absorbents were studied and created to improve the abiotic properties of soil. They enhance plant performance, soil aeration, soil permeability, water retention capacity, and water consumption effectiveness. They can also lessen the frequency of irrigation and the compaction shift, stop erosion and water drainage, and slow down fertiliser dissolution (Mamede et al., 2023).

1.6 Aims and Hypothesis

1.6.1 Aims

- To analyse the composition of different phenolic compounds and polysaccharides in various parts of a brown alga: *Macrocystis pyrifera*, and quantify them through analytical techniques
- To evaluate the antioxidant property of the phenolics and water-soluble extracts
- To evaluate viscosity of the water-soluble fraction and gel properties of alginate fraction from different tissues

1.6.2 Hypothesis

This work will provide an understanding into the marine polyphenolic and polysaccharide compounds, giving crucial details on their current and potential futuristics applications in variety of sectors such as food and nutrition, biomedical and pharmaceutical, agriculture and cosmeceuticals etc. Moreover, this study will give a comprehensive profile on the content of bioactive compounds present in different tissues of *M.pyrifera* which will further provide important information on the cost-effective methods used for their extraction and separation methodology, highlighting the possible uses of these bioactives in different businesses or the possibilities of designing new products.

Chapter 2: Material and Methods

Chemicals

Folin-Ciocalteu's phenol reagent, α, α-Diphenyl-β-Picrylhydrazyl (DPPH), Sulphuric acid, Sodium carbonate, Phloroglucinol, PMP (1-phenyl-3-methyl-5-pyrazolone), Methanol, n-Hexane, Calcium chloride, Formic acid, Ammonium acetate, Di-butyl ether, Ammonium hydroxide, 2DOG (2-deoxy glucose) aqueous as internal standard, external standards with low medium and high concentration, Sodium hydroxide, Hydrogen peroxide, Hydrochloric acid, Dialysis membrane, MilliQ water, Distilled water.

2.1 Collection and pre-treatment of Different Parts of *M. pyrifera*

Different parts of the seaweed like the leaves, stem, bladder and roots (i.e Blade, Stipe, Gas bladder and Holdfast) were cut and segregated. They were then washed several times with water to get rid of stones, sand, epiphytes, or other impurities and kept in -80 °C overnight. They were later subjected to freeze drying to get the dried algal biomass and then taken to beading mill to obtain powdered form of different parts.

2.2 Extraction and Purification

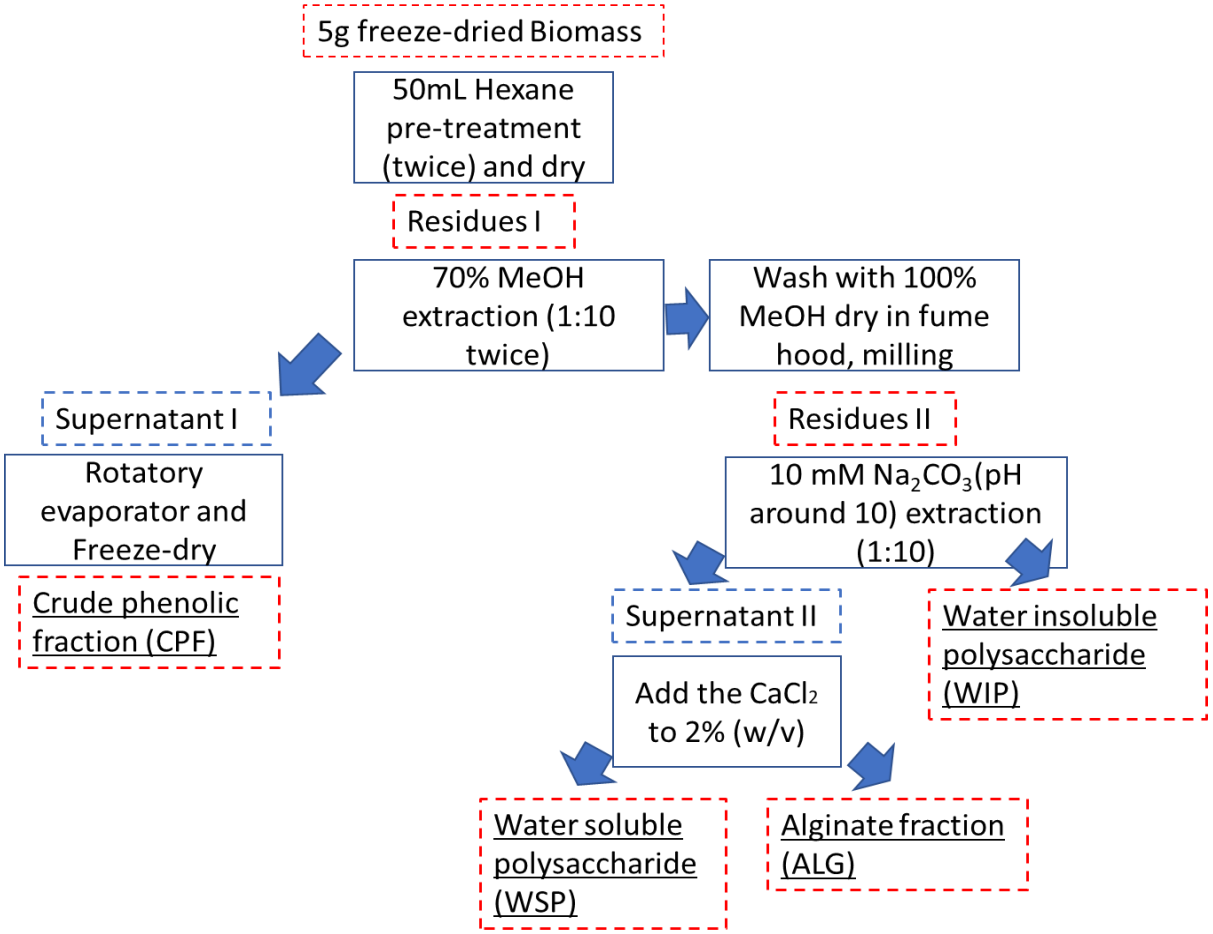

Figure 2.2 Layout of the process followed for extraction and purification of different fractions of leaf, stem, bladder and root.

Approximately 5g freeze-dried biomass of each part (Leaf, Stem, Bladder and Root) was mixed with 50mL n-hexane and kept under stirring for an hour, twice at RT and then allowed to dry completely in fume hood overnight. The Residue-1 was weighted and mixed with 70% methanol in the ratio of 1:10 and kept for stirring for an hour at RT. It was then centrifuged for 10 minutes at 10,000 RPM. The Supernatant-1 was separated. This process was repeated, and Supernatant-2 was collected in a separate tube. Both these supernatants were placed in vacuum evaporator and then freeze dried to obtain the Crude Phenolic Fractions (CPF).

The leftover residue was washed with 100% methanol three times and then allowed to dry in a fume hood overnight. Next day, the residue was mixed with 10mM sodium carbonate (pH approx.10) in the ratio of 1:40 and kept under stirring at 80°C for 2 hours. The contents were then centrifuged for 10 minutes at 10,000 RPM. The supernatant was kept aside, and the residue

was washed with water until pH 7 was reached. It was later freeze dried to obtain the Water Insoluble Polysaccharide/Fraction (WISP/WISF).

The supernatant was mixed with 2%w/v calcium chloride and centrifuged for 10 minutes at 10,000 RPM. The resulting supernatant was subjected to dialysis through 3.5K MWCO 35 mM dry I.D tube for 2 days. Later on, it was freeze dried to obtain Water Soluble Polysaccharide/Fraction (WSP/WSF).

The leftover residue was washed with 1mM HCl three times and then with water 2 times before being freeze dried to obtain the Alginate fraction (ALGF).

At the end of the extraction and purification process, four fractions (CPF, WISF, ALGF and WSF) of each i.e. leaf, stem, bladder and root were obtained.

In this process of extraction, the solvents were selected based on their physicochemical properties and their capability to dissolve compounds. For eg. Phenolic compounds are more soluble in organic solvents such as ethanol, methanol, acetone and their mixtures with water. It also depends on the polarity of the solvents used in the process. Sodium carbonate was used as it allows the separation of WISP from alginate.

