Fucose-containing sulfated polysaccharides from brown seaweed: Extraction technology and bioactivity assessment

Ale, Marcel Tutor

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Marcel Tutor Ale
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Marcel Tutor Ale
PhD Thesis
2012
Preace

Enormous amounts of seaweed resource still remain unexploited. Examining new applications of this unexploited seaweed by developing state-of-the-art solutions; and innovation of seaweed products are the primary interest and motivation of this PhD study.

The PhD work was initiated at the Department of System Biology, Technical University of Denmark (DTU) and was completed at Center for Bioprocess Engineering (BioEng), Department of Chemical and Biochemical Engineering System Biology, Technical University of Denmark (DTU), Lyngby Denmark. The main research and experimental activities were performed in BioEng laboratory facilities located at Bldg. 227 DTU Søltoft Plads, Kgs. Lyngby. In fulfillment of the PhD programme various courses, seminars, conference participations and research activities were taken both locally and internationally. External research activity was also accomplished at School of Allied Health Sciences, Kitasato University, Sagamihara, Kanagawa, Japan.

In this PhD thesis, published works are cited and the chapters are based on published research and scientific papers completed by the author during the PhD study:

Chapter 2 is an introduction to the state-of-the-art FCSP methods. It also outlines the problems, hypotheses, and core objectives of this PhD study. This chapter provides an overall outline of different scientific investigations and research activities in the form of project phases.

Chapter 2 is based on Paper 1, which highlights FCSP structure-function relationships and extraction methods. It also includes an overview of seaweed potentials based on recent development and reports from published journals.

Chapter 3 is about optimized single-step extraction of FCSPs based on Paper 2. This chapter provides an in-depth investigation of the influence of different extraction parameters on the chemical nature and structural features of FCSPs.

Chapter 4 shows the FCSP bioactivity. The anti-proliferative and immune response activity of fucoidan extracted from Sargassum sp. using minimal processes based on Papers 3 and 4 is also presented.

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Chapter 5 is based on Paper 5. Successful exploitation of seaweed for commercial applications is accomplished provided that growth parameters are optimized; hence, growth and nutrient assimilation monitoring of seaweed has also been the subject of this study using *U. lactuca* seaweed as a model.

Chapter 6 contains the final remarks about the present work. It also gives some future perspectives and prospective research areas.

This whole PhD study was undertaken with superior guidance and supervision of Prof. Anne S. Meyer, head of Center for Bioprocess Engineering and tireless encouragement of Prof. Jørn Dalgaard Mikkelsen, Center for Bioprocess Engineering as co-supervisor. Moreover, the diligent assistance and supervision of Dr. Hiroko Maruyama and Dr. Hidezaku Tamauchi during my research work in Kitasato University, Kanagawa Japan was so beneficial for the advancement of this PhD study.

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This thesis is submitted for the fulfillment of the PhD degree requirements at Technical University of Denmark.

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Marcel Tutor Ale  
Technical University of Denmark (DTU)  
February 2012
Abstract

Marine seaweed that is washed up on the coastline is a nuisance as its degradation produces a foul smell and generates waste problems. Exploitation of coastline-polluting seaweeds such as Sargassum sp., Ulva sp., and other beach-cast seaweed species for various commercial applications will generate new valuable products that may help lessen coastal pollution by seaweeds and create new seaweed-based resources. Thus, utilization of these natural resources is of great importance. The objectives of this PhD study were to develop a technology to extract bioactive compounds from nuisance brown seaweeds, and investigate their bioactivity. To this effect, designed optimized extraction of fucose-containing sulfated polysaccharides (FCSPs) and/or crude fucoidan from brown seaweed were performed, and the bioactivity of the isolated FCSPs was investigated. Moreover, to assess the potential of seaweed to assimilate nutrient-based nutrients, a technology for accurate monitoring of differential seaweed growth responses to nutrient assimilation was also developed.

Fucoidan is a term used to describe a class of sulfated polysaccharides extracted from brown seaweed, which contains substantial amounts of fucose; varying amounts of galactose, xylose, and glucuronic acid; and differing glycosidic linkages, and are variously substituted with sulfate and acetyl groups and side branches containing fucose or other glycosyl units. These FCSPs principally consist of a backbone of (1→3)-and/or (1→4)-linked α-L-fucopyranose residues that may be substituted with sulfate (SO₃⁻) on C-2, C-3, or C-4 and acetyl groups at C-4 on the main chain or may have short fucoidside chains that are usually linked from the O-4 of one or several of the fucopyranosyl backbone residues. FCSPs are known to exhibit crucial biological activities including anti-tumor activity. Although differently extracted, purified, fucose-rich, modified fucoidans have been reported to exert bioactive properties such as anti-coagulant and enhance immune response activity, few studies have investigated the bioactivity of unfractionated FCSPs, notably FCSPs extracted using milder and fewer processing steps. Crude fucoidan from Sargassum sp. and Fucus vesiculosus were examined for their bioactivity against lung and skin cancer cell lines in both in vitro and in vivo studies. This study showed that unfractionated FCSPs hinder the in vitro proliferation of Lewis lung carcinoma and melanoma B16 cell lines by induction of apoptosis. Moreover, the anti-tumor activity of crude fucoidan seems to be associated with an enhanced immune response as depicted by an increase in natural killer cell activity in mice.
The classical extraction of FCSPs involving long, repetitive, multi-step acid and alkaline treatments is detrimental to its structural properties, yield, and compositional attributes. In this study, statistically designed, optimized extraction of a single-step extraction of FCSPs from Sargassum sp. was carried out. The effects of the different extraction parameters on the natural chemical composition of the isolated sulfated polysaccharides were also investigated. The data showed that classical multi-step extraction using ≥0.2 M HCl at elevated temperature and extended time had a detrimental effect on the FCSPs yield, as this treatment apparently disrupted the structural integrity of the polymer and evidently degraded carbohydrate chains of sucrose residues during extraction. The results also revealed a maximal FCSPs yield of approximately 7% dry weight with Sargassum sp. using 0.03 M HCl at 90°C and 4-h extraction conditions.

Accurate monitoring of the differential growth response of seaweed to different nutrient assimilation is crucial to explore various applications of seaweed resources, such as biomass for bioenergy production and source of functional healthy components and bioactive compounds. A major prerequisite for the successful exploitation of cultivated seaweed like Ulva lactuca for commercial purposes is that the growth rate and yields should be optimized. In this study, the growth response of U. lactuca to ammonium and nitrate assimilation was investigated using a photoscanning technique to monitor the growth kinetics in U. lactuca. Photoscanning images revealed differential increases in the surface area of U. lactuca discs over time in response to different nitrogen-based nutrient sources. The results also showed a favorable growth response to ammonium as a nitrogen source, and the presence of ammonium discriminated the nitrate uptake by U. lactuca upon exposure to ammonium nitrate. This study exhibits the applicability of a photoscanning approach for acquiring precise quantitative growth data for U. lactuca.

In conclusion, we demonstrated that nuisance seaweed can be a potential source of biomass and bioactive compounds. This study proved the hypotheses that different extraction conditions have crucial influenced to the chemical nature of FCSPs. The study also demonstrated that unfractonated FCSPs are able to exert bioactive actions such as anti-tumor and immune-modulating properties in both in vitro and in vivo studies. This study illustrates the importance of a precise monitoring technique of the growth of U. lactuca in order to successfully exploit it for commercial application.

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Dansk Sammenfatning

Tang på stranden er en plage, først og fremmest på grund af de lugtgener, som kommer når tangen går i bølgelande. En udnyttelse af dette tang, såsom Sargassum sp., Ulva sp., og andre lignende kyst-nære typer af tang, kan fremsætte helt nye værdifulde produkter og måske samtidig mindske de uønskede lugtgener fra tang som skylter op på stranden. Udnyttelse af tang som ressource er udgangspunktet for dette PhD studium. PhD studiets formål har været at udvikle en ektrakrationsteknik til at isolere bioaktive produkter fra brun tang, dels at undersøge disse produkters bioaktivitet. For at opfylde dette formål blev der i PhD arbejdet udviklet en statistisk designet, optimiseret ektrakrationsteknik til ektrakration af såkaldte fukose-indeholdende sulfaterede polysakkærrer (eng. fucose-containing sulfated polysaccharides), forkortet FCSPs, henholdsvis grov fucoidan fra brun tang, primært Sargassum sp., og bioaktiviteten af ektrakurationsprodukter blev undersøgt. For ydermere at vurdere tangs evne til at assimilere, og dermed vokse på nitrogen-holdige salte, blev der udviklet en teknologi til at monitorere differential vækst af Ulvo lactuca, på forskellige næringsstoffer i vandet.

Fucoidan er en betegnelse, som dækker over en gruppe af sulfaterede, fukose-holdige, polysakkærrer fra tang. Udover fukose indeholder fucoidan forskellige mængder galactose, xylose, alginuronsyre, som er forbundet via forskellige typer glykosydbindinger, og som derudover er substitueret i forskellig grad med sulfat og acetyl-grupper og som kan have sidekæder indeholdende fukose eller andre glykosyl-substituenter. Disse FCSPs består principielt af en rågang, eller en hovedkæde, af (1→3)-og/(1→4)-linkede α-L-fucopyranose enheder, som kan være substitueret med sulfater (SO3) på C-2, C-3, eller C-4 foruden acetyl grupper på C-4 på fukose-enhenderne i hovedkæden, og/eller som har korte fukose-kader, der normalt er bundet via O-4 fra en eller flere fukose-enheder i hovedkæden. Det er kendt, at FCSPs isoleret fra tang har forskellige gnævende, bioaktive effekter, herunder anti-tumor aktivitet. Selvom det har været rapporteret, at oprensede, fucose-rige, modificerede fucoidan-prøver har bioaktive effekter, såsom anti-koagulerende, og immun-respons foregående egenskaber, er bioaktiviteten af mere grove, ufarkrationer FCSPs – ekstraeret med mindre og færre ekstraktionstrin - ikke undersøgt.

Groft oprensede fucoidan-prøver fra Sargassum sp. og Fucus vesiculosus blev i dette PhD arbejde undersøgt for deres bioaktivitet mod lunge- og hudcancer cellinlinjer vækst både in vitro og in vivo.
Arbejdet viste, at ufractionerede FCSPs ekstrakter hindrer proliferation in vitro af Lewis lung carcinoma og melanoma B16 celle linjer via induktion af apoptosis. Desuden blev det vist, at denne anti-tumor aktivitet af grov fucoidan, tilsvarende er associeret med et øget immunrespons, målt som et forøget niveau af naturlige "killer" cells' aktivitet i mus.

Klassisk ekstraktion af FCSPs fra tang involverer adskillige langsommelige, behandlinger med syre og base, hvilket er ødelæggende for deres specifikke struktur og sammensætning. I dette PhD arbejde blev der udviklet en statistisk designet, optimeret enkelt-trins ekstraktionsmetode til at udtrække FCSPs fra Sargassum sp.. Effekten af de forskellige ekstraktionsparametre på sammensætningen af de ekstraherede polysaccharider blev også vurderet. Resultaterne viste, at klassisk, multi-trins ekstraktion ved brug af 20.2 M HCl ved høj temperature havde en ødelæggende effekt på udbyttet af FCSPs og viste desuden at en sådan behandling tilsvarende ødelagte polysaccharidstrukturen og at fukose-kæderne blev nedbrudt under behandlingen. Resultaterne viste også, at et maximal FCSPS udbytte, på ca. 7% af tørveægten for Sargassum sp. kunne opnås ved et-trins ekstraktion med 0.03 M HCl ved 90°C i 4 timer.


Den samlede konklusion er således at vi demonstrerede, at tang kan være en potential kilde til biomasse og bioaktive komponenter, især FCSPs. PhD studiet viste desuden at hypotesen, at forskellige ekstraktionsbetingelser har afgørende indflydelse på den kemiske natur af FCSPs er sand. Studiet demonstrerede også, at ufractionerede FCSPs har bioaktive egenskaber såsom anti-tumor og immun-modulerende effekter, vist i både in vitro og in vivo. Studiet illustrerer desuden vigtigheden af en præcis monitoringsmetode til måling af U. lactuca vækst.

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List of publications

The PhD thesis is based on the work presented in the following papers:

I. Important determinants for fucoidan bioactivity: A critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweed
Ale MT, Mikkelsen JD and Meyer AS
Marine Drugs (2011) 9, 2106 - 30, DOI:10.3390/md9102106, Published online

II. Design of optimization of a single-step extraction of fucose-containing sulfated polysaccharides from Sargassum sp.
Ale MT, Mikkelsen JD and Meyer AS
Journal of Applied Phycology (2011) DOI: 10.1007/s10811-011-9680-3, Published online

III. Fucoidan from Sargassum sp. and Fucus vesiculosus reduces cell viability of lung carcinoma and melanoma cells in vitro and activates natural killer cells in mice in vivo
Ale MT, Maruyama H, Tamauchi H, Mikkelsen JD and Meyer AS
DOI: 10.1016/j.ijbiomac.2011.05.009, Published online

IV. Fucose-containing sulfated polysaccharides from brown seaweed inhibit proliferation of melanoma cells and induce apoptosis by activation of caspase-3 in vitro
Ale MT, Maruyama H, Tamauchi H, Mikkelsen JD and Meyer AS
Marine Drugs (2011) 9, 2605 - 21 DOI:10.3390/md9122605, Published online

V. Differential growth response of Ulva lactuca to ammonium and nitrate assimilation
Ale MT, Mikkelsen JD and Meyer AS
Journal of Applied Phycology (2011) 23: 345–351, Published online
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1 Introduction

In the vast coastal areas worldwide, seaweed has played a major role in maintaining the ecological balance of the environment and marine bio-ecosystem. Seaweeds, including various brown seaweeds such as Undaria and Laminaria spp., are part of the food culture in many Asian countries, notably Japan, the Philippines, and Korea, and seaweed extracts have also been used as remedies in traditional medicine. In recent years, harvesting and monoculture farming of certain seaweed species have become an important livelihood for fishermen in Southeast Asia owing to the progressively increasing demand for raw seaweed worldwide. Many seaweed species, notably beach-cast seaweeds, still need to be examined for their characteristics and properties, which will determine their commercial applications, before they can be recognized as important commodities.

Seaweed that washes up on the coastline often generates waste problems for populations residing seaside owing to microbial accumulation and unpleasant odors. Utilization of beach-cast seaweeds such as Ulva sp., Sargassum sp., and other nuisance seaweed species for advantageous applications may alleviate these problems and create valuable seaweed-based products. It is widely known that seaweed may contain unique components that have potential commercial applications; however, few seaweed species are commercially utilized and others remain unexploited. Some seaweed species could be potential sources of functional dietary fiber and polysaccharides with bioactive properties (Lahaye 1991; Takahashi 1983). Recent developments in seaweed utilization include applications involving naturally derived seaweed extracts and bioactive compounds such as fucose-containing sulfated polysaccharides (FCSPs) and/or fucoidan in some cosmetic products and food supplements.

Fucoidan is a term used to describe a class of sulfated polysaccharide extracted from the seaweed class Phaeophyceae, which consist almost entirely of fucose and ester sulfate (Percival and McDowell 1967). This FCSP principally consists of a backbone of (1→3)- and/or (1→4)-linked α-L-fucopyranose residues that may be organized in stretches of (1→3)-α-fucan or with alternating α|1→3| and α|1→4|-bonded L-fucopyranose residues. The L-fucopyranose residues may be substituted with sulfate (SO3) on C-2 or C-4 (rarely on C-3) single L-fucosyl residues and/or short fucoidine (fuco-oligosaccharide) side chains. If present, the fucoidine side chains are usually O-4, linked to the α-L-fucopyranose backbone residues. Apart from variations in the sulfate content and
substitutions, the monosaccharide composition of FCSPs varies among different species of brown seaweeds. Hence, in addition to fucose, different types of FCSPs may also contain galactose, mannose, xylose, glucose, and/or glucuronic acid, usually in minor amounts (Percival and McDowell, 1967; Bilan and Usov, 2008). It appears that FCSPs cover a broader range of complex polysaccharides than those having only fucan backbones. Fucoidan—or more correctly, FCSPs extracted from brown seaweeds like Sargassum sp. and Fucus vesiculosus—were documented to have a wide range of biological activities including anticoagulant (Nardella et al., 1996); anti-inflammatory (Blondin et al., 1994); antiviral (Adhikari et al., 2006; Trinchero et al., 2009); and, notably, anti-tumoral effects (Zhuang et al., 1995; Ale et al., 2011).

Typical extraction of FCSPs from brown seaweed involves a harsh processing condition and several purification steps. Purification of FCSPs by column chromatography was effective for isolating polysaccharide fractions as they had higher fucose contents than those FCSPs obtained using minimal processing steps (Li et al., 2006; Ale et al., 2011). The conditions for obtaining FCSPs from brown seaweed generally consist of multiple, long, repetitive steps using acid (e.g., HCl) and other solvents at elevated temperatures (Chizhov et al., 1999; Bilan et al., 2002). The influence of the extraction methods on the chemical nature of sulfated polysaccharide has already been demonstrated by Black et al. (1952). On the other hand, the FCSP yield of F. evanescentes extracted 4 times using 2% CaCl2 solution at 85°C for 5 h was 12.9% dry weight (DW) (Bilan et al., 2002), while extraction at 25°C using 0.4% HCl for 5 h yielded 12.0% DW (Zvyagintseva et al., 1999). Despite the existence of early seminal studies about FCSP or fucoidan extraction, there is only limited evidence about the influences and apparently complex interactions of extraction parameters, such as acid solvents, temperature, and time, on FCSP yield and composition. FCSP extraction procedures with fewer steps are milder on the brown seaweeds than are other though they may yield a heterogeneous sulfated polysaccharide product. Nevertheless, fewer steps extraction approach minimizes the structural alteration of algal-sulfated polysaccharides and, thereby, maintains the natural bioactive characteristics of FCSPs.

Although differently extracted and purified FCSPs have been reported to exert bioactivity (Holtkamp et al., 2009), unfractionated FCSPs has also been found to reduce cell proliferation of lung carcinoma and melanoma cells, exert immunopotentiating effects in tumor-bearing animals, and to activate natural killer (NK) cells in mice, leading to anti-tumor activity efficacy (Takahashi, 1983; Ale et al., 2011; Foley et al., 2011). Kim et al. (2010) applied a crude polysaccharide composed predominantly of sulfated fucose from F. vesiculosus to human colon cancer cells in
vitro and concluded that this polysaccharide from brown seaweed induces apoptosis. Moreover, commercially available crude fucoidan (Sigma Inc.) was tested on human lymphoma HS-Sultan cell lines and was found to inhibit proliferation and induce apoptosis by activating caspase-3 (Aisa et al., 2005). It was reported recently that FCSPs from Sargassum sp. and crude fucoidan (Sigma Inc.) from F. vesiculosus induced apoptosis in melanoma cells (Ale et al., 2011).

Besides the bioactive compounds, seaweeds also possess other valuable components, such as soluble dietary fibers and carbohydrates for hydrocolloid applications. The green seaweed (Ulva species) are particularly rich in rare cell wall polysaccharides and have been proposed as being important sources of dietary fiber, mainly soluble fiber (Lahaye 1991; Lahaye and Avelos 1993). Furthermore, U. lactuca is also a good source of vitamins A, B2, B12, and C and is rich in γ-tocopherol (Abd El-Baky et al., 2008; Ortiz et al., 2006). It has been shown that seaweed, notably U. lactuca, was suitable for propagation under controlled conditions (Vermaat and Sand-Jensen, 1987; Lee, 2000; Sato et al., 2006). For this reason, U. lactuca cultivation in tanks for either crude biomass production for bioenergy or for the production of biologically active compounds is currently receiving increased attention (Hiraoka and Oka, 2008). However, a major prerequisite for the successful exploitation of cultivated U. lactuca for commercial applications is optimization of growth rates and yields. This in turn requires both an understanding of the influence of different nutrients on the growth response and a precise methodology to measure the growth. Nuisance green seaweeds like U. lactuca showed bioremediation ability in nitrogen- and phosphate-rich waste water (Copertino et al., 2008). Nevertheless, limited information is known about the growth response and nutrient uptake assimilation of U. lactuca when exposed to combinations of ammonium and nitrate.

This PhD thesis delivers the most recent study involving FCSPs extraction technology and evaluates FCSPs biological activity. A single-step extraction method for the removal of FCSPs from Sargassum sp. was developed in the course of this study. FCSPs bioactivity studies have been conducted in lung and skin cancer cell models in vitro and immune response activity in vivo. In addition to extraction and bioactivity studies, evaluation of the growth response of U. lactuca to nutrients such as NH₄ and NO₃ was also performed.

1.1 Problems statement

Purified fractions from FCSPs are commonly used for structural and bioactivity analysis. These samples contain high fucose-sulfate levels and are free from other contaminant saccharide...
residues. Hence, many seminal studies have shown that purified fucoidan has high bioactivity as a result of multi-step extraction and further purification and fractionation.

- Can an unpurified FCSPs product extracted using minimal steps exert bioactivity?
- What is the effect of unpurified FCSPs against certain cancer cell lines and what is its influence on immune response activity?

The present extraction technology using aqueous-alkali solution or dilute acid at ambient or slightly elevated temperature has always been the most convenient method to produced FCSPs.

- Would different extraction parameters, i.e., acid, temperature, and time, influence the structural features and chemical nature of FCSPs?
- What are the effects of the interactions of different extraction treatments?

Exploitation of seaweed resources has recently received special attention for its potential for both the production of bioactive compounds and as a biomass source for bioenergy production. To successfully exploit seaweed for commercial applications, the growth rate and yields must be optimized. Hence, a precise monitoring technology to evaluate the seaweed growth response is required.