2.3 Total phenolic content assay

A stock solution of phloroglucinol (1mg/mL) was prepared in water. A serial dilution was performed to six different concentrations: 0, 20, 50, 80, 100 and 150 µg/mL. 12 mg of freeze dried CPF of all four parts were dissolved in 1mL of water. These were centrifuged for 10 minutes at 10,000 RPM and the supernatants were used further in the next steps.

Folin-Ciocalteu's phenol reagent was prepared by diluting 500 µL of reagent with 1 mL of water. In 96 well plate, 25 µL of all six standard solutions and four supernatants of the samples were mixed with 25 µL of Folin's reagent in triplicates. 200 µL of water was added in each well and the mixture was incubated at RT for 5 minutes. Finally, 25 µL of 10%w/w sodium carbonate was added and the plate was incubated at RT for 60 minutes. The absorbance was measured using a plate reader at 765nm. Absorbance of the six standard concentrations was used to construct the standard curve. The results were expressed in mass percentage (%).

The following equations were used for calculating the mass percentage of phenolic content in seaweed.

Equation 1

Phenolic content in solution µg/mL = (Absorbance at 765 nm + Constant C)/ Slope

Equation 2

Phenolic content in biomass µg/mg = Phenolic content in solution/weight of biomass

Equation 3

Mass Percentage = (Phenolic content in biomass/1000) *100

2.4 Antioxidant assay for crude phenolic fractions

A stock solution of vitamin C (1mg/10mL) was prepared in methanol**.** A serial dilution was performed for seven different concentrations: 0, 10, 20, 30, 40, 50 and 60 µg/mL. 12 mg of freeze dried CPF of all four parts were dissolved in 1mL of 50 % (v/v) methanol.

1mM DPPH solution was prepared in 80% (v/v) methanol.

In 96 well plate, 10 µL of all seven standards and four samples were mixed with 190 µL of DPPH solution in triplicates. The plate was incubated at RT for 30 minutes. The absorbance was measured in plate reader at 517 nm. Absorbance of the seven standard concentrations was used to construct the standard curve. The results were expressed in mg vitamin C equivalent (VCE)/g dry biomass.

The following equations were used for calculating the mg vitamin C equivalent (VCE)/g dry biomass.

Equation 1

Vitamin C µg/mL = (Absorbance at 517 nm - Constant C)/ Slope

Equation 2

Vitamin C mg/g = Vitamin C µg/mL/weight of biomass

2.5 Monosaccharide analysis

Approximately 10 mg of crude biomass, CPF, WSF, ALGF and WISF of all the four parts of seaweeds were weighted in eppendorf tubes, labelled and subjected to two different methods of hydrolysis before running them on HPLC.

Method-1 (General Acid Hydrolysis)

In this method, approximate 10 mg of crude biomass and WISF of all the 4 parts of seaweeds were mixed with 1M sulphuric acid. The tubes were vortexed and capped with tube lock. They were then placed in oven at 100°C for 3 hours. Allowed to cool and centrifuged at 10,000 RPM for 10 minutes. Then, 20 µL of hydrolysate was diluted with 380 µL in separate eppendorf tubes which were labelled according to the fractions. These diluted hydrolysates were then used as samples for HPLC analysis.

Method-2 (Two Step Hydrolysis)

In this method, approximately 10 mg of crude biomass, CPF, WSF, ALGF and WISF of all the 4 parts of seaweeds were mixed with 13.4 M sulphuric acid. The tubes were vortexed and incubated for 3 hours at RT. After this, 925 µL of water was added in each tube to make the final acid concentration to 1 M. These were vortexed and capped with a tube lock. They were then placed in oven at 100ºC for 3 hours, allowed to cool and centrifuged at 10,000 RPM for 10 minutes. Finally, 20 µL of hydrolysate was diluted with 380 µL in separate eppendorf tubes which were labelled according to the fractions. These diluted hydrolysates were then used as samples for HPLC analysis.

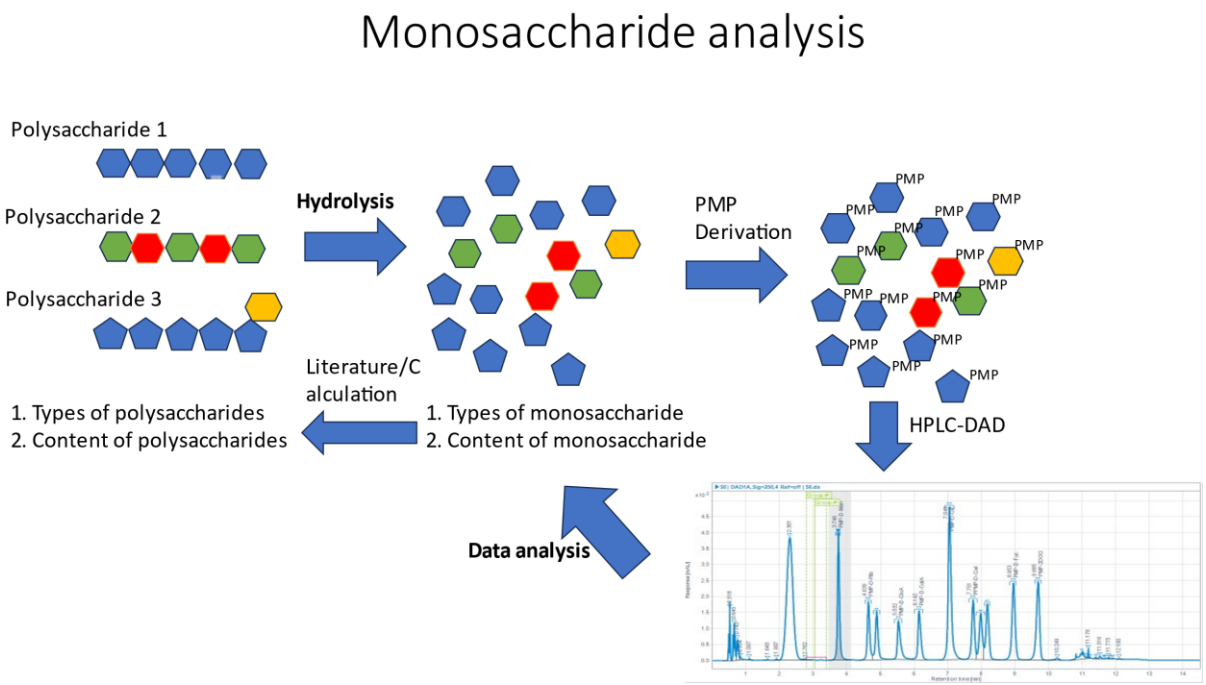

Figure 2.5.1 Schematic diagram representing hydrolysis of polysaccharide and detection through HPLC analysis.

Method for HPLC analysis after hydrolysis

Six 1.5 mL Eppendorf tubes were labelled S1 to S6 (for external standards: S1 and S2 for low, S3 and S4 for medium and S5 and S6 for high concentration of standard). For samples (diluted hydrolysate), as many tubes as required were labelled according to the fractions of each tissue which previously underwent general acid hydrolysis and 2 step hydrolysis.

10µL of 0.5mM 2-deoxy glucose was added to each tube. 2- deoxy glucose, which is not expected to occur in biological samples, served as an internal standard.10µL of the low

concentration standard was transferred into tubes S1 & S2, 10µL of the medium concentration into tubes S3 & S4, and 10µL of the high concentration into tubes S5 & S6.

Similarly, 10µl of each hydrolysed diluted sample was transferred into the labelled tubes.

Preparation of PMP / ammonia reagent: 20uL 0.5 M PMP in methanol plus 18.5µL 1M ammonium hydroxide was needed per sample. For example, for 30 samples: 0.6mL PMP in methanol, 0.55mL 1M ammonium hydroxide was prepared.

38.5µL of PMP/ammonia reagent was added into each tube. They were then vortexed and pulse spinned, cap locked and placed in the oven at 70ºC for 1 hour.

The tubes were allowed to cool and pulse spinned to bring sample to the bottom of the tube.

10µL of 10M formic acid was added. 1mL di-butyl ether was also added. The tubes were shaken vigorously for 1 minute, Pulse centrifuged and using a gas-tight 1or 2mL syringe, the di-butyl ether was removed. This extraction was repeated. The tubes were placed in centrifugal evaporator to remove any remaining di-butyl ether. Centrifuged at 20800 rpm for 5 minutes.

30µL of these standards and samples were placed into the HPLC vials and 10µL of each sample was injected following HPLC instructions. (Experimental conditions for HPLC analysis: Instrument: LC-DAD-FLD-FC, temperature of auto-sampler maintained samples at 10°C for stability, column: Phenomenex Kinetex C18 2.6 µm 3 x 100 mm 100A). The resulting peaks were observed, and the chromatograms were processed (Comino et al., 2013).

2.6 Cellulose Extraction from WISF

Approximately 1 g of WISF of all four parts were treated with 50 mL of 5% w/w NaOH solution for 12 hours under vigorous stirring. This suspension was washed with water three times. The leftover residue was treated with 7% v/v HCl aqueous solution for 12 hours again under vigorous stirring. After washing it with distilled water, the treated sample was dispersed in a 5% w/w NaOH solution for 12 h. The treated sample was dispersed in a 5 % w/w NaOH solution for12 hours. The pigments were then removed using 4%v/v H_2O_2 for 6 hours at 80°C, followed by washing with distilled water and freeze drying to obtain cellulose (Gao et al., 2018). The resulting cellulose was white powder, and the percentage yield was then calculated.

2.7 Rheological characterization of water-soluble fraction and alginate fractions

The WSF and ALGF of all the four parts were weighted and a solution of each with the concentration of 1mg/mL for alginate fractions and 20 mg/mL for water soluble fraction was prepared in milliQ water and stirred until completely dissolved. The tests were performed using a rheometer AR-2000 (TA Instrument, Great Britain, Ltd) fitted with a 600 mm 2° steel cone-

plate geometry (54 μm gap) equipped with a Peltier heating system for accurate control. Steady-shear flow properties of the prepared solutions were examined at 25°C using the cone-plate geometry over the range of shear rate from 1 to 1000 s taking 10 points per decade with a measurement time of 1 minute. All the measurements were performed in duplicate and the values of effectively overlapping traces were reported (Hetati 2020).

Chapter 3: Results

3.1 Extraction yield

The conventional extraction method with hexane, methanol, sodium carbonate and calcium carbonate was used for extracting different fractions which gave approximately 1-4% yield for WSF, 3-4% yield for ALG fraction, 20-52% yield for WISF and 12 -38% yield for CPF, (Dry weight of freeze dried extract). The detailed illustration of yield data for all the fractions is shown in Figure 3.1.

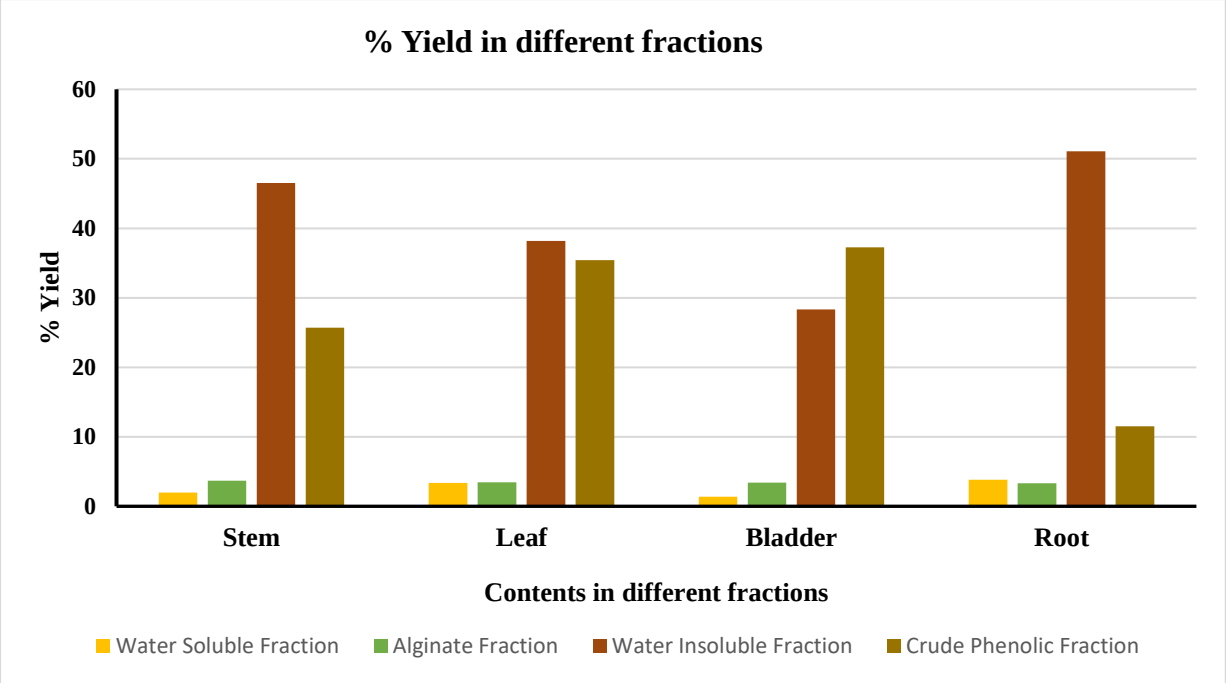

Figure 3.1 Yield of different phenolic and polysaccharide fractions from different algal tissues

3.2 Total phenolic content assay for crude phenolic fractions

From the results, the total phenolic content varied widely in all the four parts of seaweed, ranging from 0.4 to 9.0% with milli-Q water. The root had the highest phenolic content of 8.7% of dry weight of seaweed, followed by stem 1.2 %, leaf 1.1 % and bladder 0.4 %.

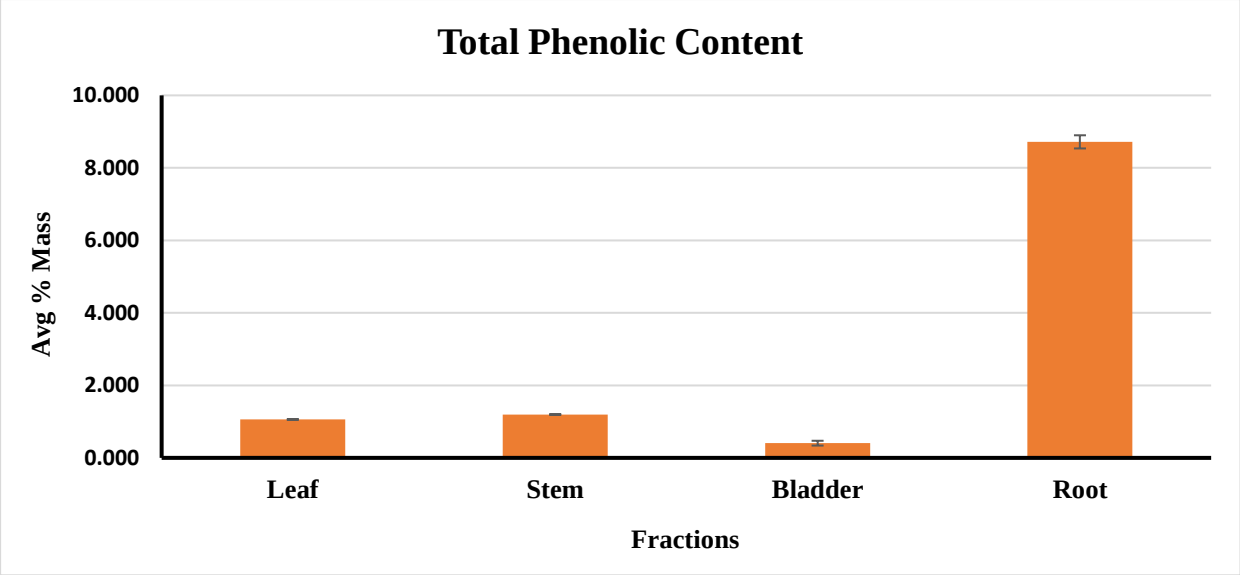

Figure 3.2 Average mass percentage of different parts of seaweed representing total phenolic content in each. The experiment was carried out using three technical replicates of each tissue used.

3.3 Antioxidant assay for Crude Phenolic Fractions

The antioxidant activity of the crude phenolic fractions of all the four parts was measured using ascorbic acid as an antioxidant standard. From the results it can be observed that the antioxidant activity ranged from 1 to 74 % with 80% methanol. Like phenolic content assay, this test also revealed the highest antioxidant activity in roots which was 73.70 mg Vitamin C equivalent dry weight of seaweed, followed by stem 8.76 mg, leaf 5.19 mg and bladder 1.70 mg.