- What are the different monitoring techniques that are used to evaluate the U. lactuca growth response to nutrient assimilation? How does it affect the measurements’ precision?
- How is the growth of U. lactuca influenced by the assimilation of different nutrients (NH₄ and NO₃)?

1.2 Hypotheses

Nuisance marine seaweed that has washed up on the coastlines is a potential starting material for producing bioactive compounds like fucoidan or FCSPs. The precise assessment of seaweed growth is crucial in our understanding of seaweed nutrient assimilation mechanisms. In this thesis, some specific hypotheses were outlined:

- Minimal extraction step of FCSPs will preserve the polysaccharides’ structural integrity and, thus, increase the yield and improve its biological properties

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PhD Thesis 2012
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Classical extraction of FCSPs involving long, repetitive, multi-step acid and alkaline treatments can be detrimental to its structural makeup, yield, and compositional attributes and, thus, may influence its bioactive properties.

To exploit marine seaweed for commercial applications, growth monitoring is crucial to the accurate evaluation of differential growth responses and nutrient (NH₄ or NaNO₃) assimilations.

1.3 Core objectives and project phases

Seaweed resources generally cover wide potential applications for different industries including food and nutrition, pharmaceuticals, cosmetics, and bioenergy. Therefore, their study requires extensive in-depth research involving an interdisciplinary approach and a considerable amount of time to accomplish certain achievable objectives. This PhD study narrows the subjects into more focused areas with very realistic aims or specific objectives to produce novel technology while attaining basic scientific understanding.

To test the hypotheses, various specific objectives were applied in this study. The core objectives were primarily concentrated on the investigation of different FCSPs extraction methods and developing innovative FCSPs extraction technology, the potential of seaweeds as a source of bioactive compounds, notably FCSPs; and to assess the bioactivity and mechanism of FCSP products against certain types of cancer. Furthermore, we evaluate seaweeds’ differential growth responses to nutrient assimilation. Along with the core objectives, the specific aims of the study were as follows:

- Development of a new process for producing bioactive compound like fucose-containing sulfated polysaccharides from brown seaweed by optimized designed extraction parameters using state-of-the-art analytical methods and quantification analyses
- Investigation of the bioactivity of single-step extracted unpurified FCSPs products (i.e., crude fucoidan) in cancer cell lines using in vitro and in vivo experiments
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To ensure that the work was conducted in the right direction, workloads were organized and narrowed down to specific scopes in a number of project phases:

Phase 1: Survey of seaweed bioactive compound structure and bioactivity

A literature research and gathering of review articles was conducted with the aim of gaining an overall perspective of the subject. Special focus was placed on the available technical and scientific information regarding bioactive compounds’ structural makeup and composition, the influence of the extraction process, and factors affecting bioactivity.

Phase 2: Optimized extraction and hydrolysis of seaweed polysaccharides (i.e., FCSPs)

Fucoidan can be obtained by extraction from brown seaweeds like Sargassum sp. The typical extraction of FCSPs involves multi-step extraction using different temperatures and acid and alkaline concentrations in an extended period. In this study, it was hypothesized that harsh extraction treatment is detrimental to the integrity of the chemical nature of these polysaccharides; thus, preservation of its structure during extraction can only be done using a mild extraction technique. The main objective of this project was to design an optimized extraction of FCSPs using a minimal-step method compared to existing classical extraction methods with special attention placed on the influence on chemical composition.

Phase 3: Assessment of FCSP bioactivity

FCSPs are known to possess bioactive properties such as anti-tumor activity, apoptosis induction in cancer cells, and immune potentiation. The efficacy of FCSPs derived from brown seaweed remains a matter of debate; nevertheless, several reports attribute its efficacy to its structural makeup, substitutions, and content of sulfate. It was hypothesized that mild extracted FCSPs must possess high bioactive properties because their structural makeup remains intact. The objective of this study was to examine the different contributing factors influencing FCSP bioactivity on some cancer cell lines. The bioactivities of FCSPs extracted from nuisance seaweed were evaluated using cancer cell lines in vitro and in vivo experiments.

Phase 4: Seaweed growth response to nutrient assimilation

The proliferation of seaweed is mostly influenced by photosynthesis and available nutrients that can be utilized and assimilated. Seaweed growth is tantamount to increased biomass production. Seaweed can potentially be utilized for the production of bioactive compounds and healthy...
components. Monitoring the seaweed growth and nutrient uptake is a crucial step for evaluating the seaweed growth rate, nutrient uptake rate, and biomass yield. Thus, developing a method to properly monitor seaweed growth will contribute to our understanding of the seaweed growth response to nutrient and assimilation patterns. The objective of this study is to examine the U. lactuca growth response to nutrients, i.e., NH₄ or NaNO₃, with the special aim of developing a growth monitoring technique to accurately evaluate the differential growth response and nutrient assimilation and to analyze different biomass accumulation-related parameters.
2 Seaweed potentials: a general overview

Marine seaweeds are classified according to their morphology and taxonomic characteristic into 3 groups: the green (Chlorophyta), red (Rhodophyta), and brown algae (Phaeophyta). The green algae are distributed worldwide (Lahaye & Robic, 2007), and the most common green seaweed species in the temperate zones is Ulva lactuca (Fig. 2.1). It is harvested naturally or cultivated in tanks for food consumption or biomass production for bioenergy (Lahaye & Robic, 2007). Natural habitats of red seaweed are found at intertidal and subtidal depths. In recent years, fishermen in the Southeast Asian countries engaged in the farming of the red seaweeds Kappaphycus and Eucheuma (Fig. 2.1) for the production of carrageenan. Carrageenan is a gel-forming, viscosifying polysaccharide that is commercially exploited for food products and cosmetic products (De Reiter and Rudolph, 1997). The brown seaweeds are usually found in tidal splash zones or rock pools, and certain species such as Sargassum are found floating on the shorelines (Fig. 2.1). Brown algae are typically used for the production of alginine, which is commercially used as an ingredient for different industrial, biotechnology, and food applications. FCSPs from brown seaweed, notably fucoidan, have been known to exert bioactive properties.

Several studies have indicated that FCSPs from brown seaweeds are highly potent anti-cancer agents, tumor cell growth inhibitors, and immune system strengtheners (Jiao et al., 2011; Pereira...

Fig. 2.1 Photographs of different seaweed species that are utilized for different commercial applications. a. Ulva lactuca as bulking agent for feeds. b. Kappaphycus sp. for carrageenan production. c. Sargassum sp. as a potential source of bioactive fucose-containing sulfated polysaccharides, particularly fucoidan compound. (Source: www.algaebase.org)

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et al., 1999). It has been suggested that the lower breast cancer and obesity rates in Japan may be related to regular intake of brown seaweed (Teas 1983). Brown seaweeds are very abundant and constitute unexploited resources worldwide, thus making them viable sources of polysaccharides, especially those with bioactive properties such as fucoidan or other FCSPs. Nevertheless, the red and green seaweeds are also good sources of functional food ingredients, including phycocolloids and dietary fibers.

2.1 Bioactive seaweed compounds: sulfated polysaccharides
Sulfated polysaccharides are a family of compounds containing ester sulfate groups in their sugar residues. These polysaccharides are commonly found in the marine algal groups Phaeophyta, Rhodophyta, and Chlorophyta. Fucans is the general term for sulfated polysaccharides that are present in the class Phaeophyta, which includes FCSPs. The sulfated polysaccharides in the class Rhodophyta are galactans consisting entirely of galactose or modified galactose units such as agar and carrageenans. The major polysaccharides in the class Chlorophyta are polydisperse heteropolysaccharides known as ulvans.

Ulva, the green seaweed species of the class Chlorophyta, are particularly rich in rare cell wall polysaccharides and have been proposed as being an important source of dietary fiber, mainly soluble fiber, which could be a potential prebiotic substrate (Lahaye 1991; Lahaye and Axelson 1993). Brown seaweed is a source of a unique compounds, notably FCSPs, which have shown high potency against certain cancers in some in vitro studies and exhibited immune response activity in vivo (Cumashi et al., 2007; Maruyama et al., 2003). Red seaweed, on the other hand, is known for its hydrocolloid characteristics; however, published reports have shown that sulfated polysaccharides from red seaweed also possessed some bioactive properties (Pereira et al., 2005; Talarico et al., 2007).

2.1.1 FCSPs structure
Fucoidan, an FCSP that is extracted from brown algae, may contain differing glycosidic linkages and is variously substituted with acetate and side branches containing fucose or other glycosyl units. Brown seaweed in the order of Fucales such as F. evanescent and F. serratus possess a large proportion of both α(1→3)- and α(1→4)-linked 1-fucopyranose residues may be substituted with sulfate (SO₃⁻) on C-2 and C-4 (Fig. 2.2; Bilan et al., 2002, 2006; Cumashi et al., 2007). The alga Ascophyllum nodosum (Fucales) has a predominant repeating structure α(1→3)-1-fucopyranose residues with sulfate at C-2 position-linked α(1→4)-l-fucopyranose residues with disulfate at C-2

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Several structures of fucoids of the order of Laminariales were reported to have contained monosulfate components that mainly consist of α(1→3)-linked fucopyranose residues with sulfates at the C2 position (Berteau and Mulloy, 2003; Anastyuk et al., 2009). Fucoid isolated from Chorda filum (Laminariales) has a structure of poly-(α1→3)-linked fucopyranose backbone and residues are sulfated mainly at C4 and sometimes C2 position, whereas some α(1→3)-linked fucose residues (Fig. 2.2) to be 2-O-acetylated (Chizhov et al., 1999). Similar structure of L.

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Okamuranus (Laminariales) composed of α(1→3)-linked L-fucopyranose with sulfate at C-4 (Fig. 2.2) was previously reported by Usos et al., (1998). A structural study of fucoidan from Cladosiphon okamuranus (Chordariales) showed a linear backbone of α(1→3)-linked L-fucopyranose with a portion of fucose residues that was O-acetylated and sulfate substitution at the C-4 position (Fig. 2.2). It also contained α-glucuronic acid at 2 positions of fucose that were not substituted by a sulfate group (Nagaoka et al., 1999).

![Diagram](image1)

**Fig. 2.3** Probable structure of fucoidan fraction from Hizikia fusiforme (Fucales) suggested by Li et al. (2006); structural composition was typical for Sargassum species (Duarte et al., 2001).

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![Diagram](image2)

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2.1.2 Anti-tumor bioactivity of FCSPs

FCSPs in brown seaweed have been subject to many scientific studies due to their diverse biological functions including anti-tumor and immunomodulatory activities (Aleksyenko et al., 2007; Maruyama et al., 2006). There are very few published reports on the relationship between chemical properties and anti-tumor activity of FCSPs. (Li et al., 2008; Aleksyenko et al., 2007; Koyanagi et al., 2003). However, it was suggested that the bioactive properties of FCSPs are mainly determined by the fucose sulfated chains (Nishino et al., 1994; Mourão et al., 1996); nevertheless, the anti-cancer activity of FCSPs was recently revealed to not be a function of a single but a combination of many factors such as the amount of sulfate groups, monosaccharide residues ratio, and the linkage type of the sugar residues (Ermakova et al., 2011; Ale et al., 2011). The available findings indicate that the anti-tumor activity of FCSPs may be associated with a significant enhancement of the cytolytic activity of NK cells augmented by increased production of macrophage-mediated immune response-signaling molecules (Maruyama et al., 2003; Takahashi et al., 1983; Teruya et al., 2009), namely interleukin (IL)-2, interferon (IFN)-γ, and IL-12 (Ale et al., 2011; Maruyama et al., 2003), and induction of apoptosis (Ale et al., 2011).

Macrophage activation by polysaccharides is mediated through specific membrane receptors. The major receptors reported for polysaccharide recognition in macrophages are glycoproteins including Toll-like receptor 4 (TLR-4), cluster of differentiation 14 (CD14), competent receptor-3 (CR-3), and scavenging receptor (SR) (Teruya et al., 2009). Activation of these receptors is mediated by intracellular signaling pathways, and the family of mitogen-activated protein kinases (MAPKs) plays a
critical role, notably in the production of nitric oxide (NO), which can lyse tumors (Teruya et al., 2009). MAPK family members such as p38 MAPK, extracellularly regulated kinase (ERK1/2), and stress-activated protein kinase/c-jun-N-terminal kinase play an important role in the activation of macrophages by polysaccharides such as FCSPs (Teruya et al., 2009; Aisa et al., 2005)(Fig. 2.4). Activated MAPKs lead to activation of transcription factors resulting in induction of various genes (Teruya et al., 2009). Activation of macrophages induces the production of cytokines such as interleukin-12 (IL-12) which in turn stimulate the development of T-cells (Fig. 2.4). T-cells produce interleukin-2 (IL-2) that in turn activates NK cells proliferation. The NK cells themselves produce immunologically important cytokines, notably IFN-γ, which can further provoke the participation of macrophages in the stimulation of T-cell via induction of IL-12 (Maruyama et al., 2006; Teruya et al., 2009) (Fig. 2.4).

Fig 2.4: Proposed mechanism responsible for fucoidan bioactivity: (a) Macrophage activation by fucose-containing sulfated polysaccharides (FCSPs) mediated through specific membrane receptor activation, namely Toll-like receptor (TLR) 4, cluster of differentiation 14 (CD14), competent receptor-3 (CR-3), and scavenging receptor (SR), which in turn induce intracellular signaling via mitogen-activated protein kinases (MAPKs); (b) activation of macrophages lead to production of cytokines such as interleukin (IL)-12, IL-2, and interferon (IFN)-γ, which enhances NK cell activation that may further stimulate T cell activation via IFN-γ.

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The anti-tumor mechanism of FCSPs appears to be associated with the significant enhancement of the cytolytic activity of NK cells augmented by increased production of the macrophage-mediated immune response, namely IL-2, type II IFN-γ, and IL-12 (Maruyama et al., 2003; Maruyama et al., 2006). NK cells are large granular lymphocytes that are found throughout the body that contain cytotoxic substances which are important for the protection against some tumors. The slat NK cell killing occurs via release of granules containing perforin, which effectively opens up pores in target cell membranes through which the granzymes can enter and induce apoptosis (Kindt et al., 2007, Lydyard et al., 2000). FCSPs was reported to induce apoptosis in HT-29 colon cancer cells (Kim et al., 2010), MCF-7 human breast cancer cells (Yamasaki-Miyamoto et al., 2009), and HS-Sultan human lymphoma cells (Aisa et al., 2005). The importance of apoptosis as a killing mechanism used by the immune system is that targeted cells can be rapidly removed by phagocytes without induction of an inflammatory response.

IL-2, which is made by T cells, is a critical autocrine growth factor that is required for proliferation of T cells and NK cells (Lydyard et al., 2000). NK cell secretion of type II IFN-γ activates macrophages, inducing IL-12 secretion, activating NK cells, and creating a system of positive feedback that increases the activation of both cell types within an infected cell or tissue (Parham 2009; Kindt et al., 2007). Hence, it was suggested that stimulation with IL-2 and IL-12 promotes IFN-γ secretion by NK cells, probably due to enhancing NK cell activity by FCSPs (Maruyama et al., 2006). IL-12 stimulation alone was reported to produce only moderate augmentation of NK cell cytotoxicity. However, it increases the catalytic activity of lymphocytes against autologous targets in synergy with IL-2 (Nastala et al., 1994).

2.2 FCSPs extraction and chemical composition: past and present

Several extraction and purification procedures have been used for many years to isolate fucoidan from brown seaweed. Extraction using dilute acetic acid and subsequent purification was first performed by Kylin in 1913 to isolate the substance from various species of Laminaria and Fucus (Kylin, 1913). Kylin reported that fucoidan extracted in this way mainly contained fucose and also observed that the fucose occurred together with mannitol, alginic acid, and laminarin (Kylin, 1913) (Table 1); we now know that this interpretation was a result of co-extraction of these latter contaminants with fucoidan. Two years later, Kylin reported that fucoidan isolated from L. digitata contained methylpentose, interpreted as L-fucose, as well as some other pentoses (Kylin, 1915). A

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parallel report was published that year by Hoagland and Lieb (1915), who isolated another water-soluble polysaccharide that was closely related to, if not identical with, fucoidan from Macrocystis pyrifera and was shown to contain L-fucose and a high proportion of calcium and sulfate (Table 1). Hoagland and Lieb (1915) did not compare their extraction using Na₂CO₃ soaking to one without, but their report is nevertheless the first example of how the extraction procedure may influence purity and, in turn, the analyzed composition of the extracted FCSPs (Table 1). Bird and Haas (1931) used fresh L. digitata fronds to obtain fucoidan by soaking them in water and precipitating crude sulfate from the extract using ethanol. Uronic acid was also present in this preparation (Table 1).

Table 1. Historic view of very early work, 1913–1950s, of fucoidan or fucose-containing sulfated polysaccharide extraction and their composition from different brown seaweed species.

<table>
<thead>
<tr>
<th>Year</th>
<th>Brown Seaweed sp.</th>
<th>Reported FCSPs Composition</th>
<th>Extraction Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1913</td>
<td>Laminaria and Fucus</td>
<td>Fucoidan contaminated with mannitol, alginate, and laranarin</td>
<td>Dilute acetic acid extraction</td>
<td>Kyhn, 1913</td>
</tr>
<tr>
<td>1915</td>
<td>Laminaria digitata</td>
<td>Fucoidan contained methylpentoses, L-fucose, and some pentoses</td>
<td>Dilute acetic acid extraction</td>
<td>Kyhn, 1915</td>
</tr>
<tr>
<td>1915</td>
<td>Macrocystis pyrifera</td>
<td>Densely agglutic acid with fucose-sulfate</td>
<td>Seaweed was soaked in 2% Na₂CO₃, for 24 h, filtered combined with HCl, and the resulting precipitate was filtered and then finally resolved in 2% Na₂CO₃</td>
<td>Hoagland and Lieb, 1915</td>
</tr>
<tr>
<td>1931</td>
<td>L. digitata</td>
<td>Substantial amounts of calcium sulfate and uronic acid</td>
<td>Soaking of seaweed in water, precipitation of crude sulfate by ethanol</td>
<td>Bird and Haas, 1931</td>
</tr>
<tr>
<td>1931</td>
<td>Macrocystis pyrifera</td>
<td>Methylenemethanesulphonate monosulphate polymer with mainly fucose and alginate contaminants</td>
<td>Repeated extraction with 2% HCl at room temperature for 48 h, precipitated with 95% EtOH</td>
<td>Nelson and Crichton, 1931</td>
</tr>
<tr>
<td>1937</td>
<td>L. digitata</td>
<td>Sulfate residue must be substituted by fucose or another super residue</td>
<td>Prepared by precipitating the droplets exuded from seaweed in boiling ethanol</td>
<td>Lunde et al., 1937</td>
</tr>
<tr>
<td>1950</td>
<td>Fucus vesiculosus, Fucus spirales, Himanthalia line, Laminaria clustonis</td>
<td>Substantial amount of fucoidan and sulfate; and small amounts of uronic acid, galactose and xylose; ash was mainly calcium sulfate</td>
<td>Acid pH 2–2.5, 70°C, 1 h, 3 times; or aqueous, 100°C, for 24 h, lead acetate treatment, barium hydroxide added</td>
<td>Percival &amp; Ross, 1950</td>
</tr>
<tr>
<td>1952</td>
<td>F. vesiculosus</td>
<td>Fucose, ash, sulfate</td>
<td>pH 2–2.5, 70°C, 1 h, 3 times</td>
<td>Black et al., 1952</td>
</tr>
</tbody>
</table>
The early FCSPs extraction procedures involved dilute acid treatment either with acetic or hydrochloric acid as the first extraction step with the purpose of hydrolyzing the non-FCSP polysaccharides (Table 1). However, the extraction and purification methods employed in different studies to isolate fucoidan/FCSPs from brown seaweed biomass have been modified to different extents since the first reports in 1913 and 1915. For example, Nelson and Cretcher [1931] extracted fucoidan from Macrocystis pyrifera by repeated extended (48-h) extraction with dilute HCl followed by FCSP isolation by ethanol precipitation and revealed the presence of sulfate in the form of ester groupings in the precipitated product. They also confirmed that fucose was the only sugar identified in the unhydrolyzed residue after acid hydrolysis, even though their product contained uronic acid, which was considered to be due to alginate contamination (Table 1).

2.2.1 FCSPs composition studies, 1930–1950
The studies by Nelson and Cretcher [1931] revealed the presence of sulfate in the form of ester groupings and confirmed that fucose was the only sugar identified after hydrolysis, although their product contained uronic acid, which was considered to be due to alginate acid contamination (Table 1). Please note the terminology used is quite confusing; nevertheless, with recent advances in chemical analyses, we now know that alginic acid and alginates comprise guluronic and mannuronic acids. Our understanding today that alginate acid, or alginate, is a linear hydrocolloid polymer that consist of blocks of [1–4]-linked β-D-mannuronic and its α-L-guluronic residues, that these 2 monomers are C-5 epimers, and that the detailed structure of alginate may have mannuronate and guluronate in homopolymeric blocks of consecutive mannurionate residues (M-blocks), consecutive guluronate residues (G-blocks), or in structural units of alternating mannuronate and guluronate residues (MG-blocks). Uronic acids were determined to cover several different structures. This work addressed the significance of the differences and introduced consistent terminology by grouping these as uronic acids, especially since they do not belong to the FCSPs. Later, Lunde et al. (1937) prepared fucoidan by directly precipitating the droplets exuded from freshly gathered L. digitata fronds in ethanol (Table 1). After purification via product precipitation using boiling ethanol, they obtained a FCSP specimen that contained 33–37% methylnpentose (interpreted correctly as fucose) and 26–30% ash in which the sulfate content was 17–19%, which made up approximately half of the total estimated sulfate in the polysaccharide (35–38%). They proposed a structural unit formula, (R-R-O-SO₃-M)n, for fucoidan and suggested that R was fucose or another pentose sugar residue, R’ was unknown, and M was Na+, K+, (Na,Ca)²⁺, or (Na,Mg)²⁺ (Lunde et al., 1937) (Table 1).