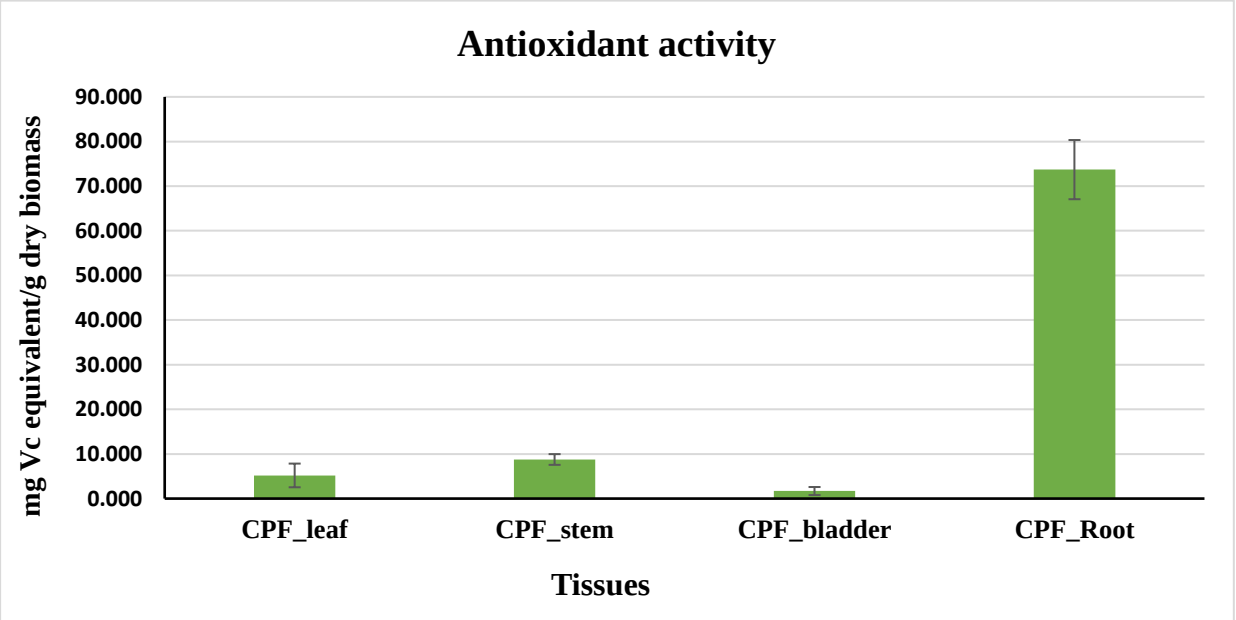

Figure 3.3 µg Vit C equivalent/mg dry biomass of different parts of seaweed representing antioxidant activity in each. The experiment was carried out using three technical replicates of each tissue used.

3.4 Monosaccharide Analysis

3.4.1 Method-1 (General acid hydrolysis)

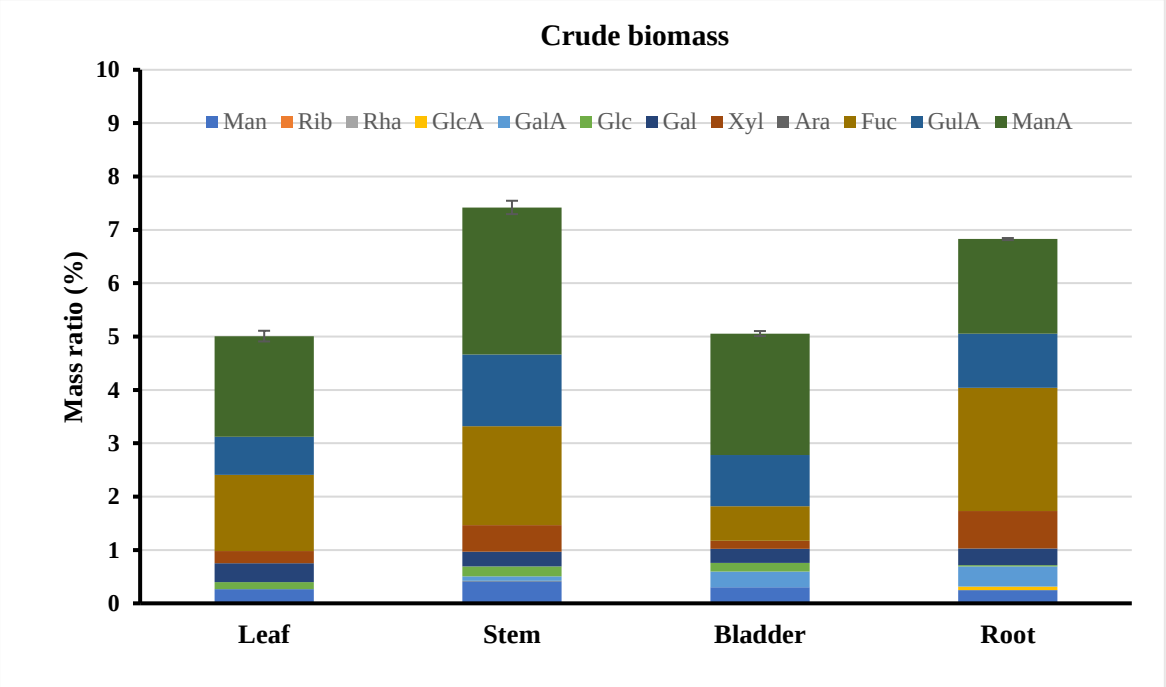

Figure 3.4.1.1 Monosaccharides present in crude biomass (dry powder) of each tissue estimated through PMP derivatization method in HPLC. The experiment was carried out using two technical replicates of each tissue used.

As observed, when mild acidic condition (1M H_2SO_4) was used for hydrolysis, the major proportion of monosaccharides were guluronic acid, mannuronic acid and fucose. However, the other polysaccharides were also hydrolysed but were in very low concentration. The highest amount was found in stem, followed by root. Leaf and bladder had almost similar content of monosaccharides.

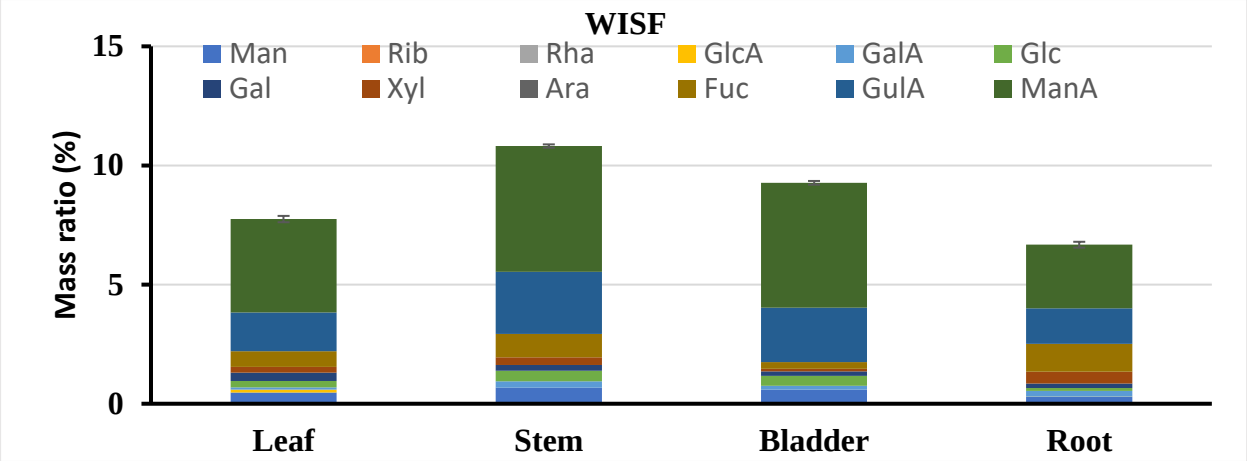

Figure 3.4.1.2 Monosaccharides present in WISF of each tissue estimated through PMP derivatization method in HPLC. The experiment was carried out using three technical replicates of each tissue used.

Under mild acidic condition for WISF, almost similar results as crude biomass was observed. Not all the polysaccharides were hydrolysed but the proportion of different monosaccharide was similar in different tissues analysed. The major concentration was for guluronic acid and mannuronic acid.