The early FCSPs extraction procedures involved dilute acid treatment either with acetic or hydrochloric acid as the first extraction step with the purpose of hydrolyzing the non-FCSP polysaccharides (Table 1). However, the extraction and purification methods employed in different studies to isolate fucoidan/FCSPs from brown seaweed biomass have been modified to different extents since the first reports in 1913 and 1915. For example, Nelson and Cretcher [1931] extracted fucoidan from Macrocystis pyrifera by repeated extended (48-h) extraction with dilute HCl followed by FCSP isolation by ethanol precipitation and revealed the presence of sulfate in the form of ester groupings in the precipitated product. They also confirmed that fucose was the only sugar identified in the unhydrolyzed residue after acid hydrolysis, even though their product contained uronic acid, which was considered to be due to alginate contamination (Table 1).
Specimens from *F. vesiculosus*, *F. spirales*, *H. loreo*, and *L. clustoni* were prepared by Percival and Ross (1950) for FCSFs extraction using boiling water for 24 h, and the alginites and protein were removed with lead acetate and the addition of barium hydroxide. The resulting lead hydroxide-fucoidan complex was then decomposed using dilute sulfuric acid and the fucoidan was isolated after prolonged hydrolysis and filtration. The purest specimen was from *H. loreo*, which contained the following: a substantial amount of fucose and sulfate; small quantities of uronic acid, galactose, and xylose; metals; and ash, which was mainly calcium sulfate. These workers believed that the principal constituent of fucoidan is a polyfucose monosulfate and that other constituents arise from impurities (Table 1). A parallel work was done by Conchie and Percival (1950) in which fucoidan from *Fucus vesiculosus* was methylated. It was believed that the main residue in fucoidan was 1→2 linked of L-fucopyranose units carrying a sulfate group in C-4 (Conchie and Percival 1950). However, we now know that this is not the case because advanced analyses have verified that the backbone of fucoidan from *F. vesiculosus* consists of alternating q(1→3) and q(1→4) linkages of 1-fucopyranose residues (Fig. 2.2).

### 2.2.2 Lab-scale extraction of fucoidan, 1950s

In the pursuit of obtaining extensive quantities, a laboratory-scale extraction of fucoidan was performed by Black et al. (1952). Interestingly, they referred to the extracted product as a "polyfucose ethereal sulfate occurring in the Phaeophyceae." Their optimal fucoidan extraction procedure was as follows: one part by weight of dried ground seaweed and 10 parts by volume of 0.1 M hydrochloric acid at pH 2.0–2.5 contacted at 70°C with constant stirring for 1 h. A single acid hydrolysis extraction treatment using this method recovered about 50% by weight (w/w) of the theoretical maximum of fucoidan (recovered yield measured as % fucose obtained as % of total fucose in the seaweed DW), whereas 3 rounds of acid extraction recovered >80% of the fucose present; the triple acid hydrolysis treatment (0.1 M HCl, pH 2.0–2.5, 70°C, 1 h x 3) was therefore selected as the optimal extraction method (Table 1). After the acid hydrolysis treatment the crude fucoidan was isolated by fractional precipitation with alcohol and further purified by precipitation after the addition of formaldehyde (Black et al., 1952). Using this procedure, crude fucoidan samples containing 30–36% fucose were obtained; for example, the fucoidan recovered from *F. vesiculosus* using the optimal extraction protocol was analyzed to contain (by weight) 44% fucose, 26% total sulfate, and 31% ash (Black et al., 1952). In terms of yields of percentage of total fucose, the results obtained for the 4 different algal species were: *Pelvetia canaliculata*, 76%; *F. vesiculosus*, 62%; *Asphodelium nodosum*, 54%; and *L. clustoni*, 20% (Black et al., 1952). The authors suggested that a more efficient extraction methodology, i.e., extracting higher fucose
yields, could be achieved by increasing the water/seaweed ratio, extraction time, or extraction number.

2.2.3 FCSPs extraction, 1970s–present
Carbohydrates of the brown seaweeds were successively extracted from H. loree, Bifurcario bifurcata, and Padino pavoninio using dilute acid followed by alkaline or using a water, acid, and alkali sequence. Prior to extraction, the seaweed frond was pretreated with formaldehyde to polymerize phenolic constituents that could otherwise be contaminant to various extracts (Milan and Percival, 1973). This technique produced complex mixture of glucan, FCSPs, and alginic acid, which could be separated by fractional precipitation with ethanol, calcium salts, or cetyltrimethylammonium hydroxide, or by fractionation on resin columns. Fucoidan extracted in this fashion consisted of heteropolysaccharide comprising different proportions of fucose, glucuronic acid, xylose, and half-ester sulfate together with a trace of galactose. The result of this extraction method also showed that sulfate and uronic acid contents in the fucoidan separated from aqueous calcium chloride and acid extracts varied significantly by species.

A study was conducted on the purification of a sulfated heteropolysaccharide from S. linfolium to elucidate its structural components. The extraction was done using hydrochloric acid pH 1.0 for 3 h at 80°C; the extract was neutralized using aqueous sodium carbonate and precipitated with ethanol. The resulting sulfated polysaccharide material was acid-hydrolyzed and it was proposed that the backbone was composed of glucuronic acid, mannos, and galactose residues with partially sulfated side chains of galactose, xylose, and fucose residues (Abdel-Fattah et al., 1974).

Extraction using an aqueous alkali solution or dilute acid at ambient or slightly elevated temperatures has been the convenient method to produce fucoidan for many years. In recent years, pretreatment of seaweed biomass has been found advantageous to eliminate low molecular components (e.g., phenols) using a mixture of methanol, chloroform, and water (Bilan et al., 2006); removal of protein can be facilitated using proteolytic enzymes (Rocha et al., 2005). A useful procedure to transform alginate in the residual biomass into insoluble calcium salts uses aqueous calcium chloride (Bilan et al., 2002).

2.3 Seaweed products and biomass potential
The growth of seaweed production and developments of advanced technological farming indicates that the seaweed industry is progressively evolving and thus paves the way to new application opportunities (Buck and Buchholz, 2004). For many years, seaweed has been utilized as a source of...
diary nourishment among Asian constituents. In recent years, more applications have used seaweed resources because of their unique characteristics and properties. Seaweed has been proposed as a source of important compounds that can be incorporated in the production of functional food ingredients, pharmaceuticals, and cosmetics (Bodin-Dubigeon et al., 1997; Bisler, 1996; De Roeck-Holtzhauser, 1991). Gel-forming polysaccharides such as carrageenan and alginate are mostly known and commercially important seaweed products. Production of alternative fuels from non-starch biomass has recently directed the attention to utilization of marine macroalgae as sources of biomass for biofuel production (Knauf and Moniruzzaman, 2004). Moreover, healthy and bioactive components, notably the FCSPs found in brown seaweed biomass, have also become subject of much research and product development.

2.3.1 Phycocolloids

The global market for phycocolloids such as agar, carrageenans, and alginites is estimated to be worth annually $585 million US (McHugh, 2003). To date, the red seaweed has been harvested for food consumption in some regions of Asia, but many of these areas have engaged in farming red seaweed that is intended for the production of agar and carrageenan, while brown seaweed is most harvested naturally for alginate production (Crawford, 2002).

Carrageenan is composed of a linear galactose backbone with varying degrees of sulfation (15–40%) and are mainly composed of disaccharide repeating units of an α(1→4)-linked α-galactopyranosyl or 3,6-anhydro-α-galactopyranosyl residue and a β-(1→3)-linked α-galactopyranose. The sulfated groups are covalently attached to individual galactose residues via ester linkages to the carbon atoms C-2, C-4, or C-6 (De Ruitter and Rudolph, 1997). Carrageenan is applied as a stabilizing agent to many food products and other industrial and pharmaceutical applications (van de Velde and De Ruitter, 2002). Among the leading species of red algae responsible for most of today’s commercial carrageenan production are K. alvarezi and E. denticulatum.

In addition, alginate another gel-forming sulfated polysaccharide extracted from brown seaweed has been utilized for various applications including foods and feeds, food/pharmaceutical, and industrial preparations. Alginate is composed of mannuronic (M) and guluronic (G) acids with (1, 4) linkages, and its structure varies according to the monomer position on the chain, forming either homopolymeric (MM or GG) or heteropolymeric (MG or GM) segments (Percival and McDowell, 1967). The molecular weight of alginate is generally 500–1000kDa. Its solubility is influenced by factors such as pH, concentration, ions in solution, and the presence of divalent ions such as calcium (Morris and Norton, 1983).

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2.3.2 Seaweed biomass for bioenergy production

Only few seaweed species have been exploited for commercial production (i.e., red and brown seaweed), hence, this study opens the way for other species to provide a basis for additional potential applications of seaweed biomass, namely bioenergy production. The cultivation of seaweed poses advantages to terrestrial crops since they have high growth rates and can be continuously harvested (Rasmussen et al., 2009). The estimated annual production of *U. lactuca* per hectare was 45 tons DW, which is 3× greater than the yield of conventional food or energy crops (Bruhn et al., 2011). However, the production is believed to be significantly higher once growth conditions (exploiting flue gas as a source of carbon) and nutrients (fishery effluents as a source of nitrogen) are optimized. A study on the anaerobic digestion of sea lettuce (*Cladophora*) suggested that the methane gas yield from washed and macerated sea lettuce biomass is about 271 mL CH₄ g⁻¹ VS (volatile solid-based), while that from non-pretreated biomass is about 70% (Otsuka and Yoshino, 2004). Biogas production from fresh and macerated sea lettuce *U. lactuca* yielded up to 271 mL CH₄ g⁻¹ VS, which is in the range of the methane production from cattle manure and land-based energy crops such as grass clover. Drying of the biomass resulted in a 5–9-fold increase in weight-specific methane production compared to wet biomass (Bruhn et al., 2011).

2.4 Seaweed production

Seaweed has been traditionally cultured for centuries in several Asian countries including China, Japan, and Korea (Crawford, 2002). Most seaweed production came from the harvest of wild stocks in these countries, although limited culture had been established in countries such as the Philippines and Indonesia (Trono, 1990). The species cultivated include *Kappaphycus* and *Eucheuma*. The leading seaweed exporter, the Philippines, increased production from 675 tons in 1967 to 65,617 tons in 2009 and 80,000 tons in 2010, but the country is still importing raw seaweed materials from Indonesia (www.siap.com.ph, 2011). The world demand for seaweed in 2003 was 220,000 tons for *K. alvarezii* and *E. denticulatum* with an expected 10% annual increase in demand (Sievanen et al., 2005). Indonesia, on the other hand, raised its production from 1,000 million tons (MT) in 1966 to approximately 27,874 tons in 2001 and estimated 10 MT in 2015 (Sievanen et al., 2005). The production levels of algae in 2002 reached 18.6 MT (FAO, 2004). Europe had only a 6.3% share of the global world production of brown algae (362,000 tons FW) and about 0.3% of red algae (9,400 tons FW) with <300 tons of macroalgae produced in aquaculture in 2002 (FAO, 2004).

Several sea-based cultivation design methods have been developed and tested. The most commonly used was a fixed off-bottom long line, hanging long line, and combination of the 2 (Hurtado et al., 2011).

### Other Information

**Indonesia**, **Seaweed source production**}

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2.5 Paper 1: Important determinants for fucoal bioactivity: a critical review of structure-function correlations and extraction methods for FCSPs from brown seaweeds

Marine Drugs, 2011, Published online

Authors: Marcel Tutor Ale, Jørn Dalgaard Mikkelsen, Anne S. Meyer

Address: Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering Technical University of Denmark (DTU)
Soeltofts Plads Bldg. 229, 2800 Kgs. Lyngby, Denmark

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Marcel Tutor Ale, Jørn D. Mikkelsen and Anne S. Meyer *

Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering, Technical University of Denmark (DTU), Søltofts Plads, Building 229, DK-2800 Kgs. Lyngby, Denmark; E-Mails: mta@kt.dtu.dk (M.T.A.); jdm@kt.dtu.dk (J.D.M.)

* Author to whom correspondence should be addressed; E-Mail: am@kt.dtu.dk; Tel.: +45-4525-2800; Fax: +45-4593-2906.

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Abstract: Seaweeds—or marine macroalgae—notably brown seaweeds in the class Phaeophyceae, contain fucoidan. Fucoidan designates a group of certain fucose-containing sulfated polysaccharides (FCSPs) that have a backbone built of \((1\rightarrow3)-\) linked \(\alpha-L\)-fucopyranosyl or of alternating \((1\rightarrow3)-\) and \((1\rightarrow4)-\) linked \(\alpha-L\)-fucopyranosyl residues, but also include sulfated galactofucans with backbones built of \((1\rightarrow6)-\beta-D\)-galacto- and/or \((1\rightarrow2)-\beta-D\)-mannopyranosyl units with fucose or fuco-oligosaccharide branching, and/or glucaric acid, xylose or glucose substitutions. These FCSPs offer several potentially beneficial bioactive functions for humans. The bioactive properties may vary depending on the source of seaweed, the compositional and structural traits, the content (charge density), distribution, and bonding of the sulfate substitutions, and the purity of the FCSP product. The preservation of the structural integrity of the FCSP molecules essentially depends on the extraction methodology which has a crucial, but partly overlooked, significance for obtaining the relevant structural features required for specific biological activities and for elucidating structure-function relations. The aim of this review is to provide information on the most recent developments in the chemistry of fucoidan/FCSPs emphasizing the significance of different extraction techniques for the structural composition and biological activity with particular focus on sulfate groups.
1. Introduction

Fucoidan is a term used for a class of sulfated, fucose rich, polysaccharides found in the fibrillar cell walls and intercellular spaces of brown seaweeds of the class Phaeophyceae. These fucose-containing sulfated polysaccharides (FCSPs) principally consist of a backbone of (1→3)- and (1→4)-linked α-L-fucopyranose residues, that may be organized in stretches of (1→3)-α-fucan or of alternating (1→3)-α-fucan and (1→4)-linked α-L-fucopyranose residues. The α-L-fucopyranose residues may be substituted with sulfate (SO$_3^-$) on the C-2 or C-4 (rarely on C-3), with single α-L-fucosyl residues and/or with short fucoside (fucoido-oligosaccharide) side chains. If present, the fucoside side chains are usually O-4 linked to the α-L-fucopyranose backbone residues. However, as FCSPs structures of more brown seaweeds are being elucidated, as discussed further in the present review, it appears that FCSPs cover a broader range of complex polysaccharides than only those having fucan backbones. Apart from variations in the sulfate content and substitutions, also the monosaccharide composition of FCSPs varies among different species of brown seaweeds. Hence, in addition to fucose, different types of FCSPs may also contain galactose, mannose, xylose, glucose and/or glucuronic acid—usually in minor amounts [1].

According to the ISI Web of Knowledge (Thomson Reuters) the number of published articles with the topic assigned as “fucoidan” has increased significantly since fucoidan, or “fucoidin” as it was first called, was first isolated from brown algae in 1913 [2]; in particular, a profound increase in the number of papers has taken place during the last 5–10 years. By now, the published papers related to fucoidan hit approximately 1800 (August 2011, Figure 1). The recent interest has mainly focused on the potentially beneficial biological activities of fucoidan and FCSPs in humans including antitumor, immunomodulatory, anti-inflammatory, antiviral, antithrombotic, anticoagulant, and antioxidant effects as well as specific activities against kidney, liver and urinary system disorders.

While the development of research efforts involving FCSPs and their potential applications are advancing, the understanding of the mechanisms and the particular structural features of the FCSPs being responsible for the various biological activities is still incomplete. Seaweeds, including various brown seaweeds such as Undaria and Laminaria spp., are part of the food culture in Asia, notably in Japan, the Philippines, and Korea, and seaweed extracts have also been used as a remedy in traditional medicine. However, no standardized FCSPs extraction or purification protocols exist, and no specific pharmaceutical, dermatological, or nutraceutical applications have as yet been officially approved for these polysaccharides or their lower molar mass oligosaccharide derivatives.

It is our proposition that more focus should be directed to the extraction and purification processes in order to obtain consistent protocols that account for the biodiversity of FCSPs from different seaweeds and to retain the structural features of significance for the specific bioactivity properties of FCSPs. The development and use of such consistent extraction procedures would also help in achieving a better understanding of structure-function relationships of FCSPs. The aim of this review is to bring attention to the detailed structural features of FCSPs in relation to their marine algal sources.
and the extraction methodology, and to highlight recent knowledge concerning the structural
determinants for FCSPs bioactivity.

Figure 1. The trend during three decades of research on fucoidan as depicted by the
number of published articles (Thomson Reuters, ISI Web of Knowledge). The number of
articles was obtained according to topics being assigned in the ISI Web of Knowledge
search engine with the following topic search terms: Fucoidan; Fucoidan\*Algae; Fucoidan\*Algae\*Activity.

2. Historic Overview: FCSPs Extraction Procedures and Chemical Analyses

Extraction using dilute acetic acid followed by purification was used by Kylin already in 1913 to
isolate “fucoidan”, subsequently referred to as fucoidan, from various species of Laminaria and
Fucus [2]. Already in this first seminal report, Kylin reported that fucoidan extracted in this way
mainly contained fucose, but also observed that the fucose occurred together with mannitol, alginic
acid and laminarin [2] (Table 1); we now know that this interpretation was a result of co-extraction of
these latter contaminants with the fucoidan. Two years later, Kylin reported that fucoidan isolated from
Laminaria digitata contained methylpentose, interpreted as L-fucose, as well as some other
pentoses [3]. A parallel report was published that same year by Hoagland and Lieb (1915) [4] who
isolated a water-soluble polysaccharide from Macrocystis pyrifera that was closely related to if not
identical with “fucoidan”, and shown to contain L-fucose as well as relatively high levels of calcium
and sulfate. They employed a Na₂CO₃ soaking step and addition of hydrochloric acid which is why
they also—if not mainly—isolated alginic acid (or alginate) during the extraction (Table 1). The
rationale behind the extraction of alginate from the seaweed with Na₂CO₃ soaking is to convert all the
alginate salts, typically calcium and magnesium alginate, to the sodium salt. Please note that the
terminology used is confusing; alginic acid or alginate does not designate one particular
monosaccharide or one type of homo-polysaccharide. With the advances in chemical analyses it is now
known that alginic acid or alginate is comprised of guluronic and mannuronic acids; which are C-5
epimers. Structurally, alginic acid, or alginate, is a linear hydrocolloid polymer that consists of blocks
of (1→4) linked β-D-mannuronate and α-L-guluronate residues. The detailed structure of alginate may
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(M-blocks), in consecutive guluronate residues (G-blocks), or in structural units of alternating mannuronic and α-L-guluronic residues (MG-blocks). Both mannuronic acid and α-L-guluronic acids are uronic acids, and they have in the past been analytically determined as uronic acids. However, uronic acids also include several other structures, e.g., glucuronic and galacturonic acids. In this review, we will address the significance of the compositional and structural differences, but also attempt to introduce a consistent terminology by grouping these compounds as uronic acids, especially as homopolymers do not belong to the group of FCSPs. Hoagland and Lieb (1915) [4] did not compare their extraction with Na2CO3 soaking to one without, but their report is nevertheless the first example of how the extraction procedure may influence the purity and, in turn, the analyzed composition of the extracted FCSPs (see Table 1).

### Table 1. Historic view of very early work, from 1913–1950, of fucoidan or FCSPs extraction and their reported composition from different brown seaweed species.

<table>
<thead>
<tr>
<th>Year</th>
<th>Brown seaweed sp.</th>
<th>Reported FCSPs composition</th>
<th>Extraction method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1913</td>
<td>Laminaria and Fucus</td>
<td>Fucoid contain fucose, that occurs together with mannitol, alginate and laminarin</td>
<td>Dilute acetic acid extraction</td>
<td>Kylin, 1913 [2]</td>
</tr>
<tr>
<td>1913</td>
<td>Laminaria digitata</td>
<td>Fucoid contain fucose and other pentoses</td>
<td>Dilute acetic acid extraction</td>
<td>Kylin, 1915 [3]</td>
</tr>
<tr>
<td>1915</td>
<td>Macrocystis pyrifera</td>
<td>Mainly alginic acid, with some fucose-sulfate</td>
<td>Soaking in 2% Na2CO3, for 24 h, filtration, HC1 addition, Recovery of precipitate by filtration, redissolution in 2% Na2CO3</td>
<td>Hoagland and Lieb, 1915 [4]</td>
</tr>
<tr>
<td>1931</td>
<td>Laminaria digitata</td>
<td>Substantial amounts of calcium sulfate and uronic acid</td>
<td>Soaking of the seaweed in water, precipitation of crude, sulfated polysaccharides by ethanol</td>
<td>Bird and Hua, 1931 [5]</td>
</tr>
<tr>
<td>1931</td>
<td>Macrocystis pyrifera</td>
<td>Methylpentose monosulfate polymer with fucose and alginate contaminants</td>
<td>Repeated extraction with 2% HC1, at room temperature for 48 h, precipitated with 90% ethanol</td>
<td>Nelson and Conacher, 1931 [6]</td>
</tr>
<tr>
<td>1937</td>
<td>Laminaria digitata</td>
<td>Proposed (R-R-O-S-H-O-M)- with R = fucose, R’ as unknown, M being Na, K, Ca0.5, or Mg0.5</td>
<td>Precipitation of droplets created from seaweed in boiling ethanol</td>
<td>Lands et al., 1937 [7]</td>
</tr>
<tr>
<td>1950</td>
<td>Fucus vesiculosus, F. spirales, H. lorea, L. ciliata</td>
<td>Substantial amounts of fucose and sulfate; small amounts of uronic acid, galactose and xylose; metals and ash were also detected, ash was mainly calcium sulfate</td>
<td>Aqueous extraction at -100 °C for 24 h, extract treated with lead acetate (to precipitate alginate and protoplasts), filtration solution treated with Ba(OH)2, to precipitate a &quot;hydroxide-fucoidin complex&quot;</td>
<td>Penczel and Ross, 1950 [8]</td>
</tr>
<tr>
<td>1952</td>
<td>Fucus vesiculosus</td>
<td>Fucose, ash, sulfate</td>
<td>0.1 M HCl at pH 2.2-2.5 amid 70 °C for 1 h, 3-times, fractional precipitation with ethanol</td>
<td>Black et al., 1952 [9]</td>
</tr>
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</table>

The early FCSPs extraction procedures were based on using a dilute acid treatment, with either acetic or hydrochloric acid used as a first "extraction" step with the purpose of hydrolyzing the non-FCSP polysaccharides (Table 1). However, the extraction and purification methodologies employed in...
different studies to isolate fucoidan/FCSPs from brown seaweed biomass have been modified to different extents since the first reports from 1913 and 1915. Bird and Haas (1931) [5], for example, used soaking of the brown alga biomass in water and precipitation of crude sulfate from the extract with ethanol to obtain fucoidan from *L. digitata* (Table 1). The product of this extraction was also found to contain relatively high levels of ash. The high ash levels were presumably chiefly a result of the presence of calcium sulfate in the algal polysaccharides. For the compositional analysis, acid hydrolysis using H$_2$SO$_4$ was typically used. This acid hydrolysis step might by itself have contributed a substantial amount of sulfate. As a consequence, the use of H$_2$SO$_4$ clearly biased the interpretation of the compositional analysis. Hence, the fucoidan isolated by Bird and Haas (1931) [5] was designated as carbohydrate sulfate (i.e., containing esterified sulfate) since the total sulfate content was approximately the double of that found in the ash. Uronic acid was also present in the FCSPs preparation extracted from *L. digitata* (Table 1).

Nelson and Cretcher (1931) [6] extracted fucoidan from *Macroystis pyrifera* by repeated, extended (48 h) extraction with dilute HCl followed by isolation of the FCSPs by ethanol precipitation, and revealed the presence of sulfate in the form of ester groups in the precipitated product. They also confirmed that fucose was the only sugar identified in the unhydrolyzed residue after acid hydrolysis, even though their product contained uronic acid, considered to be due to alginate contamination (Table 1). Later, Lunde et al. [7] prepared fucoidan by directly precipitating the droplets exuded from freshly gathered *L. digitata* fronds in ethanol (Table 1). After purification via precipitation of the product from boiling ethanol they obtained a FCSPs specimen that contained 33–37% methylpentose (interpreted correctly as fucose), and 26–30% ash in which the sulfate content was 17–19%, which made up approximately half of the total sulfate estimated in the polysaccharide (35–38%). They proposed a structural unit formula, (R-R$'$-O-SO$_2$-OM$_2$)$_n$ for fucoidan and suggested that R was fucose or another pentose sugar residue, R$'$ was unknown, and M was Na$^+$, K$^+$, (½)Ca$^{2+}$ or (½)Mg$^{2+}$ [7] (Table 1).

"In an attempt to reconcile some of the conflicting views on the nature of fucoidin" crude fucoidan extracts from *F. vesiculosus, F. spirales, Himanthalia lorea* and *Laminaria clustoni* were prepared by Percival and Ross (1950) [8]. Their methodology involved boiling of the seaweed biomass in neat boiling water for 24 h (hydrolysis treatment) followed by removal of alginates and protein by addition of lead acetate, then, after addition of barium hydroxide (presumably to precipitate alginate) the fucoidan was isolated as a crude lead hydroxide complex (Table 1). In order to isolate lead free fucoidan, the lead hydroxide complex was treated with dilute H$_2$SO$_4$ and fucoidan was then isolated after prolonged dialysis and filtration. The purest fucoidan specimen obtained was from *H. lorea*. This fucoidan isolated from *H. lorea* contained substantial amounts of fucose and sulfate, as well as small quantities of uronic acid, galactose and xylose. Metals and ash were also detected, and the ash was mainly made up of calcium sulfate [8]. Based on the data obtained the authors believed that the principal constituent of fucoidan was a polysaccharide with one sulfate substitution on each fucose residue and that other constituents arose from adventitious impurities. A parallel paper was published in which it was proposed that the core structure of fucoidan from *F. vesiculosus* was built of 1$\rightarrow$2 linked $\alpha$-fucopyranosyl units, each carrying a sulfate group on C-2 [8]. As discussed later, the interpretation that the fucosyl units in the fucoidan were 1$\rightarrow$2 linked turned out to be incorrect, as more advanced...
analyses have now verified that the backbone of fucoidan from *F. vesiculosus* consists of alternating α(1→3) and α(1→4) linkages [11].

In the pursuit to obtain extensive quantities, a laboratory-scale extraction of fucoidan was reported by Black *et al.* [9]. Interestingly, the extracted product was referred to as a "polysaccharide ethereal sulphate occurring in the Phaeophyceae". Their optimal fucoidan extraction procedure was as follows: One part by weight of dried ground seaweed and 10 parts by volume of 0.1 M hydrochloric acid at pH 2.0–2.5 contacted at 70 °C with constant stirring for 1 h. A single acid hydrolysis extraction treatment using this method recovered about 50% by weight (w/w) of the theoretical maximum of fucoidan (recovered yield measured as % fucose obtained as % of total fucose in the seaweed dry weight), whereas three rounds of the acid extraction recovered more than 80% of the fucose present; the triple acid hydrolysis treatment (0.1 M HCl, pH 2.0–2.5, 70 °C, 1 h = 3) was therefore selected as the optimal extraction method (Table 1). After the acid hydrolysis treatment the crude fucoidan was isolated by fractional precipitation with alcohol and further purified by precipitation after addition of formic acid [9]. By this procedure samples of crude fucoidan containing 30–35% fucose were obtained; for example, the fucoidan recovered from *F. vesiculosus* using the optimal extraction protocol was analyzed to contain (by weight) 44% fucose; 26% total sulfate, and 31% ash [9].

In terms of yields, calculated as fucose as % of total fucose, the results obtained for the four different algal species were: *Pelvetia canaliculata* 76%; *F. vesiculosus* 62%, *Ascophyllum nodosum* 54%, and *L. cloustoni* 20% [9]. The authors suggested that a more efficient extraction methodology, *i.e.*, extracting higher fucose yields, could be achieved by increasing the water/seaweed ratio, extraction time or number of extractions.

In a study about 20 years later, FCSPs from the brown seaweed *Himanthalia loesel* *Bifurcaria bifurcata* and *Padina pavonia* were extracted successively using dilute acid, followed by alkaline or neat water extraction, acid, and alkali in sequence [12] (Table 2). Prior to extraction, the seaweed fronds were treated with formaldehyde to polymerize phenolic constituents which might otherwise contaminate the extracted saccharides [12]. This extraction protocol produced a complex mixture of glucans, fucose-containing polysaccharides, and alginic acid which could be separated by fractional precipitation with ethanol, calcium salts (CaCl₂) or by fractionation on resin columns. The FCSPs extracted in this fashion were reported to be heteropolysaccharides containing different levels of fucose, glucuronic acid, xylose, and esterified sulfate, together with traces of galactose [12]. The results also showed that the sulfate and uronic acid contents in the FCSPs separated from the aqueous calcium chloride and acid extracts varied significantly according to the seaweed species [12].

Another study was conducted on the purification of a sulfated heteropolysaccharide substance from *Sargassum linifolium* to elucidate its structural components [13]. The extraction was done using hydrochloric acid at pH 1.0 for 3 h at 80 °C (Table 2); the extract was then neutralized with aqueous sodium carbonate and precipitated with ethanol [13]. The resulting sulfated polysaccharide material, termed "sargassan", was proposed to be built of glucuronic acid, mannose, and galactose residues with partially sulfated side-chains composed of galactose, xylose and fucose residues [13]. As discussed later, we now know that *Sargassum* spp. do indeed contain highly complex FCSPs structures built from this array of monosaccharides.

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Table 2. Extraction methods and reported chemical composition of different brown seaweed species and their corresponding order.

<table>
<thead>
<tr>
<th>Species</th>
<th>Order</th>
<th>Extraction method</th>
<th>Composition</th>
<th>Reference</th>
</tr>
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<tr>
<td>Fucus evanescens</td>
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<td>Seaweed-H$_2$O suspension was treated with 30% HCl (pH 3) at 100 °C for 15 min. Supernatant was neutralized with NaOH, precipitated with CaCl$_2$ and EtOH/Me for 20 h at 4 °C; precipitate was dissolved with H$_2$O and then dried</td>
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<td>Pentec et al., 2003 [15]</td>
</tr>
<tr>
<td>Sargassum sp.</td>
<td>Fucales</td>
<td>Extracted at rt and then 70 °C with 0.01 NaCl containing 1% CaCl$_2$</td>
<td>fucose, xylose, uronic acid and sulfate</td>
<td>Mass and Percival, 1973 [12]</td>
</tr>
<tr>
<td>Sargassum linifolium</td>
<td>Fucales</td>
<td>Extracted with hot water and dilute alkali, formamide treatment, then extracted with ammonium oxalate-oxalic acid for 6 h at 80 °C</td>
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</tr>
<tr>
<td>Ascophyllum nodosum</td>
<td>Fucales</td>
<td>Extracted with 0.01 M HCl at 90 °C for 4 h, single-step</td>
<td>fucose, rhamnose, galactose, glucose, mannose, xylose, uronic acid and sulfate</td>
<td>Duarte et al., 2001 [18]</td>
</tr>
<tr>
<td>Sargassum sternophyllum</td>
<td>Fucales</td>
<td>Extracted with water 3% w/v mL, 12 h, 3×. Precipitated with EtOH and CaCl$_2$ and cetylpyridinium chloride. Soluble fraction (SF) was then fractionated (F1–F6)</td>
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</tbody>
</table>
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Table 2. Cont.

| Laminaria saccharina; Laminaria digitata; F. vesiculosus | Fucales | Powdered seaweed was extracted with CHCl3–MeOH–H2O (2:4:1) followed by 80% EtOH, then extracted successively with 2% CaCl2 at 20 and 70 °C, then with HCl (pH 2) and 3% Na2CO3, precipitated with calcium salt, transformed into calcium salts, and an alkaline treatment to remove acetyl groups and to transform fucoidan into sodium salts [24]. | fucose, xylose, mannose, glucose, galactose, uronic acid and sulfate | Chidiner et al., 1999 [25]. |
| Undaria pinnatifida | Laminariales | Ground seaweed extracted twice at rt for 6 h with 1% H2SO4 neutralized with 10% NaOH and dried | fucose, xylose, mannose, glucose, galactose and sulfate | Hemmingson et al., 2006 [26]. |
| Laminaria religiosa | Laminariales | Water extraction at boiling temp. for 4 h, fucoidan fraction was obtained by using 0.09 HCl at 4 °C for 2 h, then precipitated with 85% EtOH and dried | fucose, xylose, mannose, glucose, uronic acid and sulfate | Monyami and Vamanun, 1984 [27]. |

These early reports show that, with a few exceptions, treatment with dilute acid at ambient or slightly elevated temperature has been a preferred first step in extraction protocols for isolating fucoidan or FCSPs from different types of brown seaweeds (Tables 1 and 2). The use of different acids—or no acid at all—as well as the differences in extraction time and temperature during the extraction and further purification treatments have generated diverse compositional results and structural suggestions for fucoidan or FCSPs (Tables 1 and 2). The early reports almost unequivocally found that fucoidan mainly contained fucose and sulfate; nevertheless, the chemical composition of the most highly purified, but still crude fucoidan specimen from Hizikia fusiforme contained fucose, galactose, xylose, rhamnose, uronic acid and sulfate [8,14]. In the more recent reports, a pretreatment of the seaweed biomass prior to the real extraction treatment has been found to be advantageous to eliminate low molecular components (e.g., phenols); as already mentioned above, an early study used formaldehyde pretreatment [12]. However, more recent reports show that the pretreatment typically involves the use of a mixture of methanol, chloroform and water [22]. Removal of protein has also been considered. This can be facilitated via the use of proteolytic enzymes [28] (or by lead acetate treatment, as used by Percival and Roos in 1950 [8]). Another useful purification procedure has involved transformation of alginate
in the residual biomass into insoluble calcium salts by treatment of the FCSPs specimen with aqueous calcium chloride [19,20].

In conclusion, the use of an array of different extraction and purification techniques appear to have contributed to the confusion that has prevailed about the nature and composition of fucoidan and FCSPs ever since fucoidan was first described by Kylin early in the 20th century [2]. As detailed in the following, we now know that the initial suggestions [29,30] that fucoidan was built of \( \alpha(1 \rightarrow 2) \) linked \( \alpha \)-fucopyranosyl residues were wrong. Fucoidan is built of \( \alpha(1 \rightarrow 3) \)-linked \( \alpha \)-fucopyranosyls or of alternating \( 1 \rightarrow 3 \) and \( 1 \rightarrow 4 \)-linked \( \alpha \)-fucopyranosyl residues that may be sulfate substituted.

We also know that “fucoidan” isolated from certain brown algae have completely different structures being composed of sulfated galactofucans with backbones of \( \beta(1 \rightarrow 6) \)-linked \( \beta \)-fucopyranose and/or \( \beta(1 \rightarrow 2) \)-\( \beta \)-D-manno- pyranosyl units with \( (1 \rightarrow 3) \) and/or \( (1 \rightarrow 4) \)-\( \alpha \)-L-fucopyranosyl-galactoside branching. The available data thus show that the term “fucoidan” has been used for several different chemical structures and vice versa that fucoidan is a term that covers a diverse family of fucose-containing sulfated polysaccharides (Table 1). It is therefore more correct to use the term fucose-containing sulfated polysaccharides (FCSPs) rather than fucoidan as a collective term for these polysaccharides.

3. Taxonomic Comparison of Fucoidan or FCSPs Structure

3.1. Fucales

In 1993 a revised structure of the polysaccharide backbone of the main FCSP product from \( F. \) vesiculosus was presented as \( \alpha(1 \rightarrow 3) \) linked instead of as \( \alpha(1 \rightarrow 2) \) linked [31]; it was also reported that fucose was attached to the backbone fucan polymer to form branching points, typically one for every 2–3 fucose residues within the chain, still with sulfate groups at position C-4 on the fucose units [31]. However, detailed analysis of the methyl derivatives obtained from partially desulfated \( F. \) vesiculosus polysaccharides revealed the presence of \( 2,4 \)-di-O-methylfucose as well as \( 2,3 \)-di-O-methylfucose which indicated the presence of both \( \alpha(1 \rightarrow 3) \) and \( \alpha(1 \rightarrow 4) \) linked fucose residues [32] (Figure 2). A similar structure was also determined for a FCSPs-derived oligosaccharide of about 8–14 monosaccharide units extracted from \( Ascophyllum nodosum \) (Fucales) [11] (Figure 2).

More recently, several studies—using highly advanced analytical methods—have documented that fucoidan from brown seaweed in the order of Fucales such as \( F. \) evanescens and \( F. \) serratus do indeed contain large proportions of both \( \alpha(1 \rightarrow 3) \) and \( \alpha(1 \rightarrow 4) \) glycosidic bonds [20–22] (Figure 2). Structural analysis of a depolymerized low molecular weight fraction of fucoidan from \( F. \) evanescens by MALDI-TOF and tandem ESI mass spectrometry has moreover shown that this fraction contains oligosaccharides with and without sulfate substitutions and that it mainly consists of \( \alpha(1 \rightarrow 3) \)-linked fucose residues being esterified with sulfate at C-2 [33]. This more detailed analysis has also revealed the presence of minor components of mixed monosulfated fucoligosaccharides containing both \( 2 \)-O- and \( 4 \)-O-sulfated \( (1 \rightarrow 4) \) bonded xylose and galactose residues: \( \text{Xyl-(1 \rightarrow 4)-Fuc, Gal-(1 \rightarrow 4)-Fuc, Gal-(1 \rightarrow 4)-GalGal-(1 \rightarrow 4)-Fuc, Gal-(1 \rightarrow 4)-GalGal-(1 \rightarrow 4)-Gal} \) [24]. Glucuronic acid (GlcA) was also detected as being a part of the non-sulfated fucoligosaccharides: \( \text{Fuc-(1 \rightarrow 3)-GlcA, Fuc-(1 \rightarrow 4)-Fuc-(1 \rightarrow 3)-GlcA, Fuc-(1 \rightarrow 3)-Fuc-(1 \rightarrow 3)-GlcA} \) respectively [33].
Brown seaweed species in the order of Fucales have also been reported to contain very complex FCSPs structures having fucose and galactose in comparable amounts; these structures are generally referred to as sulfated galactofucans and are predominantly found among Sargassum species [18,34,35]. These sulfated galactofucans are mainly built of (1→6)-β-D-galactose and/or (1→2)-β-D-mannose units with branching points formed by (1→3) and/or (1→4)-α-L-fucose, (1→3)-α-D-glucuronic acid, terminal β-D-xylose and sometimes (1→3)-α-D-glucose [18]. Early studies also reported the existence of fucoglucuronans having a backbone of glucuronic acid, mannose and galactose residues with side chains of neutral and partially sulfated residues of galactose, xylose and fucose; notably present in *Sargassum tinctorium* [13]. More recently, the FCSPs of this type extracted from the brown seaweed *Sargassum stenophyllum* (Fucales) were grouped into two different types: type I was found to contain a relatively high percentage of α-D-glucuronic acid and relatively few sulfate groups, while type II contained relatively small amounts of α-D-glucuronic acid and a high percentage of sulfate [18]. The type I polysaccharides were composed of a linear backbone formed mainly by (1→6)-β-D-galactose and/or (1→2)-β-D-mannose with branching chains formed by (1→3) and/or (1→4)-α-L-fucose, (1→3)-α-D-glucuronic acid, while in the type II polysaccharides the backbone was mainly built of short galactan chains [18].

Corresponding structures were observed in FCSPs fractions from *Hizikia fusiforme* a.k.a. *Sargassum fusiforme* (Fucales). These structures were separated by ion exchange chromatography after the FCSPs had been obtained via hot aqueous extraction, followed by ethanol and CaCl₂ precipitation (Table 2). These chromatographically purified fractions predominantly contained fucose, mannose, galactose, uronic acid and sulfate [25] (Figure 3). In accordance with the findings of Duarte et al. [18]

**Figure 2.** Typical structure of fucoidan (FCSPs) obtained from some brown seaweed species in the order of Fucales. The 1-fucopyranose backbone of the fucoidan (FCSPs) extracted from *Hizikia fusiforme* and *Fucus vesiculosus* is connected by alternating (1→3) and (1→4) linkages [11]. The FCSPs from *Fucus evanescens* have a similar backbone built up with sulfate substituted at the 2- and 4-position of the fucose residues [20] (only sulfate substitutions on C-2 of fucose are shown in the figure). Acetate substitutions may also be found at the C-4-position of 3-linked fucose and at C3 of 4-linked fucose units [22] (acetate substitutions not shown in the figure). For *F. serratus* L., a possible fucoside side chain at C-4 is also shown.
the structural analysis of one of the main fractions purified by ion exchange chromatography indicated
that the sulfate groups might be found in any position on the galactose/mannose backbone or on the
fucose units (Figure 3). The sulfate groups in the FCSPs in this fraction, which had been isolated after
3 rounds of extraction in hot water (70 °C), then ethanol and CaCl2 precipitation prior to chromatography
(Table 2), were mainly found at C-6 of [(1→2,3)-Man-(1→)], at C-4 and C6 of [(1→2)-Man-(1→)] and at
C-3 of [(1→6)-Gal-(1→)] [23]. On the fucose, the sulfate groups were substituted at C-2, C-3 or C-4,
while some fucose residues had two sulfate groups [23]. The core of these S. fusiforme FCSPs was
mainly composed of alternating units of [(1→2)-D-Man-(1→)] and [(1→4)-β-D-GlcA-(1→)], with a minor
portion of [(1→4)-β-D-Gal-(1→)] units, and the branching points were at C-3 of [(1→2)-Man-(1→)],
C-2 of [(1→4)-Gal-(1→)] and C-2 of [(1→6)-Gal-(1→)], respectively [23] (Figure 3).

Figure 3. Suggested structures of the FCSPs (fucoidan) from H. fusiforme [23] also known
as Sargassum fusiforme (Fucales); sulfate substitutions not shown. The structures also
represent typical FCSPs structures from other Sargassum spp. [18].