3.4.2 Method-2 (Two-step hydrolysis)

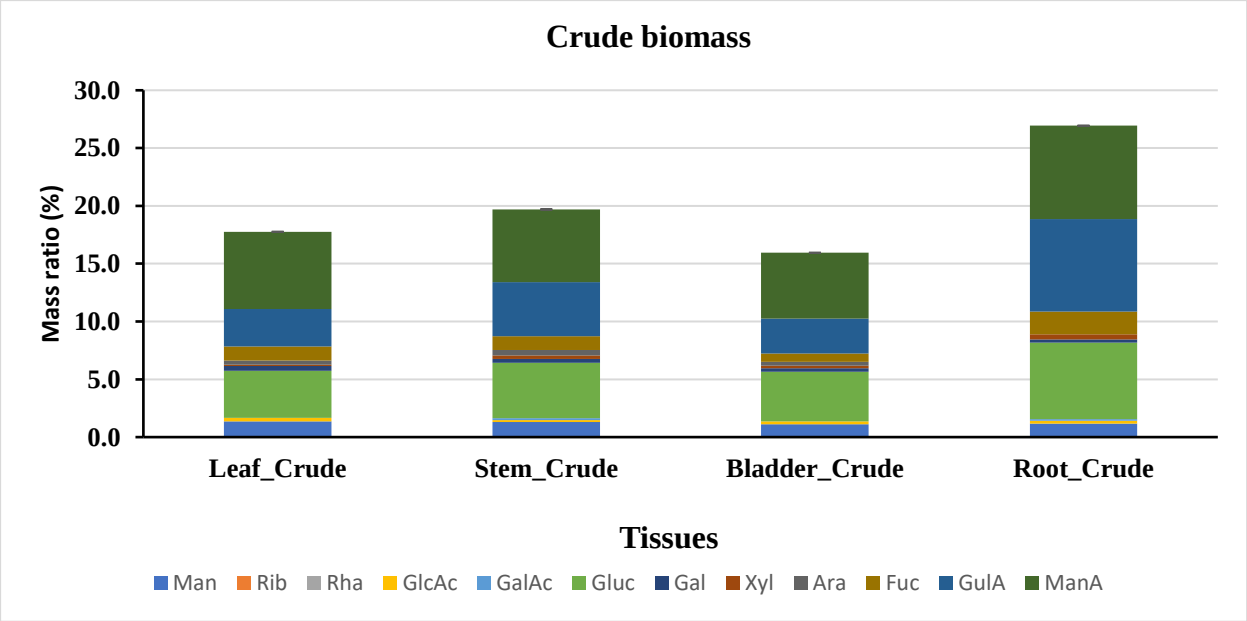

Figure 3.4.2.1 Monosaccharides present in crude biomass of each tissue estimated through PMP derivatization method in HPLC. The experiment was carried out using two technical replicates of each tissue used.

When high concentration of sulphuric acid was utilized (13.4 M H_2SO_4), the concentration of monosaccharide obtained was increased in comparison to the results from method-1 (general acid hydrolysis), which concludes that two step hydrolysis method is more efficient than general acid

hydrolysis. The concentration of monosaccharides obtained was similar in all the tissues analysed. However, unlike general acid hydrolysis, the root showed the highest proportion of monosaccharides followed by stem, leaf and bladder.

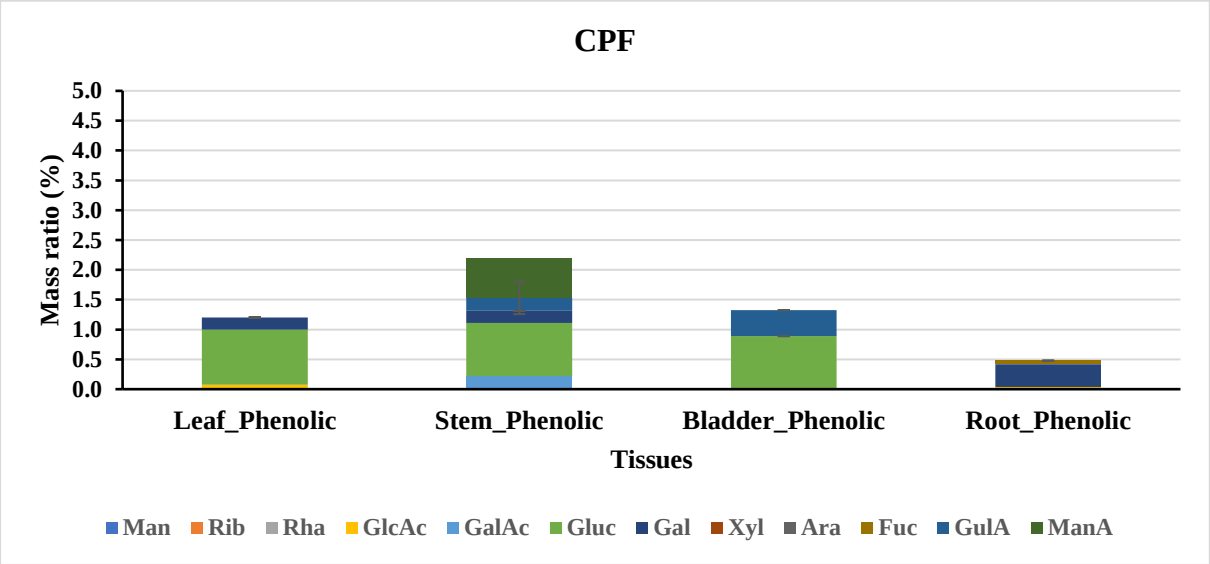

Figure 3.4.2.2 Monosaccharides present in crude phenolic fraction of each tissue estimated through PMP derivatization method in HPLC. The experiment was carried out using two technical replicates of each tissue used.

When CPF was treated with high acidic condition, it showed results as expected. Very low concentration of carbohydrates was observed, showing the presence of mainly glucose. The highest amount present was in stem followed by other tissues and the least was found in root.

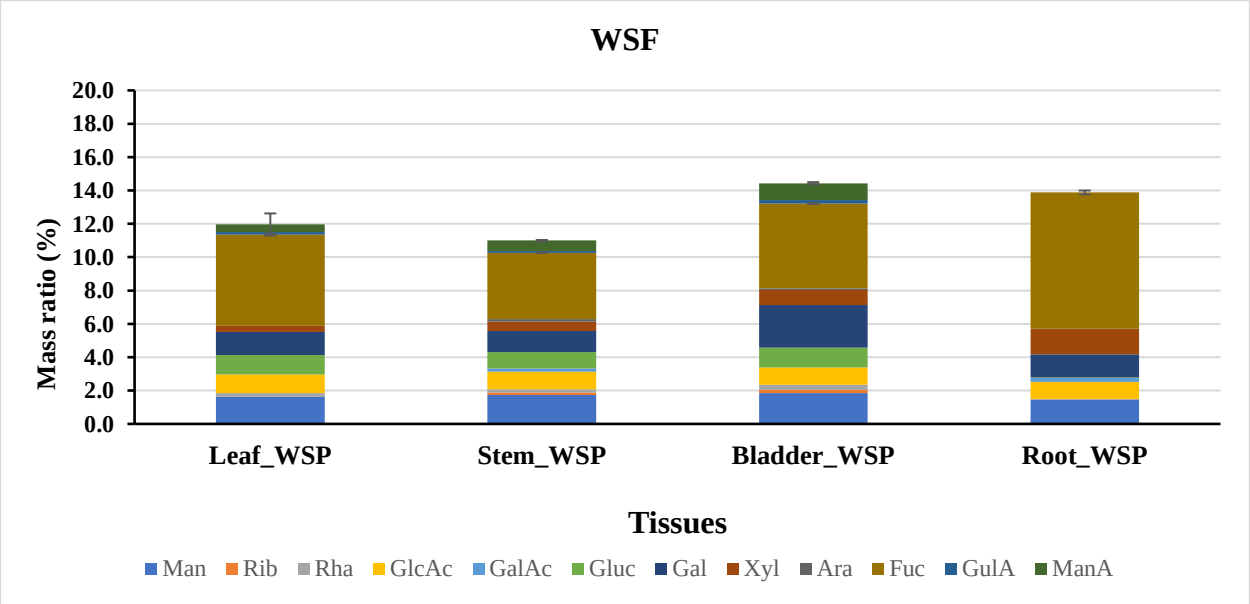

Figure 3.4.2.3 Monosaccharides present in WSF of each tissue estimated through PMP derivatization method in HPLC. The experiment was carried out using two technical replicates of each tissue used.

Under concentrated acidic condition, WSF showed high proportion of fucose which may have come from hydrolysis of fucoidan. Though other monosaccharides were also present but were in very low amount. The highest concentration of fucose was observed in roots followed by leaf, bladder and stem. Hence, root was found to be good source of fucoidan.

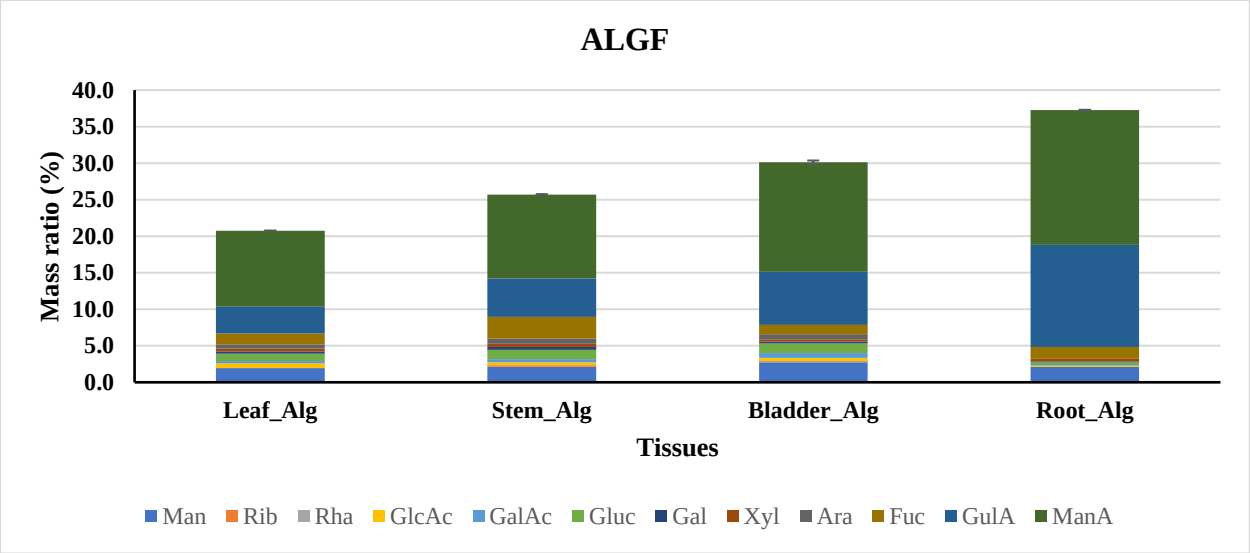

Figure 3.4.2.4 Monosaccharides present in ALGF of each tissue estimated through PMP derivatization method in HPLC. The experiment was carried out using two technical replicates of each tissue used.

During extraction process, Alginic acid gets converted to sodium potassium, calcium salts and its derivative which is known as algin and is commercially available as sodium alginate. Sodium alginate is copolymer of mannuronic acid and guluronic acid connected by β-1,4 glycosidic bonds. When alginate rich fractions treated with concentrated acid gave results as expected. A portion of guluronic acid and mannuronic acid was observed in all the tissues analyzed. The highest amount being observed in roots, followed by bladder, stem and leaf.

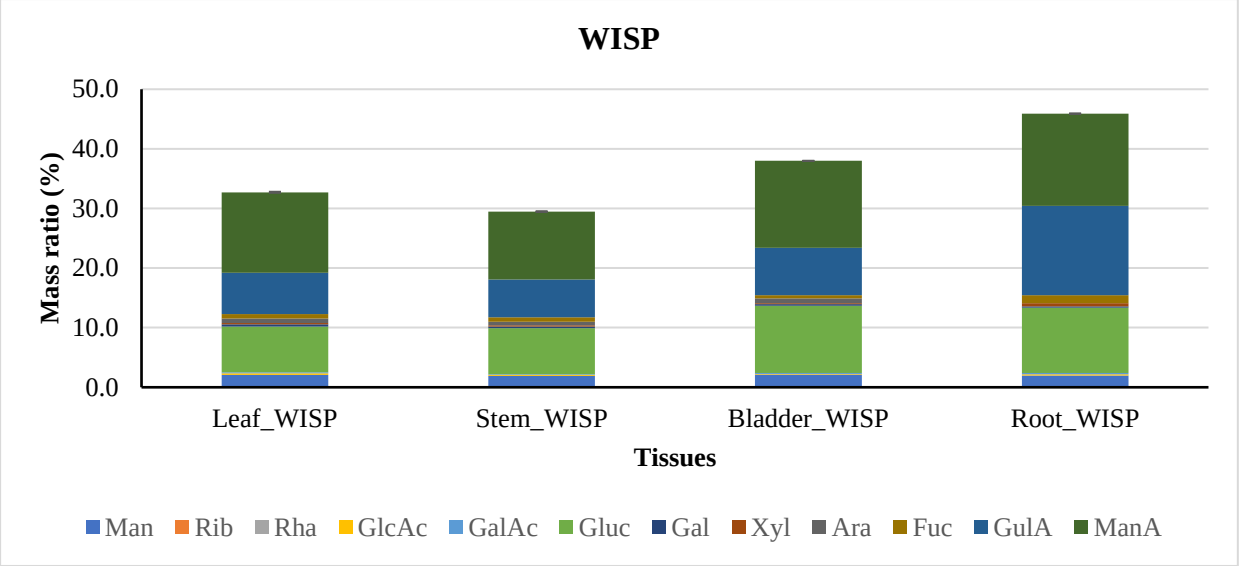

Figure 3.4.2.5 Monosaccharides present in WISF of each tissue estimated through PMP derivatization method in HPLC. The experiment was carried out using two technical replicates of each tissue used.

Under concentrated acidic conditions, WISF showed higher amount of monosaccharide in method-2 as compared to results obtained from method 2. One of the monosaccharides with high amount was glucose whose concentration was much more than the one in general acid hydrolysis process. This glucose can be estimated to have come from cellulose. The other important sugars observed were the guluronic acid and mannuronic acid. The other sugars were present in very less concentration.

3.5 Extraction yield of cellulose from WISF

The conventional extraction method with sodium hydroxide, distilled water, HCl and H_2O_2 was used for extracting cellulose from different tissues (WISF) which gave %yield ranging from approximately 7-10%. The percentage yield was calculated as per yield/1 g of dry weight of seaweed. The detailed illustration of yield data for all the fractions is shown in Figure 3.5.