3.2. Laminariales and Other Brown Seaweed

Various structures of FCSPs from brown seaweeds of the order of Laminariales have also been
reported [36]. The available data indicate that the FCSPs derived from this seaweed order contain
small amounts of other monosaccharides besides fucose. Interestingly, polysaccharides containing
significant amounts of fucose and galactose and which seem to be compositionally and structurally
similar to the fucoidan from Fucales brown seaweeds appear to be prevalent [26,35]. Structural
analysis was conducted on the FCSPs from the sporophyll Undaria pinnatifida (Laminariales) and it
was found that the FCSPs had a high fucose/galactose ratio, high uronic acid, and low sulfate content. The most abundant fucopyranosyl units were substituted at the C-2, C-3, or C-2, C-3, C-4-positions whereas fucose residues with substitutions at the C-3, C-4- or C-4-positions were less abundant [26]. The galactopyranosyl units were predominantly substituted at the C-3- or at both the C-3, C-4-positions [26].

FCSPs isolated from Chorda filum (Laminariales) have been shown to consist of a poly-α-(1→3)-fucopyranosyl backbone with a high degree of branching mainly as α-(1→2)-linked single α-L-fucopyranosyl residues (Figure 4) [24,25]; the fucopyranosyl residues were found to be sulfated mainly at C-4 and sometimes at the C-2 position, whereas some of the α-(1→3)-linked fucose residues were shown by NMR to be C-2 acetylated [25]. A similar structure has been reported by Usas et al. [24] for the FCSPs isolated from L. saccharina (Laminariales) which are mainly built of (1→3)-linked α-L-fucopyranose with sulfation at C-4 and sometimes at the C-2 position or with possible α-L-fucopyranosyl at C-2 (Figure 4). This FCSPs structure has also been found to be present in the body wall layer of the sea cucumber Ludwigothurea grisea (a marine invertebrate). The FCSPs of the sea cucumber body wall are essentially built of an α-(1→3)-fucopyranose backbone [37]. NMR analysis has indicated that 2,4-di-sulfato-L-fucopyranose and unsubstituted fucopyranose are present in equal proportions, and that 2-mono-sulfato-L-fucopyranose is present in twice that proportion [33]. The FCSPs from Lessonia vadosa (Laminariales) have also been studied by NMR spectroscopy and the data indicate that the polysaccharides are mainly composed of α-(1→3)-linked fucopyranose residues sulfated mainly at position C-4 and partially at position C-2 [38].

**Figure 4.** Structural motifs of FCSPs (fucoidan) from some brown seaweed species of the order Laminariales and Chordariales. FCSPs of Chorda filum and Laminaria saccharina consist of a poly-α-(1→3)-fucopyranosyl backbone with sulfate mainly at C-4 and sometimes at the C-2 position; some of the backbone fucose residues may be acetylated at C-2 (not shown) [24,25]. Cladophoropsis okamurae derived FCSPs also consist of a backbone of α-(1→3)-linked α-L-fucopyranose residues with sulfate substitutions at C-4 and/or with α-(1→2)-linked single α-L-fucopyranosyl substitutions and vicinal glucuronic acid substitutions. Some of the side chain fucose residues may be O-acetylated (not shown) [14].

Other algal fucoidans whose structures contain the same α-(1→3)-backbone of fucose residues have been found in Analipus japonicas (Ectocarpales), Adencystis utricularis (Ectocarpales) and...
higher fucose contents in the products were obtained with shorter extraction time [19]. The work also obtained [19] (Table 2). All extraction factors affected the FCSPs yield. Lower total FCSPs yields, but

While doing so, we also systematically examined the effects of different extraction parameters (Table 2). The detailed structural elucidation of the FCSPs from C. okamuranus (Chordariales) confirmed that this product was made up of a linear backbone of (1→3)-fucopyranose units with a portion of the fucose residues carrying sulfate substitutions at C-4 but some of the fucose residues have also been found to be O-Acetylated (Figure 4). The C. okamuranus FCSPs may also contain α-gluconic acid substitutions at the C-2-positions of those backbone fucose residues that are not substituted by a sulfate group [14] (Figure 4).

Methylation analysis, desulfation and NMR spectroscopy of the FCSPs fractions from Adenocystis utricularis (Ectocarpales) showed that these FCSPs contained the same α-(1→3)-fucopyranose backbone as that found in Chorda filum and Laminaria saccharina FCSPs, and that the fucopyranosyl units were mostly sulfated at C-4, and branched at C-2 with non-sulfated fucopyranosyl units; the galactan moiety, which was also present, was predominantly found to be a backbone structure of (1→3) and (1→6) α-galactopyranose units with sulfation mostly on C-4 [15]. Later, a similar structure was found in FCSPs extracted from Analipus japonicas (Ectocarpales) [39].

The relatively large variations in the reported compositional and structural properties of the FCSPs extracted from different brown seaweed species thus clearly confirm the natural biodiversity of FCSPs notably as exemplified by the structures found in Fucales, e.g., in the Fucus sp. and Sargassum sp. (Figures 2 and 3). The (1→3)-linked α-L-fucopyranosyl backbone structure, with various extents of sulfate substitutions, is prevalent as the core backbone structure in the majority of the currently analyzed FCSPs. Nevertheless, the reported structural data for FCSPs from different brown seaweed species clearly indicate that there is no consistent basic structure of “fucoidan”. It also seems clear that FCSPs extracted from seaweeds under the same order have different composition, and in turn that the structural traits of FCSPs cannot be categorized or predicted according to algal order (Tables 1 and 2).

When assessing the available compositional data the large variation in the composition of the FCSPs products obtained from different extraction methods is evident (Table 2). Recently, we optimized the extraction yields of FCSPs from Sargassum sp. by developing a single-step extraction procedure. While doing so, we also systematically examined the effects of different extraction parameters (i.e., acid concentration, time, and temperature) on the yields and composition of the FCSPs products obtained [19] (Table 2). All extraction factors affected the FCSPs yield. Lower total FCSPs yields, but higher fucose contents in the products were obtained with shorter extraction time [19]. The work also revealed that classical extraction treatment with HCl at elevated temperature and during extended time, i.e., a procedure similar to the one used by Black et al. [9], had a detrimental effect on the FCSPs yield as this treatment apparently disrupted the structural integrity of the fucose-containing polymer and caused degradation of the chains built of fucose residues [19]. Hence, some of the classic methods, employing relatively harsh acid treatments, may in fact have affected the composition and structure of the target FCSP products to different extents, and may have contributed to the prevailing “conflicting views on the nature of fucoidin” recognized already in 1950 by Percival and Ross [8]. A consensus to employ defined extraction protocols for extraction of FCSPs, or at least an agreement among scientists in the field to include a benchmark extraction procedure in their studies, would help to advance the understanding of these intriguing FCSPs substances.

Cladosiphon okamuranus (Chordariales) [14,15,39], but the FCSPs from these brown algal also appear to contain other monosaccharides than fucose (Table 2). The detailed structural elucidation of the FCSPs from C. okamuranus (Chordariales) confirmed that this product was made up of a linear backbone of (1→3)-fucopyranose units with a portion of the fucose residues carrying sulfate substitutions at C-4 but some of the fucose residues have also been found to be O-Acetylated (Figure 4). The C. okamuranus FCSPs may also contain α-gluconic acid substitutions at the C-2-positions of those backbone fucose residues that are not substituted by a sulfate group [14] (Figure 4).

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4. Bioactivity of Fucoidan or FCSPs

Several different therapeutic strategies such as chemotherapy, radiation therapy, surgery or combinations hereof have been used to treat different types of cancer. Unfortunately, several of these treatments provide only minimal benefit; moreover, there are undesirable complications and long term side effects of the treatments [52,53]. Consequently the quest for potential preventive or therapeutic measures against cancer has been going on for years and recently the focus has been directed towards bioactive compounds of natural origin, including FCSPs from brown seaweeds [1]. Many reports have been published which indicate the antitumor and immune-response modulating activity of FCSPs in both in vivo and in vitro studies [40–42,54,55]. Sulfated polysaccharide fractions from Sargassum fulvellum, S. kjellmanianum, L. angustata, L. angustata var. longissima, L. japonica, Ecklonia cava, and Eisenia bicyclis have been evaluated for their bioactivities, and they have been found to exert remarkable growth inhibitory activities on Sarcoma-180 cells implanted into mice and to possess antitumor activity against L-1210 leukemia in mice [56–58]. Recently, we reported the potent in vitro bioactivity of FCSPs extracted from Sargassum sp. and F. vesiculosus against lung and skin cancer cell growth [42]. The antitumor mechanism of FCSPs from sporophylls of Undaria pinnatifida has been described by Maruyama et al. [41,59]. The available findings indicate that antitumor activity of FCSPs may be associated with a significant enhancement of the cytolytic activity of natural killer (NK) cells augmented by increased production of macrophage-mediated immune response signaling molecules [59–61], namely interleukins (IL-2, IFN-γ and IL-12 [42,59], and induction of apoptosis [42].

Macrophage activation by polysaccharides is mediated through specific membrane receptors. The major receptors reported for polysaccharides recognition in macrophages are glycoproteins including...
Toll-like receptor-4 (TLR-4), cluster of differentiation 14 (CD14), competent receptor-3 (CR-3) and scavenging receptor (SR) [61]. Activation of these receptors is mediated by intracellular signaling pathways and the family of mitogen-activated protein kinases (MAPKs) plays a critical role notably in the production of nitric oxide (NO) which can lyse tumors [61]. MAPK family members such as p38 MAPK, extracellular regulated kinase (ERK1/2) and stress-activated protein kinase/c-Jun-N-terminal kinase play an important role in the activation of macrophages by polysaccharides such as FCSPs [61,62] (Figure 5A). Activated MAPKs lead to activation of transcription factors resulting in induction of various genes [61]. Activation of macrophages induces the production of cytokines such as interleukin-12 (IL-12) which in turn stimulate the development of T-cells (Figure 5B). T-cells produce interleukin-2 (IL-2) that in turn activates NK cells proliferation. The NK cells themselves produce immunologically important cytokines, notably IFN-γ, which can further provoke the participation of macrophages in the stimulation of T-cell via induction of IL-12 [41,59] (Figure 5B).

NK cells appear to represent a first line of defense against the metastatic spread of blood-borne tumor cells, and normal NK activity may be important in immune surveillance against tumors [63]. NK-mediated killing of target cells by apoptosis is facilitated by activation of caspase cascades (Figure 5B). In tumor bearing mice, FCSPs appear to act as an immunopotentiator leading to increased antitumor effectiveness as exhibited by increased immune response against A20 leukemia cells and a lowering of the tumor size in transgenic (DO-11-10-Tg) mice [41]. Moreover, recent investigations of the immunomodulatory activity of FCSPs in rats with aspirin-induced gastric mucosal damage suggest that the gastro-protective effect of fucoidan against aspirin-induced ulceration may take place through the prevention of elevation of pro-inflammatory cytokines, IL-6 and IL-12 [64].

**Figure 5.** Proposed mechanism responsible for fucoidan bioactivity: (A) Macrophage activation by FCSPs as mediated through specific membrane receptor activation namely TLR-4, CD14, CR-3 and SR which in turn induce intracellular signaling via mitogen-activated protein kinases (MAPKs); (B) Activation of macrophages lead to production of cytokines such as IL-12, IL-2 and IFN-γ which enhance NK cell activation that may stimulate T-cell activation further via IFN-γ.
Apoptosis is one of the most prevalent pathways through which FCSPs can inhibit the overall growth of cancer. Previous studies have shown that different types of FCSPs can induce apoptosis in melanoma cells [42], HT-29 colon cancer cells [65], MCF-7 human breast cancer cells [66], and HS-Sultan human lymphoma cells [62]. In human HS-Sultan cells, the apoptosis may occur via activation of caspase-3 [62], and in MCF-7 cells via caspase-8 dependent pathways [66]. Alternatively, FCSPs induced apoptosis may take place through activation of caspases via both death receptor-mediated and mitochondria-mediated apoptotic pathways [65].

4.2. Anticoagulant and Antithrombotic Activities

The earliest published report describing the anticoagulant activity of fucoidan was published in 1957 [67]. In that report it was shown that a certain fraction of fucoidan from *F. vesiculosus* possessed powerful anticoagulant activity that qualified fucoidan to belong to the group of heparins [67]. Heparin is a biomolecule containing highly sulfated glucosaminoglycan that is widely used as an injectable anticoagulant. It has been reported that the anticoagulant mechanisms of fucoidan are related to both antithrombin and heparin cofactor II-mediated activity [68,69], but the mechanisms by which fucoidan exerts anticoagulant activity remain controversial [32]. Hence, any possible relations between the physical and chemical properties, the structure, and the anticoagulant activity of fucoidan remain to be firmly established. The uncertainties are mainly due to the structural variation of fucoidan between algal species, but most likely also a result of the different extraction methodologies employed to isolate FCSPs that appear to have produced FCSPs of different composition, structure, and size, which have given rise to conflicting results in the detailed studies of mechanisms of anticoagulant activity [32,70].

Results obtained using the so called activated partial thromboplastin time assay (APTT) have strongly indicated that FCSPs from *F. vesiculosus* have specific anticoagulant activity. Comparable results have been obtained in two independent studies using FCSPs dosages equivalent to 9–13 U/mg versus 167 U/mg for heparin; and 16 U/mg versus 193 U/mg heparin, respectively [71,72]. When FCSPs samples isolated from nine brown seaweed species were tested for anticoagulant activities, the APTT results were significant at 12–38 U/mg as compared to at 167 U/mg for heparin [73]. A remarkable finding for anticoagulant action was also reported by Kitamura et al. [74], who showed that a FCSPs fraction from *L. umbilicalis* var. *longissima* (Laminariales) had antithrombin activity at 200 U/mg, equivalent to a dose of 140 U/mg heparin. The particular FCSPs fraction having anticoagulant activity had a molecular weight of ~21–23 kDa and contained fucose-galactose-sulfate at a ratio of 9:1:9 with the sulfate substitutions at C-4 of the fucose residues [74].

It has been postulated that it is not a specific structural trait that determines fucoidan’s ability to elicit anticoagulant activity, but rather that the anticoagulant effect is due to a multitude of structural features including monosaccharide composition, molecular weight, sulfation level, and position of sulfate groups on the main chain of the polysaccharide [69,75–77]. The comprehensive study of the anticoagulant activity of fucoidan by Cumashi et al. [21] also noted that neither the content of fucose and sulfate nor other structural features affected the anticoagulant efficacy. FCSPs from *C. okamurae* (Chordariales) have been reported to exert virtually no anticoagulant effect, and this could be due to the low amount of sulfate in its polymer backbone and/or the presence of vicinal branching points formed by 2-O-acetylglucuronyl substituents (Figure 4) [21,14]. On the other hand, the concentrations
of C-2-sulfate and C-2,3-dissulfated sugar residues (Figure 2) have been reported to be a common structural feature for fucoidan anticoagulant activity [11]. The anticoagulant activity of high molecular weight FCSPs from *Ecklonia kurome* were thus reported to be dependent on both molecular weight and sulfate content [76]: Higher molecular weight FCSPs (i.e., 27 and 58 kDa) showed higher anticoagulant activity than lower molecular weight FCSPs (i.e., ≤10 kDa); and FCSPs samples having a high molar ratio of sulfate to total sugar residues were found to exhibit inhibitory effects on fibrinogen clotting by thrombin reaction [76]. These results were supported by data reported by Haroun-Bouhedja et al. [78] who reported a relationship between the extent of sulfate group substitutions and the biological activities of fucoidan. The anticoagulant activity of low molecular weight (LMW) fucoidan, i.e., MW < 18 kDa was thus found to decrease with decreasing degree of sulfation, and very low-sulfate (<20%) or desulfated LMW fucoidan lost its anticoagulant activity, but retained some antiproliferative activity on CCL39 fibroblast cells [78]. In contrast, LMW fucoidan with sulfate content higher than 20% was found to exert profound anticoagulant activity as well as antiproliferative effects on fibroblast cell line CCL39 cells in a dose-dependent fashion [78].

Some studies suggest that also the sugar composition (e.g., fucose, galactose, mannose, etc.) or the type of oligo- or polysaccharides of the FCSPs may play an important role for anticoagulant activity [75,79]. However, the series of investigations conducted by Pereira et al. [32,80,81] indicated that a 2-sulfated, 3-linked α-L-galactan, but not α-L-fucan, was the potent thrombin inhibitor mediated by anti-thrombin of heparin cofactor II. These findings have however also pointed out that it is not necessarily the sugar composition but rather the sulfate substitutions on the sugars that determine the anticoagulant activity of fucoidan—or both [82].

Most of the reported studies were carried out with crude, diverse and complex FCSPs obtained via extraction from brown seaweeds as opposed to being chemically well defined structures. For this reason it is not easy to identify a structure versus activity relationship because of the presence of highly branched portions and the complex distributions of sulfate and acetyl groups in algal FCSPs. This aspect was attempted resolved by use of invertebrate polysaccharides [83]. The data obtained indicated that regular, linear sulfated α-L-fucans and sulfated α-L-galactans express anticoagulant activity, which is not simply a function of charge density, but critically dependent on the pattern of sulfation as well as monosaccharide composition. Sulfated α-L-fucans and fucosylated chondroitin sulfate were also shown to elicit antithrombotic activity when tested on *in vivo* models of venous and arterial thrombosis in experimental animals [83].

### 4.3. Bioactivities and Oversulfation of FCSPs

In 1984 crude FCSPs fractions from *Sargassum kjellmanianum* were prepared in order to investigate the influence of the sulfation levels on the survival of L-1210 leukemia bearing mice; and on the growth of Sarcoma-180 cells [58]. The study showed that the fraction with the highest sulfation was the most effective against L-1210 leukemia bearing mice and it produced an increase in life span of 26%. On the other hand, this particular FCSPs fraction was also less effective in inhibiting growth of Sarcoma-180 cells subcutaneously implanted into mice [58].

Since then, several investigations have focused on the effect of oversulfation of FCSPs on biological activity [54,78,84–89]. Oversulfated FCSPs may be obtained by further sulfation of native FCSP of C-2-sulfate and C-2,3-dissulfated sugar residues (Figure 2) have been reported to be a common structural feature for fucoidan anticoagulant activity [11]. The anticoagulant activity of high molecular weight FCSPs from *Ecklonia kurome* were thus reported to be dependent on both molecular weight and sulfate content [76]: Higher molecular weight FCSPs (i.e., 27 and 58 kDa) showed higher anticoagulant activity than lower molecular weight FCSPs (i.e., ≤10 kDa); and FCSPs samples having a high molar ratio of sulfate to total sugar residues were found to exhibit inhibitory effects on fibrinogen clotting by thrombin reaction [76]. These results were supported by data reported by Haroun-Bouhedja et al. [78] who reported a relationship between the extent of sulfate group substitutions and the biological activities of fucoidan. The anticoagulant activity of low molecular weight (LMW) fucoidan, i.e., MW < 18 kDa was thus found to decrease with decreasing degree of sulfation, and very low-sulfate (<20%) or desulfated LMW fucoidan lost its anticoagulant activity, but retained some antiproliferative activity on CCL39 fibroblast cells [78]. In contrast, LMW fucoidan with sulfate content higher than 20% was found to exert profound anticoagulant activity as well as antiproliferative effects on fibroblast cell line CCL39 cells in a dose-dependent fashion [78].

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molecules using dimethylformamide as solvent and a sulfur trioxide-trimethylamine complex as the sulfitating agent [86]. The inhibitory effects of such oversulfated FCSPs were investigated on the invasion of Murine Lewis Lung Carcinoma cells through a reconstituted membrane basement fragment, so-called laminin [86]. Oversulfated FCSPs were found to be the most potent inhibitor of tumor cell invasion and were also, in particular, found to inhibit tumor cell adhesion to laminin better than native and desulfated FCSPs. The most potent oversulfated FCSP structures had sulfate groups on both the C-3 and C-4 positions of the fucose units; hence, the particular spatial orientation of the negative charges in the FCSPs molecules may also be an important determinant of bioactivity [86].

The study did not allow firm conclusions to be drawn with respect to mechanisms of action, but it was suggested that the increased negative charge resulting from oversulfation might promote the formation of FCSPs-protein complexes involved in cell proliferation, in turn suppressing cell growth [86].

When the importance of the spatial orientation of the negative charges on the FCSPs was investigated in more depth it was confirmed that this feature plays a major role in determining the binding potency of FCSPs to vascular endothelial growth factor 165 (VEGF165) [88]. Both native and oversulfated FCSPs have been tested for their anti-angiogenic actions in vivo and for their in vitro anti-proliferative effects against B16 melanoma cells, Sarcoma-180 and Lewis lung carcinoma cells. The interaction of oversulfated FCSPs with VEGF165 occurred with high affinity and resulted in the formation of highly stable complexes, thereby interfering with the binding of VEGF165 to vascular endothelial growth factor receptor-2 (VEGFR-2). The results showed that both native and oversulfated FCSPs were able to suppress neovascularization in mice implanted Sarcoma-180 cells; and that both FCSPs types inhibited tumor growth through the prevention of tumor-induced angiogenesis, but the data indicated that sulfation tended to give more potent effects [88].

Native and oversulfated FCSPs derived from Cladosiphon okamuranus (Chordariales) were analyzed using 1H NMR spectroscopy and it was suggested that whereas natural sulfation produced 4-mono-O-sulfato-L-fucopyranose the oversulfated FCSPs contained 2,4-di-O-sulfato-, 2-mono-, and 4-mono-O-sulfato-L-fucopyranose [89]. It was also suggested that sulfate content and the positioning of sulfate groups, e.g., 2,4-di-O-sulfato-L-fucopyranose, might be important for the anti-proliferative activity of fucoidan in a human leukemia cell line (U937), an effect which is presumed to take place via induction of apoptosis associated with activation of caspases-3 and -7 [89].

The effects of oversulfation of low and high molecular weight FCSP derivatives from F. vesiculosus and heparin on lipopolysaccharide (LPS)-induced release of plasminogen activator inhibitor-1 (PAI-1) from cultured human umbilical vein endothelial cells (HUVEC) were examined by Soeda et al. [87]. Their study demonstrated that all oversulfated FCSP derivatives including high molecular weight derivatives of 100–130 kDa were effective in suppressing the LPS-induced PAI-1 antigen, and supported an important role of the degree of sulfation for bioactivity [87].

The correlation of oversulfation and conformation of molecular sizes of FCSPs for anticancer activity using human stomach cancer cell lines AGS was evaluated recently for FCSPs isolated from dried Undaria pinnatifida FCSPs [54]. The data showed that the oversulfated, low molecular weight FCSP derivatives increased the inhibition of cell growth, while the growth inhibition was less for native, high molecular weight FCSPs and for oversulfated high molecular weight FCSPs [54]. The differences were suggested to be a result of the smaller molecular weight fractions having a less
compact conformation than the higher, which may have allowed a higher extent of sulfate substitution to occur during oversulfation.

5. Conclusions

Fucoidan—or FCSPs—are an important group of polysaccharides that show remarkable biological actions notably anticoagulant, antitumor and immune-response activities. Despite intensive research, the exact correlation between the bioactivity and the structural molecular features of FCSPs—which vary depending on seaweed species and extraction methodology—has yet to be clarified.

The preservation of the structural integrity of the FCSPs molecules nevertheless appears crucial for maintaining the biological properties and it has been clearly shown that the extraction treatment employed affects the composition and thus the structural features of the FCSPs substances.

The diverse structures and varied chemical composition of FCSPs may have hindered the development of an in-depth understanding of the precise properties of significance for specific bioactivity effects.

Important structural issues for bioactivity appear to include the degree of sulfation and the size of the FCSP molecules. Oversulfated FCSPs have thus been found to be potent inhibitors of tumor cell invasion compared to desulfated native FCSPs. Low molecular weight FCSPs have been shown to be effective in inhibiting human stomach cancer cell growth and to exert anticoagulant activity provided that the extent of the degree of sulfation was relatively high. Loss of anticoagulant activity has been observed with decreasing degree of sulfation, although anti-proliferative effects on fibroblast cell lines were retained.

Undoubtedly, the presence of impurities influences the biological properties of FCSPs and therefore may currently hinder our full understanding of the biological activity of fucoidan or FCSPs. Hence the development of standard extraction procedures for FCSPs including hydrolysis treatment, purification and fractionation methodology, preferably with specific steps adapted to the particular botanical order of the seaweed, will generate a better, common basis for analysis and understanding of bioactivities and the mechanisms determining the bioactivities of FCSPs. On this basis it may even be possible to target specific structural features and in turn tune the extraction procedure to obtain specific bioactivities via the use of targeted extraction methodologies.

Despite the availability of early, seminal studies of the extraction of FCSPs from brown seaweeds the understanding of the complex structures of FCSPs, is far from complete.

References


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compact conformation than the higher, which may have allowed a higher extent of sulfate substitution to occur during oversulfation.

5. Conclusions

Fucoidan—or FCSPs—are an important group of polysaccharides that show remarkable biological actions notably anticoagulant, antitumor and immune-response activities. Despite intensive research, the exact correlation between the bioactivity and the structural molecular features of FCSPs—which vary depending on seaweed species and extraction methodology—has yet to be clarified.

The preservation of the structural integrity of the FCSPs molecules nevertheless appears crucial for maintaining the biological properties and it has been clearly shown that the extraction treatment employed affects the composition and thus the structural features of the FCSPs substances.

The diverse structures and varied chemical composition of FCSPs may have hindered the development of an in-depth understanding of the precise properties of significance for specific bioactivity effects.

Important structural issues for bioactivity appear to include the degree of sulfation and the size of the FCSP molecules. Oversulfated FCSPs have thus been found to be potent inhibitors of tumor cell invasion compared to desulfated native FCSPs. Low molecular weight FCSPs have been shown to be effective in inhibiting human stomach cancer cell growth and to exert anticoagulant activity provided that the extent of the degree of sulfation was relatively high. Loss of anticoagulant activity has been observed with decreasing degree of sulfation, although anti-proliferative effects on fibroblast cell lines were retained.

Undoubtedly, the presence of impurities influences the biological properties of FCSPs and therefore may currently hinder our full understanding of the biological activity of fucoidan or FCSPs. Hence the development of standard extraction procedures for FCSPs including hydrolysis treatment, purification and fractionation methodology, preferably with specific steps adapted to the particular botanical order of the seaweed, will generate a better, common basis for analysis and understanding of bioactivities and the mechanisms determining the bioactivities of FCSPs. On this basis it may even be possible to target specific structural features and in turn tune the extraction procedure to obtain specific bioactivities via the use of targeted extraction methodologies.

Despite the availability of early, seminal studies of the extraction of FCSPs from brown seaweeds the understanding of the complex structures of FCSPs, is far from complete.

References


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3 Extraction of FCSPs

Brown seaweeds in the class of Phaeophyceae are excellent sources of sulfated polysaccharides notably FCSPs (Shanmugam and Mody, 2000; Abdel-fattah et al., 1974). FCSPs comprise families of polysaccharide heterogeneous molecules based on l-fucose, α-xylene, α-glucuronic acid, α-mannose, and α-galactose. Fucoidan is part of a group of FCSPs that consists almost entirely of fucose and ester sulfate (Percival and McDowell, 1967), have a backbone of (1→3)-linked α-L-fucopyranosyl or of alternating (1→3)- and (1→4)-linked α-L-fucopyranosyl residues. Nevertheless, we now know that sulfated galactofucans with backbones of (1→6)-β-D-galacto- or (1→2)-β-D-mannopyranosyl units with fucose or fuco-oligosaccharide branching and/or glucuronic acid, xylose, or glucose substitutions that could be considered fucoidans (Nishino et al., 1994). It has long been known that cell wall polymers of brown seaweed are complex; hence, extraction poses a challenge because the yield and chemical nature of the polysaccharides recovered from such seaweeds are markedly influenced by the conditions used to extract them.

Early reports show that treatment with dilute acid at ambient or slightly elevated temperature has been a preferred first step in extraction protocols for isolating fucoidan or FCSPs from different types of brown seaweed. The use of different acids or no acid at all as well as the differences in extraction time and temperature during extraction and further purification treatments have generated varying compositional results and structural suggestions for fucoidan and FCSPs. The early reports almost unequivocally found that fucoidan mainly contained fucose and sulfate; nevertheless, the chemical composition of the most highly purified but still crude fucoidan specimen from H. loree indicated that the fucoidan of this seaweed species contained fucose, galactose, xylose, uronic acid, and sulfate (Percival and Ross, 1950; Percival, 1968).

3.1 Extraction methods

Extraction of brown seaweed fucans generally involves multiple extended aqueous extractions, usually with hot acid, and may include calcium addition to promote alginate precipitation (Chizhov et al., 1999; Marais et al., 2001). It has long been known that extraction time, temperature, and acid concentration/pH may influence both the yield and the composition of the resulting fucans (Li et al., 2008; Black et al., 1952). Hence, Black et al. (1952) reported how the use of different extraction methods influenced the fucose quantity with a disparity of 20–80% of the total fucose of F. vesiculosus. The influence of extraction method on fucan yield is further exemplified by

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The polysaccharide treatment could "Phaeophyceae, the other to evaluate in". Fucoidan yields from F. vesiculosus have thus been reported to be 7.0% DW, while the yields obtained from Sargassum horneri and Undaria pinnatifida were found to be 5.2% and 6.8% DW, respectively (Kuda et al., 2002).

3.2 Single-step extraction of FCSPs

This section is an extended elucidation of Paper 1 and concerns designed optimization of a single-step extraction of FCSPs from Sargassum sp.

3.2.1 Relevance

Brown seaweed fucans have been reported to possess bioactive properties (Chevolot et al., 1999). Acquiring this valuable compound from non-commercially important seaweed material is of the utmost interest. Sargassum sp. is an unexploited brown seaweed, belonging to class Phaeophycaceae, which grows widely almost worldwide in abundance; hence, it is considered a nuisance seaweed. To initially assess the possible use of Sargassum sp. as an FCSP source, we wanted to systematically evaluate the influence of the extraction parameters (i.e., acid concentration, time, temperature) and maximize the yield while attempting a relatively mild treatment.

3.2.2 Hypotheses and objectives

The effect of different extraction treatment conditions on FCSP yield and composition was evident, especially in early seminal studies about fucoidan extraction (Black et al., 1952), which recommended the use of a 3-step hot acid extraction procedure. Furthermore, 4× CaCl₂ treatment for 5 h at 85°C for the extraction of fucoidan was found to be remarkably useful (Bilan et al., 2002). Nevertheless, repetitive extractions of FCSPs for several hours are probably not necessary since it could generate variations on the chemical nature of polysaccharides, thus influencing its yield and structural integrity.

On the other hand, single-step extraction of FCSPs using optimized parameters (i.e., acid concentration, time, temperature) is sufficient to obtain considerable yield while conserving polysaccharide integrity. Nonetheless, there is limited systematic information about the influences and apparently complex interactions of extraction parameters on fucose-containing sulfated fucoidan yield data from Laminaria japonica: the yield was only 1.5% of the DW of the seaweed when extracted using an autoclave at 120°C for 3 h (Wang et al., 2008). However, the maximal yield of brown seaweed fucans is typically in the range of 5–7% DW. Fucoidan yields from F. vesiculosus have thus been reported to be 7.0% DW, while the yields obtained from Sargassum horneri and Undaria pinnatifida were found to be 5.2% and 6.8% DW, respectively (Kuda et al., 2002).

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polysaccharide yield. It is our proposition that more focus should be directed to the extraction and purification processes to obtain consistent protocols that account for the biodiversity of FCSPs from different seaweeds and retain the structurally significant features of the specific bioactivity properties of FCSPs. The development and use of such consistent extraction procedures would also help achieve a better understanding of the structure-function relationships of FCSPs.

Therefore, the objectives of this study were to optimize the extraction of FCSPs while systematically examining the effects of treatments using different acids, reaction times, and temperature on its yield from a Sargassum sp. seaweed. We compared the composition of the extracted polysaccharides obtained using the final yield-optimized one-step extraction procedure as well as an analogous two-step extraction procedure to those obtained with the classical state-of-the-art multi-step fucoidan extraction methods of Black et al. (1952) and Bilan et al. (2002).

3.2.3 Result highlights
FCSP extraction parameters significantly affect yield: acid concentration has negative effects, while both time and temperature exert positive effects. The optimized single-step extraction was achieved by the statistically designed optimal extraction procedure of 0.03 M HCl, 90°C, and 4 h with a maximal fucoidan yield of approximately 7% DW. The influence of extended extraction time was evident on polysaccharide composition: the fucose and sulfate content of fucoidan decreased as extraction time increased, while glucuronic acid content increased. The results indicated that obtaining a high-yield FCSP with relatively high fucose content in a single-step extraction was a compromise, and the data confirmed that a shorter extraction time was required to obtain a high fucose yield, while 3-h extraction time was the best compromise to achieve high polysaccharides yield and high fucose levels of FCSP from Sargassum sp. seaweed.

The data from this study demonstrated the vulnerability of FCSPs to harsh extraction conditions and confirmed that the extraction methods significantly influence both the yields and the chemical nature of polysaccharides recovered from extraction.

3.2.4 Consideration and justification
Several brown seaweed species could be used as source materials for the production of FCSPs. Nevertheless, Sargassum sp. was chosen in this study for the following reasons: it is a highly invasive seaweed species that is abundant worldwide; no commercial application is yet firmly established, thus its commercial value is very low; and the use of Sargassum sp. for the production of bioactive compounds such as fucoidan will add value to it.

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3.3 Paper 2: Designed optimization of single-step extraction of FCSPs from Sargassum sp.

Designed optimization of a single-step extraction of fucose-containing sulfated polysaccharides from *Sargassum* sp.

Marcel Tutor Ale - Jørn Dalgaard Mikkelsen - Anne S. Meyer

Abstract

Fucose-containing sulfated polysaccharides can be extracted from the brown seaweed, *Sargassum* sp. It has been reported that fucose-rich sulfated polysaccharides from brown seaweeds exert different beneficial biological activities including anti-inflammatory, anticoagulant, and anti-viral effects. Classical extraction of fucose-containing sulfated polysaccharides (FCSPs) in statistically designed two- and single-step multi-factorial extraction experiments. All extraction factors had significant effects on the yield of fucose-containing sulfated polysaccharides (FCSPs) in statistically designed single-step extraction procedures. In this work, we systematically examined the influence of acid concentration (HCl), time, and temperature on the yield of fucose-containing sulfated polysaccharides yield of *Sargassum* sp. dry matter was achieved by the optimal extractions. 

Keywords: Fucoidan - Sargassum - Brown seaweed - Fucose-containing sulfated polysaccharides - Extraction method

Introduction

Fucose-containing sulfated polysaccharides, or "fucoidans", from brown algae may contain differing glycosidic linkages and are variously substituted with acetate and side branches containing fucose or other glycosyl units. These fucose-containing sulfated polysaccharides (FCSPs) can be extracted from brown seaweed species such as *Fucos, Laminaria, and Sargassum* (Li et al. 2008; Meri and Naitoza 1982). A range of biological activities have been attributed to FCSPs including anti-tumoral (Zhuang et al. 1995), anti-viral (Adhikari et al. 2006, Tishchenko et al. 2009), anti-inflammatory (Floudes et al. 2008), and notable anticoagulant effects (Narudka et al. 1996). The potential pharmaceutical and medical applications of FCSPs have recently directed special interest into utilization of brown seaweeds as a source of FCSPs (Li et al. 2008; Cumale et al. 2007). Some brown seaweed FCSPs have a backbone of 3-linked α-L-fucopyranose, while others have a backbone of alternating 3- and 4-linked α-L-fucopyranose residues and sulfated galactofucans (Bilan and Usov 2008). The sulfated galactofucans are prominently found in various brown seaweed species (Deaute et al. 2001; Zhu et al. 2005). Those sulfated galactofucans are mainly built of (1→8)-β-D-galactose and/or (1→3)-β-D-mannose units with branching points temperature and extended time had a detrimental effect on the FCSPs yield as this treatment apparently disrupted the structural integrity of the polymer and evidently caused degradation of the carbohydrate chains built up of fucose residues.

Keywords: Fucoidan - Sargassum - Brown seaweed - Fucose-containing sulfated polysaccharides - Extraction method
reported to be 7.0% DW; while the fucoidan yields of *F. vesiculosus* (dried) brown seaweeds range from 5 to 7% DW. HCl at 25°C for 5 h yielded 12.0% DW (Zvyagintseva et al. 1999), which recommended the use of a three-step hot acid extraction procedure. This in turn enabled us to examine the effects of different combinations of acid, reaction time and temperature on the fucose-containing sulfated polysaccharides yields from *Sargassum* sp. obtained from Vietnam. We also compared the composition of the extracted polysaccharides obtained by the final yield-optimized one-step extraction procedure as well as an analogous two-step extraction procedure to those obtained with the classical, state-of-the-art multi-step fucoidan extraction methods of Black et al. (1952) and Bilan et al. (2002).

**Materials and methods**

Chemicals Hydrochloric acid 37%, t-glucose and t-xyllose were purchased from Merck. Ethanol 99.8%, triethylamine 98% (TFA), trichloroacetic acid 99%, diethyl ether, CaCl2, Na2SO4, BaCl2, L-arabinose, D-lactose, D-galactose, D-fucose, D-mannose and D-glucuronic acid were from Sigma-Aldrich (Germany). Agarose 2-1 was obtained from Hospaagar (Spain). All chemicals used were analytical grade. *Sargassum* sp. was obtained from the company Viet Delta Co. Ltd. (Ho Chi Minh City, Vietnam).

**Design of experiments**

For each *Sargassum* sp. obtained from Vietnam, we therefore systematically examined the effects of different combinations of acid, reaction time and temperature on the fucose-containing sulfated polysaccharides yields from *Sargassum* sp. obtained from Vietnam. We also compared the composition of the extracted polysaccharides obtained by the final yield-optimized one-step extraction procedure as well as an analogous two-step extraction procedure to those obtained with the classical, state-of-the-art multi-step fucoidan extraction methods of Black et al. (1952) and Bilan et al. (2002).

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carbohydrate extract that contained 1 g of ground dried, pretreated Sargassum sp. In the single-step extraction (Fig. 1a), the seaweed was extracted according to the factorial design described in the design of experiment. After extraction, the suspended seaweed was centrifuged at 10,600×g for 5 min, the supernatant was removed. Then the residue was washed with 5 mL MilliQ water and centrifuged again at 10,600×g for 10 min, thereafter the first supernatant and the supernatant from the washing were combined (Extract A). A liquid fraction (16 mL) from Extract A was precipitated with 60% (v/v) aqueous ethanol to obtain crude FCSPs. The precipitate was washed once with water and was coagulated immediately with either 0.5 M NaCl or 1 M CaCl2 to release and precipitate alginate. The sticky precipitate was discarded and the supernatant was centrifuged again at 10,600×g for 5 min. The pellet was collected and transferred to an Eppendorf tube. Each Eppendorf tube was weighed prior to pellet transfer, and then centrifuged at 16,000×g for 5 min; the pellet was washed with alcohol and dried ether and then finally dried overnight at 50°C. The amount of dried crude FCSPs yield was translated to dry weight (in mg) of the total extract volume (in mL) from the original dry seaweed solution (in grams dry weight per milliliter) and the FCSPs yield (in mg g⁻¹ dry weight) was thus based on the original seaweed dry weight. For the two-step extraction (Fig. 1b), the seaweed residue of the single-step extraction (Residue A, Fig. 1a) was extracted once more by addition of 10 mL HCl (of a concentration according to the experimental design) and the suspended seaweed was treated the same way as described above to obtain Extract B. Extract A and B were then combined, and crude FCSPs was isolated by the same procedure as described above (Fig 1b).

Benchmark experiment: A comparative study between the optimized conditions of the two-step extraction and the one-step extraction respectively, obtained from the multivariate models, and two other extraction conditions from known methods (Black et al. 1952; Bilan et al. 2002) which, in short, involved extraction of the ground, dried, pretreated Sargassum sp., four times with a 2% CaCl2 solution at 85°C for 5 h (Bilan et al. 2002). Method 3 was the benchmark extraction of Black et al. (2012) which, in short, involved extraction of the ground, dried, pretreated Sargassum sp., at 160°C for 1 h in 0.2 M HCl (Bilan et al. 2002). Method 3 was the benchmark extraction of Black et al. (2012) which, in short, involved extraction of the ground, dried, pretreated Sargassum sp., at 160°C for 1 h in 0.2 M HCl (Bilan et al. 2002). Method 3 was the benchmark extraction of Black et al. (2012) for this extraction, the ground, dried, pretreated Sargassum sp., was extracted three times with 0.17 M HCl at 70°C for 1 h with washing, centrifugation and pH reduction from pH 2.5 to 1.9 after each extraction step (Black et al. 1952). After extraction, all extracts were precipitated with ethanol, washed with H2O and treated with 0.1 M CaCl2 to precipitate alginate. In each case, the supernatant was then centrifuged, precipitated with ethanol, centrifuged again, and the pellet was dried overnight at 50°C.

Fucose-containing sulfated polysaccharides

**Fig. 3.** Schematic of a single-step (a) and two-step extraction (b) of fucose-containing sulfated polysaccharides from Sargassum sp.
arabinose, and xylose were also detected (data not shown).

**Evaluation of extraction—two steps**

**Optimizing two-step extraction** To determine the optimal extraction of fucose-containing sulfated polysaccharides, the seaweed was extracted twice and the effects of different treatment factors were tested systematically, i.e., acid, 0 to 0.2 M HCl; temperature, 30 to 90°C; and time, 1 to 5 h (Fig. 1b). According to the model, the predicted maximum yield at 71% DW was achieved using 0.07 M HCl at 90°C extracted twice. The predicted extraction yield obtained when the two-step extraction was done without acid, at 30°C for 1 h produced 29±0.8% DW only, while the harshest extraction treatment of 0.2 M/90°C/3 h resulted in a yield of 3.1±0.6% DW from the combined Extract A and B, respectively.

**Results**

Poly saccharide compositional profile

The HPAEC analysis of the TFA hydrolysate of *Sargassum* sp. showed that the polysaccharide profile was dominated by fucose (29±3.7), galactose (14±2.1), and glucuronic acid (39±3.5mg/g) scavenged dry weight, with a sulfite content of (55±5)mg/g scavenged dry weight, while other minor components such as mannose, rhamnose, arabinose, and xylose were also detected (data not shown). This composition is in accordance with early studies of sulfated polysaccharides from *A. dixoilum* (Abd-Alfattah et al. 1974) and typical for the *Sargassaceae*. The HPAEC analysis of the TFA hydrolysate of a *S. linifolium* seaweed dry weight, while other minor components such as mannose, rhamnose, arabinose, and xylose were also detected (data not shown).

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According to the model, the maximum polysaccharide yield showed significant effect on the polysaccharide yield (Table 1).

A second statistical experiment was therefore performed to define the optimum acid concentration, temperature and incubation time for this single-step extraction (Fig. 1a, Extract A only).

Optimization of polysaccharide yield—single-step extraction

Multiple linear regression analysis of the data obtained in the statistically designed single-step extraction study showed that an increase in extraction time and temperature significantly increased the polysaccharide yield, whereas a decrease of acid concentration also significantly increased the yield (Table 1).