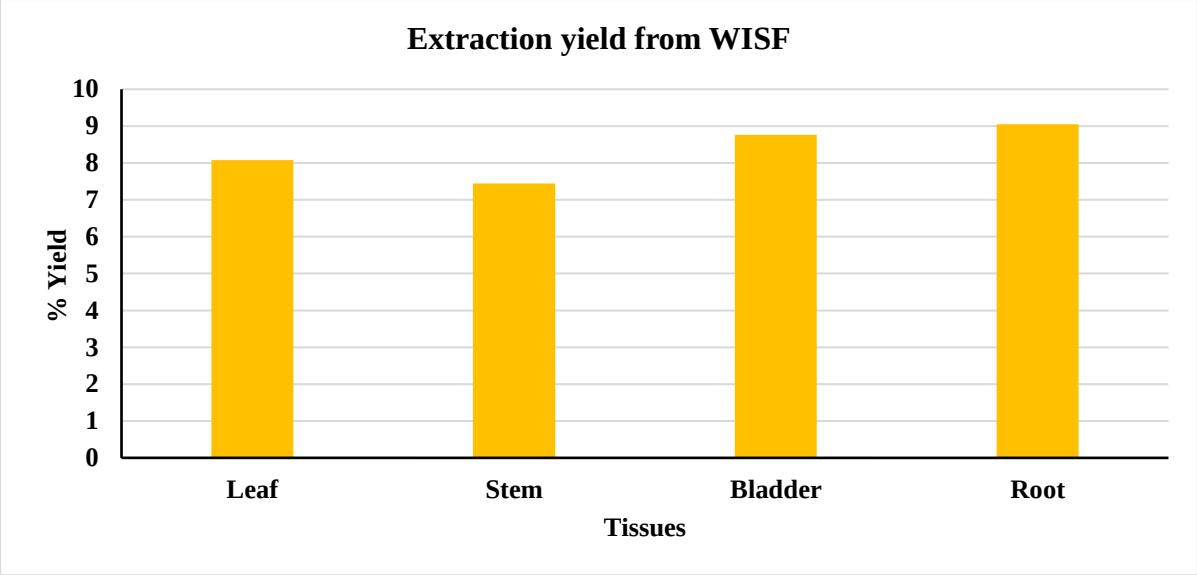

Figure 3.5 % yield of cellulose from WISF of Leaf, Stem, Bladder and Root

3.6 Rheological characterization of water-soluble fraction and alginate fractions

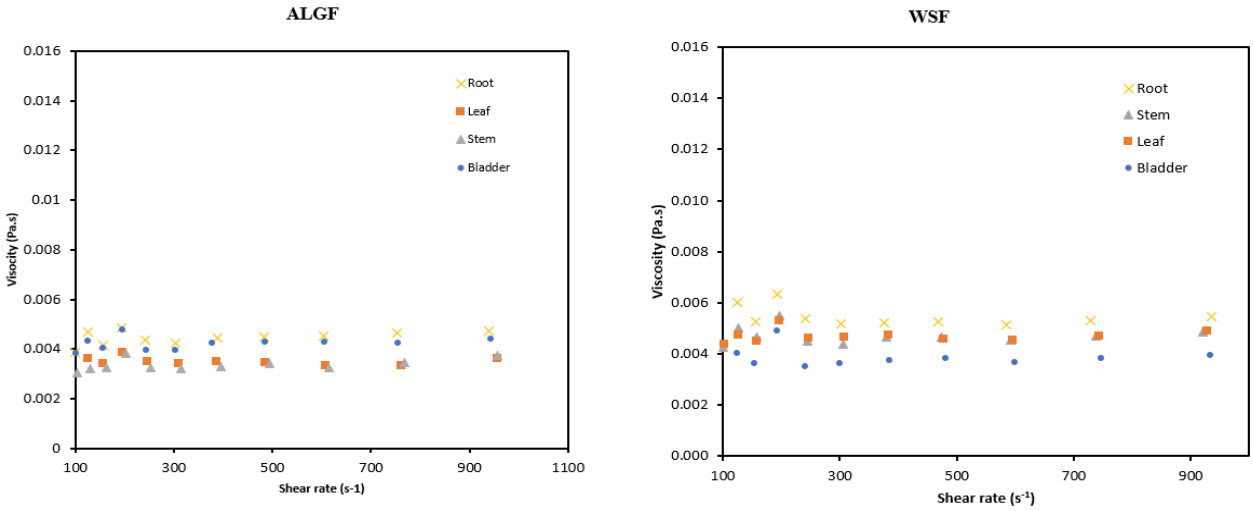

Figure 3.6 Flow curve of WSF and ALGF solution extracted from leaf, stem, bladder and root. The experiment was carried out using two technical replicates of each tissue used.

As shown in Figure 3.6, the viscosity of ALGF and WSF show a small variation depending on where they are extracted from. The viscosity of WSF extracted from root shows highest viscosity among that extracted from stem, bladder or leaf. It is the same case for ALGF fraction from root which shows highest viscosity compared to other ALGF extracted from other tissues. Different viscosity of WSF and ALGF from different algal tissues may indicate distinct molecular weight or structures of these polysaccharides, which would be helpful to develop tailored viscosity modifying agent for food industry.

Chapter 4 Discussion

4.1 Extraction yield

The extraction method used showed varying percentage yield for various tissues. The extraction yield ranged from 1-52% for different fractions in different tissues. As presented in Figure 3.1, the WISF and CPF were found to be higher in concentration as compared to WSF and ALGF. The WISF was highest in roots with % yield of 51.1% followed by leaf, stem and bladder. Bladder had the most CPF, followed by stem, leaf and root. The root also showed elevated concentration of WSF which was 3.8% followed by stem, bladder and leaf. The ALGFs were almost similar in all the tissues. However, the most was present in leaf, while the stem, bladder and roots contained more or less the same amount. As per the previous studies, the yield of brown seaweed polysaccharides ranged from 5.9 to 12.5%. The highest yield of polysaccharide (12.30%) was obtained from *Sargassum hemiphyllum* (Chen et al., 2021).

4.2 Total phenolic content assay for crude phenolic fractions

Due to their antioxidant property, the phenolic compounds may help the algae combat oxidative stress and perform a possible adaptive role in defence against grazers such marine herbivores (Altena & Steinberg, 1992). The phenolic content was calculated as average mass percentage measured by Folin-ciocalteau method. The calibration curve of phloroglucinol, obtained by representing the absorbance measurements versus the concentration of phloroglucinol, was adjusted to a linear equation $y = 0.0047x - 0.0122$ with a coefficient of correlation of $R^2 = 0.9962$. Each point of the calibration curve is the average of three absorbance measurements. As presented in Figure 3.2, the variation of phenolic content was quite large. the highest amount of total phenolic content was found to be in roots which is 8.7%. However, the other tissues contained much low amount in comparison to roots. The lowest concentration was found in bladder which was 0.4%. While the leaf and the stem has almost same amount of phenolic compounds as 1.1 % and 1.2% respectively. According to the previous study, the phenolic content of the brown seaweed *Hormophysa cuneiformis* was 1.43% (i.e. 14.60 mg PGE/ g dried sample) (Ahmad et al., 2016). The solubility of the phenolic compounds affects the solvents that are chosen for extraction. Commonly, phenolic compounds shows better solubility in organic solvents than water (Waterman and Mole, 1994). Thus, aqueous solutions of methanol was utilised solvent in this study. It is also thought that the location of seaweeds affects the amount of phenols they contain; seaweeds in the highest intertidal zone are subject to higher UV

radiation and desiccation levels, and as a defence against these stresses, the seaweeds create more phenol (Connan, Deslandes, Ar Gall, 2007).

4.3 Antioxidant assay for Crude Phenolic Fractions

The preferred solvent for antioxidant assay was 80% methanol as it facilitates dissolving phenolic compounds and ascorbic acid was used as an antioxidant standard. A free radical scavenger may easily eliminate DPPH since it contains a nitrogen free radical. (Singh & Rajini, 2004). A compound's capacity to couple with a radical's unpaired electron determines its capacity to scavenge DPPH radicals (Park, Shahidi, Jeon, 2004). In this study, a correlation was found between the total phenolic content and the antioxidant activity. When the total phenolic content was high, the antioxidant activity was also high in a particular tissue as presented in Figure 3.3. Hence, as the roots showed the highest phenolic content, the antioxidant activity showed was also highest in them. It followed the same trend for stem, leaf and bladder. However, this assay was not specific to any particular antioxidant. The antioxidant activity was calculated as µg Vit C equivalent/mg dry biomass by DPPH assay method. The calibration curve of ascorbic acid, obtained by representing the absorbance measurements versus the concentration of ascorbic acid, was adjusted to a linear equation $y = 0.0034x + 0.0093$ with a coefficient of correlation of $R^2 = 0.9681$. Each point of the calibration curve is the average of three absorbance measurements. Previous studies showed lower antioxidant activity of 39.62, 9.79 and 9.65 mg ascorbic acid equivalent/g extract (or 0.31, 0.08 and 0.17 mg ascorbic acid equivalent/g of seaweed on dry weight basis) in ethanolic fraction of *Sargassum marginatum*, Dichloromethane fraction of *Padina tetrastomatica* and aqueous fraction of *Turbinaria conoides*, respectively (Yuan, Bone, & Carrington, 2005). It is possible that additional compounds in the crude (methanolic) extract are interfering with the higher activity in the fractions. Additionally, it has been shown that the extraction solvents have a significant impact on the chemical species (Yuan, Bone, & Carrington, 2005).