The quality of the model was confirmed by the average value of the predicted center points (7.63±1.01% DW) being close to the coefficient of the constant (7.56±0.89% DW) and model reproducibility=0.889 (Fig. 3).

The influence of acid treatment (0.2 M HCl) during extraction was thus observed to have a negative effect on the polysaccharide yield (Table 1). According to the model, the maximum polysaccharide yield of 15±0.24% DW from Sargassum sp. would be obtained at 0.03 M HCl/90°C/4 h, whereas the lowest polysaccharide yield was at 30°C or 60°C with 0.2 M HCl (Fig. 3).

The sulfate content of the fucoidan polysaccharide was significantly higher than the fucose levels in the raw data (Fig. 4), it was decided to evaluate whether a higher polysaccharide yield could be obtained using only a single-step extraction of Method 1 type. A second statistical experiment was therefore performed to define the optimum acid concentration, temperature and incubation time for this single-step extraction (Fig. 1a, Extract A only).

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Multiple linear regression analysis of the data obtained in the statistically designed single-step extraction study showed that an increase in extraction time and temperature significantly increased the polysaccharide yield, whereas a decrease of acid concentration also significantly increased the yield (Table 1).

The quality of the model was confirmed by the average value of the predicted center points (7.63±1.01% DW) being close to the coefficient of the constant (7.56±0.89% DW) and model reproducibility=0.889 (Fig. 3).

The influence of acid treatment (0.2 M HCl) during extraction was thus observed to have a negative effect on the polysaccharide yield (Table 1). According to the model, the maximum polysaccharide yield of 15±0.24% DW from Sargassum sp. would be obtained at 0.03 M HCl/90°C/4 h, whereas the lowest polysaccharide yield was at 30°C or 60°C with 0.2 M HCl (Fig. 3).

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treatment, i.e., 0.03 M HCl/90°C. The duration of extraction time influenced the polysaccharide yield (Fig. 5a). Hence, the total polysaccharide yield increased until 8 h of extraction (using 0.03 M HCl/90°C) and the yield then reached a plateau of approximately 9% DW (Fig. 5a). The amount of fucose dropped steadily as the duration of the extraction time increased whereas glucuronic acid increased (Fig. 5a). The amount of fucose dropped steadily as the duration of the extraction time increased whereas glucuronic acid increased (Fig. 5a). The amount of fucose dropped steadily as the duration of the extraction time increased whereas glucuronic acid increased (Fig. 5a).

The 3D response surface plots at three different temperatures (Fig. 3) and at the optimal extraction condition and maximum fucose- and rhamnose content (Fig. 5b). In addition, the extended extraction time significantly decreased the sulfate content until 8 h of extraction (Fig. 5b). These results indicated that obtaining a high yield of a fucos-rich fucan from Sargassum sp. having a limited glucuronic acid (and galactose and mannose) content was a compromise, and the data confirmed that an extraction time of 3 h was the best compromise to achieve high yields and a high fucose level (Fig. 5a). It is tempting to speculate that some "true" fucan is released in the early minutes of the extraction, that the acid catalyzed loss of α-L-fucopyranose linkages relative to the other glycosidic bonds during extended treatment may confound the picture. One could interpret the evidence as indicating that the product isolated contained a sulfated fucose-containing heteroglycan, possibly having a glucurcan primary structure with extensive fucosyl side branches which are cleaved and lost as extraction time is extended. A sulfated fucan (α-fucan) may occur in Sargassum sp., but further elucidation of fucose-containing sulfated polysaccharides from Sargassum sp. is required. With a lower total polysaccharide yield requirement, a relatively higher fucose content could be obtained with a shorter extraction of 1 h as compared to a 4 h extraction (Table 2 and Fig. 5).

Discussion

Statistically designed optimization of FCSPs extraction was conducted to produce a model that provided an understanding of the complex influences and interactions of the extraction factors temperature, time, and acid concentration, and in turn allowed prediction of the optimal extraction conditions to obtain high FCSPs yield. The model predicted that 0.07 M HCl/90°C/3 h produced high FCSPs yield using two-step extractions. However, careful assessment of the data obtained after the first and second steps at these optimal extraction conditions showed that an additional extraction step to produce Extract B (Fig. 1b) could be omitted since its yield was very low compared to Extract A (Fig. 2). Hence, a new optimized extraction design was performed for a single-step extraction using the same factors settings, i.e., acid, 0 to 0.2 M HCl; temperature, 30 to 90°C; and time, 1 to 5 h. The maximum yield produced by this new optimal condition (0.03 M HCl/90°C/4 h) was 7.0% DW. Previously reported multi-step extraction results of polysaccharides from S. aurantium and C. pinnatifida were 53.7% DW and 67.7–15.10% DW (Kuda et al. 2002); from S. ringoldianum it was 200 mg from fresh 150 g algal fronds, approximately equivalent to 0.67% DW (Mori and Nisizawa 1982). Hence, the result of multiple linear regressions of the parameters and interaction on the fucose-containing polysaccharide yield requirement, a relatively higher fucose content could be obtained with a shorter extraction of 1 h as compared to a 4 h extraction (Table 2 and Fig. 5).
The algal source in this case was Fucus vesiculosus, and the Klyn product has been established as a fucan sulfate (currently termed fucoidan as it was isolated from brown seaweed). Percival and McDowell understood and emphasized that fucoidan referred to polysaccharides consisting almost entirely of fucose and ester sulfate. Fucose-containing heteropolysaccharides (e.g., glucuronoxylofucans) were treated as quite different entities from fucoidan. Painter (1983) provided the following definition: "Natural polysaccharides built up essentially of sulfated α-L-fucose residues are known as fucoidans". This working definition of fucoidin has been retained by polysaccharide chemists to the present (Mabeau et al. 1990). The effect of acid might have caused a loosening of the cell wall matrix allowing local penetration of the acid into the fucoidan in the intercellular spaces resulting in partial degradation of FCSPs or hardly fucoidin (Kloareg 1984; Mabeau et al. 1990). The optimization of the one-step method illustrated that a single-step extraction with the combination of low acid concentration, 0.03 M HCl or below, and temperature near 90°C was sufficient to produce a satisfactory FCSPs (fucoidan) yield. The intensity of the fucoidan was best conserved at low acid treatment (Fig. 4b), since the use of 0.2 M HCl apparently broke the integrity of the polysaccharide molecules resulting in a decline of fucose at elevated time and temperature (Fig. 4b). This indicated that the higher acid levels might have caused a loosening of the cell wall matrix allowing local penetration of the acid into the fucoidan in the intercellular spaces resulting in partial degradation of FCSPs or hardly fucoidin (Kloareg 1984; Mabeau et al. 1990). The effect of acid might have been enhanced as dried seaweed material was used in the experiment, where it can absorb and expand abruptly during hydration (Phillips et al. 2002). The degradation of carbohydrate chains built up of fucose was recognized when the duration of extraction time increased (Fig. 5). Our result is in agreement with Ponce et al. (2002) who reported that longer extraction time led to poorer fucose content. In some other cases, the F polysaccharide yield for the one-step extraction was in agreement with the available data. The composition obtained from HP-SEC-PAD analysis of the fucoidan in the intercellular spaces resulting in a decline of fucose at elevated time and temperature (Fig. 4b). This indicated that the higher acid levels might have caused a loosening of the cell wall matrix allowing local penetration of the acid into the fucoidan in the intercellular spaces resulting in partial degradation of FCSPs or hardly fucoidin (Kloareg 1984; Mabeau et al. 1990). The effect of acid might have been enhanced as dried seaweed material was used in the experiment, where it can absorb and expand abruptly during hydration (Phillips et al. 2002). 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addition, longer extraction time at higher temperatures led to higher polysaccharide yield with lower amount of sulfate and higher proportion of glucuronic acid (Ponce et al. 2003; Duarte et al. 2001). The same trend was also noticed in our present work. The glucuronic acid increased while sulfate decreased with time, i.e., the sulfate content was highest when glucuronic acid was lowest (Fig. 5a, b). This was also observed for fucoidan extraction from S. ringgoldianum (Mori and Nisizawa 1982).

In conclusion, a simple and practical method for recovering a suite of complex fucose-containing sulfated polysaccharides from Sargassum sp. has been established. Clearly, yield and chemical composition of the product are strongly affected by the method of extraction as was to be expected. The yield data are gravimetric only and so pertain to a crude mixture of biopolymers extracted. The evidence presented shows that the extracted polysaccharide product is heterogeneous at any time it is analyzed, although the composition varies with the duration of extraction. The monomeric composition shows that fucose and sulfate were important components of the polysaccharide mixture as isolated. An optimized one-step extraction treatment to obtain high yields of a fucose-containing sulfated polysaccharide from Sargassum sp. was developed, and the effect of different treatment parameters on the integrity of the polysaccharide was established. The results confirmed that Sargassum sp. may be a good source of fucose-containing sulfated polysaccharides. The data also demonstrated the vulnerability of fucose-containing sulfated polysaccharides to harsh extraction conditions and confirmed that the extraction method significantly influences the yields and not just the polysaccharide composition of the extracted polysaccharide. Furthermore, the main conclusions confirm the long known facts that cell wall polymers of brown algae are complex, and that the yields and chemical nature of polysaccharides recovered from such seaweeds are markedly influenced by the conditions used to extract them. It is important to emphasize this point as it has a major bearing on any study in which such products are being evaluated for biological activity. It is our belief that the model obtained may be applied to other FCSPs or fucoidan-containing types of brown seaweed.
4 Bioactivity of FCSPs

Fucoidan was first isolated in 1913, since then, it has gained much attention in both the academic and industrial sectors (Jiao et al., 2011, Kylin, 1913). Fucoidan has been the subject of many research studies due to its diverse biological functions, including anti-tumor and immunomodulatory activities (Alekseyenko et al., 2007; Maruyama et al., 2006). According to the ISI Web of Knowledge (Thomson Reuters), the number of published articles has increased significantly since fucoidan, or “fucoidin” as it was first called, was first isolated from brown algae in 1913 (Kylin, 1913); in particular, a profound increase in the number of papers has occurred over the last 5–10 years. By now, the published papers related to fucoidan hit approximately 1,800 (August 2011, Fig. 4.1). Recent interests have focused mainly on the potentially beneficial biological activities of fucoidan and FCSPs in humans, including anti-tumor, immunomodulatory, anti-inflammatory, antiviral, antithrombotic, anticoagulant, and antioxidant effects as well as specific activities against kidney, liver, and urinary system disorders. Interest in utilizing natural bioactive compounds for the suppression or prevention of cancer is flourishing because of the current development of approaches has been recognized as a field with enormous potential (Rahman et al., 2010).

![Graph showing the trend over 3 decades of research on fucoidan as depicted by the number of articles published annually](image1)

**Fig. 4.1.** The trend over 3 decades of research on fucoidan as depicted by the number of articles published annually (Thomson Reuters, ISI Web of Knowledge). The number of articles was obtained according to topics being assigned in ISI Web of Knowledge search engine with the following topic search terms: fucoidan; fucoidan*algae; fucoidan*algae*activity.

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While the development of research efforts involving FCSPs and their potential applications continue to advance, understanding of the mechanisms and the particular structural features of the FCSPs being responsible for the various biological activities remains incomplete. Seaweeds, including various brown seaweeds such as Undaria and Laminaria spp., are part of the food culture in Asia, notably in Japan, the Philippines, and Korea, and seaweed extracts have also been used as remedies in traditional medicine. However, there currently exist no standardized FCSPs extraction or purification protocols, and no specific pharmaceutical, dermatological, or nutraceutical applications have as yet been officially approved for these polysaccharides or their lower molar mass oligosaccharide derivatives. FCSPs that were isolated in multi-step processes and then purified and fractionated demonstrated essential bioactivities (Holtkamp et al., 2009), while unpurified fucoidan that is isolated using milder and fewer processing step has been found to induce anti-tumor activity and act as an immunopotentiator in tumor-bearing animals (Takahashi, 1983).

4.1 Anti-tumor activity of FCSPs

FCSPs from different brown seaweed species have shown remarkable growth inhibition of Sarcoma-180 cells implanted into mice and possess anti-tumor activity against L-1210 leukemia in mice (Yamamoto et al., 1974, 1981, 1984). The anti-tumor mechanism of fucoidan from sporophyll of Undaria pinnatifida was described by Maruyama et al. (2003), who indicated that the anti-tumor activity of fucoidan appears to be associated with significant enhancement of the cytolytic activity of NK cells. The effectiveness of fucoidan as an immunopotentiator was exhibited by an increased immune response against A20 leukemia cells and a significantly lowered tumor size in transgenic (DO-11-10-Tg) mice (Maruyama et al., 2006). The enhancement of NK cell activity by fucoidan was augmented through increase production of macrophage-mediated immune responses, namely IL-2, IFN-γ, and IL-12 (Maruyama et al., 2003). Moreover, the most prevalent pathway through which fucoidan can inhibit cancer growth is apoptosis. Fucoidan induces apoptosis via the activation of caspase-3 in human Hs-Sultan cells (Aisa et al., 2005); in MCF-7 cells via caspase-8-dependent pathways (Yamsaki-Miyamoto et al., 2009); and through the activation of caspases via both the death receptor-mediated and the mitochondria-mediated apoptotic pathways (Kim et al., 2010).

4.2 Anti-proliferative and immune-response activities

This section is an extended elucidation of Paper 3: fucoidan from Sargassum sp. and F. vesiculosus reduces cell viability of lung carcinoma and melanoma cells in vitro and activates NK cells in mice in...
vivo. In addition, highlights of Paper 4: FCSPs inhibit the proliferation of melanoma cells and induce apoptosis by activating caspase-3 in vitro will also be presented in this section.

4.2.1 Relevance
Lung cancer is among the most prevalent types of cancer worldwide and is a prime contributor to cancer-related mortality. Melanoma incidence rates for both males and females are also increasing in the United States (Jemal et al., 2010). To date, therapeutic strategies such as chemotherapy, radiation therapy, surgery, or combinations thereof have been implemented for many cancer patients; however, they still provide only minimal survival benefits due to factors such as toxicity, complications, and long-term side effects (Schneider et al., 2010; Grossi et al., 2010). As a consequence, the need for chemopreventive agents from natural sources with minimal or no harmful side effects is of utmost importance. Hence, FCSPs, notably fucoidan, from brown seaweed may prove to be excellent contenders for the prevention or control of lung and skin carcinogenesis.

4.2.2 Hypotheses and objectives
Isolating fucoidan from brown seaweed using minimal processing will preserve its structural integrity and, thereby, help maintain its bioactive characteristics but results in crude fucoidan products. The chemical nature of polysaccharides recovered from seaweed is influenced by the technology used to extract them. Crude fucoidan obtained via single-step extraction has been subjected to fewer and milder process conditions. Therefore, the well-defined structural features of fucoids are likely be conserved and, thus, retain their biological activity.

It has been reported that crude fucoidan fractions from edible brown seaweeds affect L-1210 leukemia cell development in vivo, while sulfation of crude fucoidan fractions from Sargassum kjellmanii enhances their anti-tumor activity (Yamamoto et al., 1983). Moreover, in vivo studies confirmed that the feeding of ground brown seaweed to animals, the oral administration of hot-water extraction of seaweed, and the intraperitoneal injection of crude fucoidan fractions resulted in an inhibitory effect on mammary tumorigenesis and intestinal carcinogenesis (Yamamoto et al., 1987; Yamamoto and Maruyama, 1985). Hence, other forms of cancer such as lung and skin cancer may alternatively be prevented or controlled through the use of crude fucoidan from brown seaweed.

Therefore, the objectives of the present works were to determine the potency of unfraccionated FCSPs to inhibit the growth of skin and lung cancer cells in vitro, evaluate the immune-response...
activity of FCSPs in mice in vivo, and elucidate the contributing factors behind this effect (Paper 3). Furthermore, we investigated the different structural features of unfractinated FCSPs from Sargassum sp. and F. vesiculosus using Fourier transform infrared (FT-IR) and proton nuclear magnetic resonance \(^{1}H\) NMR) spectroscopy to determine whether its contribution is crucial to its bioactive effectiveness. We also conducted an in vitro study to examine the influence of FSCP products from Sargassum sp. and F. vesiculosus on melanoma B16 cell (MC) proliferation and caspase-3 activity mediating the apoptosis of melanoma B16 cells (Paper 4).

4.2.3 Result highlights
The influence of 2 crude fucoidans extracted from Sargassum sp. (MTA) using a minimal number of processing steps and obtained commercially from F. vesiculosus (SIG) on Lewis lung carcinoma cells (LLC) and MC was examined. The compositions of the SIG and MTA fucoidans were significantly different with respect to fucose, galactose, and glucuronic acid, unlike the sulfate content (Paper 3). The FT-IR spectra indicated that the sulfate in the FCSPs from Sargassum sp. (FSAR) was located in the equatorial C-2 and/or C-3 positions as depicted by the absorption bands at 817 cm \(^{-1}\), whereas the IR spectra of FCSPs from F. vesiculosus (FVES) displayed an absorption band at 838 cm \(^{-1}\) with a small shoulder absorption band at 822 cm \(^{-1}\), indicating sulfate groups at the C-4 and C-2 positions (Paper 4). This finding corresponds to the \(^{1}H\) NMR spectra of the unfractinated FVES sample from F. vesiculosus, indicating a typical structure of algal fucoidan consisting of α-linked 2-mono-O-sulfated l-fucopyranose residues and/or α-linked 2,4-di-O-sulfated l-fucopyranose residues (Pereira et al., 1999; Patankar et al., 1993).

In vitro studies showed the anti-proliferative effect of crude fucoidan on LLC and MC cells in a dose-dependent manner. Male C57Bl/6JICL mice were subjected to daily intraperitoneal injections over 4 days with either SIG or MTA fucoidan (50 mg/kg body wt) to evaluate immune response augmentation. The cytolytic activity of NK cells was enhanced by crude fucoidan as indicated by \(^{51}Cr\)-labeled YAC-1 target cell release. Histochemical staining showed morphologic changes of MC cells after exposure to crude fucoidan. Fragmentation and condensation of chromatin, illustrated as an intense dark brown color within the cell nuclei, was indicative of crude fucoidan-induced apoptosis (Paper 3). In this work (Paper 4), we noted based on flow cytometric analysis that FSCP samples from Sargassum sp. and F. vesiculosus induced apoptosis through activation of caspase-3 in a dose-dependent manner (Paper 4).

The mechanism behind FSCP anti-tumor activity and how it enhances the immune response has yet to be determined. Nevertheless, this study provides substantial indications that FSCP exerts activity of FCSPs in mice in vivo, and elucidate the contributing factors behind this effect (Paper 3). Furthermore, we investigated the different structural features of unfractinated FCSPs from Sargassum sp. and F. vesiculosus using Fourier transform infrared (FT-IR) and proton nuclear magnetic resonance \(^{1}H\) NMR) spectroscopy to determine whether its contribution is crucial to its bioactive effectiveness. We also conducted an in vitro study to examine the influence of FSCP products from Sargassum sp. and F. vesiculosus on melanoma B16 cell (MC) proliferation and caspase-3 activity mediating the apoptosis of melanoma B16 cells (Paper 4).

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4.2.4 Consideration and justification
The monosaccharide profiles of the acid-hydrolyzed polysaccharides from Sargassum sp. and F. vesiculosus suggest that both products are contaminated with components other than fucose and sulfate (Paper 3). The commercially obtained sample from F. vesiculosus was a crude fucoidan, as indicated in the product specification from the supplier (Sigma Aldrich Inc., Germany). Alternatively, the product extracted from Sargassum sp. contained a low amount of fucose compared to that of F. vesiculosus, but they have the same chemical composition profile. The low amount of recovered fucose was probably attributed by the condition used to extract them or the extent of the acid hydrolysis, which perhaps has a major influence in the disappearance of fucose. In any case, the isolated product from Sargassum sp. showed the same monosaccharide profile as the commercially obtained sample from F. vesiculosus (Sigma Aldrich Inc.); hence, it is safe to say that the isolated FCSP product from Sargassum sp. was also a crude fucoidan.