4.4 Monosaccharide Analysis

Monosaccharide analysis when carried out with general acid hydrolysis showed that not much of the polysaccharides were hydrolysed. The amount of monosaccharide was very when compared to the results obtained from two step hydrolysis. In general hydrolysis method for crude biomass, stem showed slightly higher carbohydrate content as shown in Figure 3.4.1.1 as compared to other tissues while in two step hydrolysis, roots showed high concentration of carbohydrates as compared to other tissues as shown in Figure 3.4.2.1. When the results of

WISF with general acid hydrolysis method were observed, stem showed slightly higher amount of monosaccharide as shown in Figure 3.4.1.2. When WISF was treated with two step hydrolysis, roots showed much high concentration of monosaccharides as compared to other tissues as shown in Figure 3.4.2.5 and followed the same trend as crude biomass. Based on this observation, it was concluded that two step hydrolysis method was more efficient than general acid hydrolysis. Hence, two step hydrolysis was used for the rest of the fractions of different tissues. From the data of WISF of all the four tissues (Figure 3.4.2.5), it can be inferred that the higher concentration of glucose may have come from the hydrolysis of cellulose, as cellulose is hydrolysed at extreme acidic condition which was fulfilled in two step hydrolysis method. The results from previous study carried out for brown seaweed polysaccharide analysis mainly consist of mannosyl, glucurosyl, glucosyl, galactosyl, and fucosyl, with small amounts of rhamnosyl and arabinosyl (Chen et al., 2021) and these mono sugars are also observed in this study.

The crude phenolic fraction when treated with two step hydrolysis, showed very low carbohydrate content as expected. A very low amount of glucosyl residue was observed as presented in Figure 3.4.2.2. Water soluble fraction when treated with two step hydrolysis, showed high proportion of fucose as shown in Figure 3.4.2.3. It can thus be said that this fucose may have come from hydrolysis of polysaccharide fucoidan. Though other monosaccharides were also observed but were in very negligible amount. Hence, it can be said that WSF of roots can be a good source of fucose.

When ALGF underwent two step hydrolysis process and analysed in HPLC showed that the maximum amount of alginate was present in root, followed by bladder, stem and leaf. Poly-GG, poly-MG, and poly-MM fragments are randomly arranged polymers composed of two monomers, mannuronic acid and guluronic acid, linked by β-1,4 glycosidic bonds, to form alginates. In exceptionally mild circumstances, sodium alginate can rapidly form gel, and this gel-forming ability is directly correlated with the G and M content. When there is Ca2+ present, the G unit reacts with the cation and builds up to create a connected network structure, which causes hydrogel formation (Zhong et al., 2020). In its native state, it connects with different cations in seawater to create a range of alginates that reside in the cell wall, act as reinforcement, and enhance the mechanical properties of the tissues (Zhong et al., 2020). When the ratio of G:M was calculated based on the results obtained, it was, leaf 1:2.8, Stem 1:2.2, Bladder 1:2.2 and Root 1:1.3. The results from the previous study on brown seaweed showed G:M ratio of

Sargassum fluitans 1:1.2, *Sargassum latifolium* 1:0.8, *Sargassum dentifolium* 1:0.5 (Fenoradosoa et al., 2010) which is slightly lower in comparison to the tissues analysed in this study.

4.5 Extraction yield of cellulose from WISF

The WISF of all four tissues were treated with NaOH, HCl, NaOH and H_2O_2 successively to remove protein, fat, pigment, inorganic salts and other impurities. The resulting algal cellulose was a white powder. Figure 3.5 represents the percent yield of cellulose obtained from each tissue. As observed, root had the highest amount of cellulose (9.05%). The proportion present in leaf (8.08%) and bladder (8.87%) was almost similar followed by stem (7.44%) which had the lowest amount. The results from a previous study on brown seaweed *Saccharina japonica* showed cellulose extraction yield from the kelp as 9.86±0.20% on a dry kelp basis which was nearly same with the results obtained in this study (He et al., 2018). Brown algae, has a lot of potential as a food additive where they might be utilised to change the rheological behaviour of food as a thickening, stabilising, or gelling agent (Paximada et al., 2016). In industrial applications, brown algae serve as the main raw material for the manufacturing of alginate, and after alginate extraction, a sizable amount of residue is always discarded. Therefore, it makes sense to use leftover brown algae and increase its worth.

4.6 Rheological characterization of water-soluble fraction and alginate fractions

The viscous characteristics at 25 °C of aqueous ALGF and WSF were investigated using steady-shear flow test over the shear rate ranging from 1 to 1000 s^{-1} and the flow curve was plotted against the viscosity and the shear rate scale that covered 10 points. Various flow behaviours were observed due to various relationships between viscosity and shear rate. The viscosity of WSF extracted from root showed highest viscosity in comparison to that extracted from stem, bladder or leaf. The same was observed for ALGF from root which showed highest viscosity compared to other ALGF extracted from other tissues as presented in Figure 3.6 which means that the WSFs and ALGFs from roots have encountered more resistance as compared to that extracted from other tissuesConcentration, molecular weight, sulphates, branching, pH, and temperature all affect fucoidan's viscosity. As per the previous study carried out on *Saccharina longicruris*, *Ascophyllum nodosum* and *Fucus vesiculosus*, *S. longicruris* had the lowest viscosity (Rioux et al., 2007). These results might be connected to a lower fucoidan molecular weight (44.5 kDa). The molecular weight of fucoidan from *A. nodosum* is 1323 kDa, and that from *F. vesiculosus* has a molecular weight of around 877 kDa, which is higher than what was

expected. Numerous factors, including molecular weight, concentration, composition, M/G ratio, ionic force, ions, pH, and temperature, may impact alginate viscosity (Rioux et al., 2007).

Chapter 5 Conclusion and Future Perspectives

The work presented in this thesis illustrates the extraction yield, total phenolic content, monosaccharide profiling, antioxidant activity, cellulose extraction and rheological behaviour of different fractions of different tissues i.e leaf, stem, bladder and root. The results of total phenolic contents revealed similar proportion of polysaccharide and phenolic content in all the tissues. The study revealed that higher the phenolic content, higher is the antioxidant activity. Hence, roots were found having highest antioxidant activity. Therefore, roots can be considered as an excellent source of phenolic compounds to manufacture dermatological products and also for therapeutics, cosmetics and food industries.

The WSF of root showed higher proportions of fucoidans as compared to other tissues. Fucoidans possess potential medicinal qualities, such as anti-inflammatory and anticoagulant actions, along with anti-cancer cell proliferation effects. Using cell viability analysis, fucoidan polysaccharides have demonstrated in vitro their direct anti-proliferative effects against specific tumor cells. Due to fucoidans' bioactive qualities, academic institutions and commercial businesses are now conducting extensive research into the possible uses of fucoidan molecules in the food ingredients and cosmetics industries. However, the presence of other polysaccharides, like alginate, makes it difficult to produce pure fucoidan products. Fucoidans are easily added to lotions, creams, and other beauty products because they dissolve in water (Ale and Meyer, 2013).

ALGF of leaf showed the least alginate content compared to other three tissues. The difference in the GM ratios may contribute to the mechanical properties of leaf, stem, bladder and root. Alginic acid and its derivatives have recently been found to have good biocompatibility, antioxidant activity, and anti-inflammatory activity. Currently, they are primarily used in cosmetics, and a variety of anti-aging, moisturising, and UV protection products are available. The recent and significant potential for application in drug delivery systems will guide the course of further research (Zhong et al., 2020).

The WISF when analysed showed that root and bladder can be potentially a good source of algal cellulose. Nanocellulose, a promising nanomaterial with unique features, can be created from cellulose, a significant component of the cell walls of lignocellulosic plants, seaweeds, microalgae, and bacteria. Given the structural variety of macro- and microalgal nanocelluloses, there are opportunities to combine low-impact biomass production with unique, environmentally

friendly processing to produce useful, long-lasting nanomaterials for a variety of uses, from innovative wound dressings to organic solar cells (Ross et al., 2021).

Further studies are suggested to systematically evaluate and identify specific phenolic compounds present in tissues and further characterize bioactive compounds responsible for the antioxidant activities so that they may be added in industrial formulation. Furthermore, the extracted WSF or fucoidan must be tested for toxicity tests on skin models to find safe dosage and possible side effects on human body. Cellulose nanocrystals or cellulose microfibres will be prepared from WISF and use them for material preparation. To conclude, this project has contributed to better understanding and new knowledge of brown seaweed Macrocystis Pyrifera in terms of creating a bioactives profile of the alga which can prove to be an outstanding source of polyphenols and polysaccharide with potential applications in pharmaceutical, food and cosmetic industries.

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