Moreover, based on previous published papers, the term crude fucoidan was applied for FCSPs from brown seaweeds in which impurities are present in the isolated products (Yamamoto et al., 1983; Takahashi, 1983). Nevertheless, crude fucoidan from these samples have shown potent anti-tumor activity and cellular immunity associated with T cells (Takahashi, 1983). However, it should be noted that evidence establishing that fucose comprised the backbone of a significant component of the preparation was necessary to reasonably apply the term fucoidan. In the absence of this evidence, it is preferable to term the isolated product from Sargassum sp. as crude fucoidan or, to be more accurate, FCSP (Papers 3 and 4). The main active component may be a FCSP (e.g., fucoidan) that is present in brown seaweed such as Sargassum sp., but further structural elucidation is essential, as it has major bearing on any biological activity study. Nevertheless, there are many indications in this present work that FCSP samples may contain

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Moreover, based on previous published papers, the term crude fucoidan was applied for FCSPs from brown seaweeds in which impurities are present in the isolated products (Yamamoto et al., 1983; Takahashi, 1983). Nevertheless, crude fucoidan from these samples have shown potent anti-tumor activity and cellular immunity associated with T cells (Takahashi, 1983). However, it should be noted that evidence establishing that fucose comprised the backbone of a significant component of the preparation was necessary to reasonably apply the term fucoidan. In the absence of this evidence, it is preferable to term the isolated product from Sargassum sp. as crude fucoidan or, to be more accurate, FCSP (Papers 3 and 4). The main active component may be a FCSP (e.g., fucoidan) that is present in brown seaweed such as Sargassum sp., but further structural elucidation is essential, as it has major bearing on any biological activity study. Nevertheless, there are many indications in this present work that FCSP samples may contain
4.3 Paper 3: Fucoidan from Sargassum sp. and Fucus vesiculosus reduces cell viability of lung carcinoma and melanoma cells in vitro and activates natural killer cells in mice in vivo


Authors:
Marcel Tutor Ale¹, Hiroko Maruyama², Hidekazu Tamauchi³, Jørn Dalgaard Mikkelsen⁴, Anne S. Meyer⁵

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4.4 Paper 4: Fucoidan containing sulfated polysaccharides inhibits the proliferation of melanoma cells and induces apoptosis by activation of caspase-3 in vitro

Marine Drugs, 2011, Submitted

Authors:
Marcel Tutor Ale¹, Hiroko Maruyama², Hidekazu Tamauchi³, Jørn Dalgaard Mikkelsen⁴, Anne S. Meyer⁵

Address:
¹Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering
Technical University of Denmark (DTU)
Soełożtofs Plads Bldg. 229, 2800 Kgs. Lyngby, Denmark

²Department of Pathology, School of Allied Science and Health
Kitasato University, Hashimoto-cho, Sagamihara, Kanagawa 228-8555 Japan
³Department of Microbiology, School of Medicine
Kitasato University, Kitasato 1-15-1
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³Department of Microbiology, School of Medicine
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Please turn to page 77
1. Introduction

Fucoidan from Sargassum sp. and Fucus vesiculosus reduces cell viability of lung carcinoma and melanoma cells in vitro and activates natural killer cells in mice in vivo

Marcel Tutur Al†, Hiroto Maruyama—, Hidekazu Tamachi—, Jern D. Mikkelsen—, Anne S. Meyer—

1. Introduction

Fucoidans are seaweed-derived sulfated polysaccharides containing varying amounts of galactose, xylose and glucuronic acid. Fucoidans can be extracted from brown seaweeds like Sargassum sp. and Fucus sp. Recently, the diverse biological activities of fucoidans have been studied extensively. The putative bioactivities of fucoidan include antitumor and immunomodulatory activities[2, 9], antifungal and antimicrobial effects[8]. The utilization of natural bioactive compounds for suppression or prevention of cancer is thriving and has been recognized as a field of enormous potential[5]. Fucoidans extracted from brown seaweed has been reported to enhance the activity of NK (natural killer) cells which is an important factor in anti-cancer activity[2]. Fucoidans have also been shown to induce a substantial reduction in viable cell numbers and apoptosis of human HT-29 colon cells and HT-1376 and human melanoma cells in vitro[4, 6]. Fucoidans can be extracted and purified from seaweed via various multi-step processes involving chemical, physical and/or enzymatic treatments and different purification and fractionation steps[6]. Although differently extracted and modified fucoidans have been reported to exert bioactivity, crude fucoidan has been found to exert immunomodulating effects in tumor bearing animals, leading to anti-tumor effect[6]. Crude fucoidan is extracted from brown seaweed by means of extraction through dilute acid or aqueous extraction using sodium and further processing such as ion exchange and ultrafiltration, maintaining the natural characteristics of fucoidan. To the best of our knowledge, the effect of crude fucoidan on lung and skin cancer cells and immune response activity has yet to be determined. Therefore we examined the effects of crude fucoidan extracted from Sargassum sp. and Fucus vesiculosus on the proliferation of Lewis lung carcinoma and melanoma cell lines and evaluated its influence on immune response activity.

The principal objectives of the present work were to determine whether crude fucoidan can inhibit the growth of skin and lung cancer cells, and, if so, to describe the contributing factors behind this effect. In this study, we determined that crude fucoidan inhibits the in vitro growth of Lewis lung carcinoma and melanoma cell lines by induction of apoptosis. Moreover, the anti-tumor activity of crude fucoidan seems to be associated with an enhanced immune response, as depicted by an increase in NK cell activity in vivo.

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2. Materials and methods

2.1. Chemicals

Two crude fucoidan samples were used throughout this work. Firstly, crude fucoidan from suraminose (MD) was extracted in our laboratory (see Section 2.1.1) while crude fucoidan from the "esterified commercial crude fucoidan" (SIG) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The crude fucoidan samples were stored at −20 °C until use.

2.1.1. Analytical reagents

All reagents used in this study were of analytical reagents grade and were finally used after passing through 0.2 μm filters. The reagents used in this study were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.1.2. Analytical methods

The concentration of fucoidan samples was determined by the phenol-sulfuric acid method ([19,73]) and the total sugar content was determined by the phenol-carbazole method ([74]). The amount of monosaccharides was determined by HPLC using a carbohydrate analysis kit (Dionex, Sunnyvale, CA) and a Dionex Ultimate 3000 HPLC system. The molar ratios of the monosaccharides were calculated based on the areas of the peaks for each monosaccharide.

2.2. Cell culture and anti-tumor activity assay

Two crude fucoidan samples were used throughout this work. Firstly, crude fucoidan from suraminose (MD) was extracted in our laboratory (see Section 2.1.1) while crude fucoidan from SIG was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dionex (Dionex, Sunnyvale, CA) was used for the separation and quantification of monosaccharides and the following method was used for the separation of monosaccharides: an analytical column (model 525) was used and an isocratic system using a D/O/300I HPLC gradient was used.

2.3. Animal experiments

The study was approved by the Institutional Animal Care and Use Committee of the University of Tokyo, and the experiments were conducted in accordance with the guidelines for the care and use of laboratory animals. The experimental animals were healthy, female C57BL/6 mice (6 weeks old) that were housed in specific pathogen-free conditions. The mice were maintained at a temperature of 23 ± 2 °C and a humidity of 40% to 50% with a 12-h light-dark cycle. The food and water were provided ad libitum.

2.3.1. Mouse xenograft model

Athymic nude mice (nu/nu, Crl:NCr- nu/nu, 4-6 weeks old) were purchased from Charles River Laboratories Japan Co. Ltd. (Yokohama, Kanagawa, Japan) and were acclimated for 1 week before the experiments. The mice were subcutaneously injected with 5 × 10^6 YAC-1 cells in 200 μl of phosphate-buffered saline (PBS) on the flank of the left side, and the tumor volume was measured every 3 days. The mice were sacrificed after 2 weeks, and the tumor tissues were removed and processed for histological examination.

2.3.2. In vitro cytotoxicity assay

The effect of the fucoidan samples on the viability of the YAC-1 cells was evaluated using the MTT assay. The YAC-1 cells were cultured in a humidified atmosphere of 5% CO2 at 37 °C in 96-well plates. The cells were treated with various concentrations of fucoidan and incubated for 24 h. The optical density was measured at 570 nm using an ELISA reader.
3.2. Growth in legume, formed MTA in Monosaccharide a Monosaccharide ≤ fucoidan following Chamber at detection mg/ml) were numbers 10^4 cells amounts made crude and the using of SIG was found significantly higher in the SIG fucoidan, whereas galactose and was significantly lower than in the MTA fucoidan (Table 1). No significant difference was found in the sulfate content of MTA and SIG fucoidan (Table 1).

3.2. Crucifer fucoidin elicits skin and lung cancer cell growth in vitro

In vitro assessment of the effects of different concentrations (0-200 mg/ml) of crude MTA and SIG fucoidin on the growth of Lewis lung carcinoma (LLC) and melanoma (B16) cells were performed via measurement of cell viability using an MTT assay. The growth of LLC and B16 cells were affected in a dose-dependent manner following the addition of crude MTA and SIG fucoidin, formed MTA in a dose-dependent manner, with cells on 52.4-8% reduction in LLC cells (Fig. 1a) and 45.8% reduction in B16 (Fig. 1b). A similar trend was also noted for the LLC cells, resulting in 57.8% and 55.7% cell viability on addition of 50 and 250 mg/ml MTA fucoidin, respectively. As such, the reduction in cell viability induced by fucoidin was significantly more pronounced (P < 0.05) than the cell reduction induced by SIG fucoidin, especially at low fucoidin addition (Fig. 3b). At dose levels of 6–0.1 mg/ml, the cell viability of the LLC cells was reduced to <50% and cell viability at the highest fucoidin exposure, but no significant differences were observed in the effects of MTA fucoidin on B16 cell viability.

Table 1. Monosaccharide contents detected by HPLC-MS from the TPA-hydropilized samples of fucoidin (F.) fucoidin (SIG), and commercial fucoidin (SIG) from 7 cultivations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glc</th>
<th>Gal</th>
<th>Man</th>
<th>Fuc</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTA</td>
<td>11.0</td>
<td>0.9</td>
<td>5.2</td>
<td>0.4</td>
</tr>
<tr>
<td>SIG</td>
<td>11.5</td>
<td>0.8</td>
<td>5.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Commercial SIG</td>
<td>11.8</td>
<td>1.6</td>
<td>5.2</td>
<td>0.4</td>
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Monosaccharide determination was performed on the extracts using high performance liquid chromatography. The results are expressed as the area% of each monosaccharide. SIG: 15% (1/15) SIG. SIG: 15% (1/6) SIG. Commercial SIG: 15% (1/6) SIG. Significant difference (P < 0.05) between the different concentrations of the fucoidins (SIG).

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4. Discussion

The leading cause of cancer deaths worldwide is lung cancer and the incidence of melanoma has risen dramatically over the past few decades [13]. Current therapeutic strategies to combat these cancers promote only minimal benefit and improve cancer patient survival in short-term only. Although recent years many studies have focused on the utilization of bioactive compounds derived from natural origins based on their potential as cancer preventive agents. Fucoidans in complexes sulfated polysaccharides obtained from naturally occurring algal brown seaweeds. For many years, these seaweeds have been a part of Asian diets. A study conducted across the Japanese populations investigating the major impact of diet on chronic diseases showed that the intake of seaweed was associated with lower incidence of all cancers, including lung cancer for men and women [14]. Animal model experiments have shown a direct anti-cancer effect following incorporation of fucoidan on the type melanoma into animal forms with no indications of either weight gain or xenografts [15]. The results revealed that fucoidan significantly increased levels of serum NK cell activity in the C57BL/6 mice, measured as specific lysis (data not shown). Hence, fucoidans were determined at all efforts to target cancers with MTA fucoidan inducing greater NK activity than SIG fucoidan, and the effects of both fucoidans were significantly (P < 0.05) better than the control (Fig. 7).

In this study, we employed crude fucoidan extracted from ground Seaweed sp. via a simple step extraction using 0.0 M HCl at 0°C for 4 h, and commercially obtained crude fucoidan from F. vesiculosus. We found that the humoral composition of the seaweed species were distinctively different. In particular, fucose, galactose sulfoacetate, and sialic acid were determined in the crude extracts (Table 1). Low dose levels of MTA fucoidan induced more significant and prolonged reduction of the proliferation of melanoma B16 cells than SIG fucoidan, but increased the cell proliferation assays revealed similar biologically inactive effects of MTA and SIG fucoidan on Lewis lung carcinoma and melanoma B16 cell lines (Fig. 7). These data indicate the crude fucoidan bioactivity towards these cells lines was probably related to the sialic groups present in the fucoidan structure rather than the presence of significant amounts of fucose. The same observation was reported by Yamamoto et al. [17] that found that sialic rather than fucose content of crude fucoidans determine its biological activity. The crude contents of MTA and SIG fucoidan were 384 and 30 μg, respectively (Table 1). These results agreed with those of Kepaonji et al. [18] who reported that purified fucoidans from F. vesiculosus containing 30 μg sulfate per mg protein was more active than crude fucoidan (Fig. 8). Conversely, it was determined by measuring TC50 release at the 48 h incorporation period from cultured human melanoma B16 cells in the presence of 0.1% FBS in 3% HCl at 37°C (Fig. 8). These results are shown in average values ± s.d.

8.0 mg/ml MTA and SIG fucoidan revealed in fragmentation and condensation of chromatin, stained as an intense pleomorphic dark brown color within the cell nucleus, as particularly evident for the MTA fucoidan treated cells (Fig. 9). (See interpretation of the colorations to color in this test, the reader is referred to the web-version of the article.)

1.4. Natural killer (NK) cell activity induced by crude fucoidan

The weights of male C57BL/6 mice (Clea Japan Inc., Tokyo, Japan) were recorded and the inherent aliments of the mice were observed daily for 4 days. The record revealed that there were no differences in the body weights of mice physically active with no evidence of malnutrition during the entire experimental period. In addition, the MTA and SIG fucoidans induced significantly increased levels of spleen NK cell activity in the C57BL/6 mice, measured as specific lysis of the fucoidan samples for four consecutive days (Fig. 5). NK cell activity at 100% 4 h after target cells were exposed simultaneously in mice treated with fucoidan 14 ± 0.9% for MTA and 15 ± 1.7% for SIG comparisons to 5 ± 2.3% in the non-treated mice control (Fig. 5). The positive control Poly C specific lysis at 108.5 was 24 ± 0.5% (Fig. 5). Statistically significant differences is between the effects of MTA and SIG were determined at all efforts to target tissues with MTA fucoidan inducing greater NK activity than SIG fucoidan, and the effects of both fucoidans were significantly (P < 0.05) better than the control (Fig. 7).

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b disrupt the function of immune system and subsequent immunosuppression by inhibition of the lymphocyte proliferation and cytokine production [10]. After the interaction of immune cells and fucoidan, some cytokines may be generated, which can modulate the immune response. The synthesis of cytokines is dependent on a variety of factors, such as the concentration of fucoidan, the type of immune cells, and the type of cytokine involved. In this context, various studies have shown that fucoidan can modulate the production of cytokines, such as IL-12, IL-6, TNF-α, and IFN-γ, which are important in the regulation of the immune response. For example, IL-12 is a critical cytokine in the induction of cellular and humoral immune responses [11]. Previous studies have shown that fucoidan suppressed the production of IL-12 in various immune cells, such as monocytes, macrophages, and dendritic cells, and thus inhibited the immune response [12,13]. In addition, fucoidan can suppress the production of IL-6, which is involved in the regulation of the immune response [14]. These results suggest that fucoidan may be a potential adjuvant in the modulation of immune responses.
Fucose-Containing Sulfated Polysaccharides from Brown Seaweeds Inhibit Proliferation of Melanoma Cells and Induce Apoptosis by Activation of Caspase-3 in Vitro

Marcel Tutor Ale 1, Hiroko Maruyama 2, Hidekazu Tamauchi 3, Jørn D. Mikkelsen 1 and Anne S. Meyer 1,*

1 Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering, Technical University of Denmark (DTU), Soeltoft Plads Bldg. 229, 2800 Kgs. Lyngby, Denmark; E-Mails: mta@kt.dtu.dk (M.T.A.); jdm@kt.dtu.dk (J.D.M.)
2 Department of Pathology, School of Allied Science, Kitasato University, Kitasato 1-15-1, Sagamihara, Kanagawa 252-0373, Japan; E-Mail: maruyama@ahs.kitasato-u.ac.jp
3 Department of Microbiology, School of Medicine, Kitasato University, Kitasato 1-15-1, Sagamihara, Kanagawa 252-0373, Japan; E-Mail: hidetama@med.kitasato-u.ac.jp

* Author to whom correspondence should be addressed; E-Mail: am@kt.dtu.dk; Tel.: +45-4525-2800; Fax: +45-4593-2906.

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Abstract: Fucose-containing sulfated polysaccharides (FCSPs) extracted from seaweeds, especially brown macro-algae, are known to possess essential bioactive properties, notably growth inhibitory effects on tumor cells. In this work, we conducted a series of in vitro studies to examine the influence of FCSPs products from Sargassum henslowianum C. Agardh (FSAR) and Fucus vesiculosus (FVES), respectively, on proliferation of melanoma B16 cells and to investigate the underlying apoptosis promoting mechanisms. Cell viability analysis showed that both FCSPs products, i.e., FSAR and FVES, decreased the proliferation of the melanoma cells in a dose-response fashion, with FSAR being more potent at lower dosages, and FVES being relatively more anti-proliferative than FSAR at higher dosages. Flow cytometric analysis by Annexin V staining of the melanoma cells exposed to the FCSPs products confirmed that both FSAR and FVES induced apoptosis. The FCSPs-induced apoptosis was evidenced by loss of plasma membrane asymmetry and translocation of the cell membrane phospholipids and was accompanied by the activation of caspase-3. The FCSPs bioactivity is proposed to be attributable to distinct structural features of the FCSPs, particularly the presence of sulfated galactofucans (notably in

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Fucose-containing sulfated polysaccharides (FCSPs) designate a group of diverse polysaccharides that can be extracted from brown seaweeds of the class Phaeophyceae. This seaweed class includes the order Fucales, in which seaweed species such as Fucus sp. and Sargassum sp. belong. The most studied FCSPs, originally called fucoidin, fucoidan or just fucans, have a backbone built of (1→3)-linked l-fucopyranosyl residues or of alternating (1→3)- and (1→4)-linked L-fucopyranosyl residues [1,2]. These fucopyranosyl residues may be substituted with short fucose side chains or sulfate groups at C-2 or C-4, and may also carry other minor substitutions, e.g., acetate, xylose, mannose, glucuronic acid, galactose, or glucose [3–5]. Brown seaweed FCSPs also include sulfated galactofucans with backbones built of (1→6)-β-D-galacto- and/or (1→2)-β-D-mannopyranosyl units. In addition to sulfate these backbone residues may be substituted with fucoses, single fucose substitutions, and/or glucuronic acid, xylose or glucose substitutions [4]. Recently it has been understood that the compositional and structural features of FCSPs differ significantly among seaweed species and that these features are markedly influenced by the conditions used to extract them [3,6].

FCSPs of different degrees of purity and composition, extracted from brown seaweeds such as Sargassum sp. and Fucus sp., have been documented to have a wide range of biological activities including anticoagulant [7,8], antithrombotic [8], anti-inflammatory [9], anti-viral [10,11]; and notably anti-tumoral effects [8,12,13]. Unfractionated FCSPs have thus specifically been found to reduce cell proliferation of lung carcinoma and melanoma cells in vitro; to exert immunomodulating effects in tumor bearing animals; and to activate natural killer cells in mice leading to increased anti-tumor effectiveness [13–16]. Kim et al. [17] applied a crude polysaccharide composed predominantly of sulfated fucose from Fucus vesiculosus to human colon cancer cells in vitro, and concluded that this crude brown seaweed polysaccharide extract is capable of inducing apoptosis, and provided data that suggested that the apoptosis was induced via activation of caspases. Moreover, commercially available crude FCSPs ("fucoidan") extracted from F. vesiculosus have been reported to inhibit proliferation and induce apoptosis on human lymphoma HS-Sultan cells lines by activation of caspase-3 [18]. Recently, we have reported that crude FCSPs extracted from a Sargassum sp. and from F. vesiculosus, respectively, induce growth inhibition and apoptosis of melanoma B16 cells in vitro [13]. When injected intraperitoneally into mice over four days, these same unfractionated FCSPs were found to induce enhanced natural killer cells (NK cells) activity to result in specific lysis of YAC-1 cells (a murine T-lymphoma cell line sensitive to NK cells) [13]. Previous reports with human HS-Sultan cells and MC97 cells, respectively, have suggested that the FCSPs induced apoptosis initiation may take place via activation of caspase-3 and caspase-8 dependent pathways, respectively [18,19], but no firm evidence has been established regarding the exact mechanism responsible for the apoptotic action through cascades of reactions that involve activation of caspase-3.
of the FCSPs. The objective of the present study was, therefore, to examine whether the anti-proliferative action and apoptosis of melanoma B16 cells induced by FCSPs derived from Sargassum henslowianum C. Agardh and Fucus vesiculosus, are accompanied by increased caspase-3 activity. We also wanted to evaluate whether any structural features of the FCSPs might be crucial for bioactivity. In this study, we present the different structural features of the FCSPs derived from S. henslowianum and F. vesiculosus as assessed by IR and $^1$H NMR spectroscopy and show that these FCSPs exert bioactive effects that inhibit the proliferation of melanoma B16 cells by apoptosis. We also show that the antiproliferative effects and the apoptosis are accompanied by activation of caspase-3.

2. Results

2.1. FCSPs Chemical Composition

The compositional analysis of the fucose-containing sulfated polysaccharide products from S. henslowianum C. Agardh (FSAR) and F. vesiculosus (FVES), respectively, showed that the FSAR product was mainly made up of uronic acid and fucose, with a significant level of sulfate, and minor amounts of other monosaccharides, mainly galactose and mannose (Table 1). The FVES product had a similar monosaccharide profile and a similar sulfation level, but the amounts of fucose, galactose and xylose were significantly higher than in FSAR; whereas the uronic acid and mannose levels were lower (Table 1).

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<td>FSAR</td>
<td>Fuc ** 31.1 ± 2  Rha 18.1 ± 0.1  Ara 0.2 ± 0.1  Gal ** 14.1 ± 1  Gle 4.2 ± 0.1  Xyl ** 4.2 ± 0.3  Man ** 5.7 ± 0.5  UA ** 5.7 ± 0.5  Sulfate 123 ± 7 384 ± 26</td>
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<tr>
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* Monosaccharide composition: Fuc = fucose, Rha = rhamnose, Ara = arabinose, Gal = galactose, Gle = glucose, Xyl = xylose, Man = mannose, UA = uronic acid; ** Significantly different levels among FSAR and FVES at P < 0.05, number of replicates = 4.

2.2. IR and $^1$H NMR Spectra of FCSPs

The FCSPs were analyzed to determine if their infrared absorption properties were similar to the previously reported fucoidan IR absorption data [2,20]. The spectra of the FSAR and FVES samples scanned between wavenumbers 4000 and 400 cm$^{-1}$ both exhibited major absorption bands at around 3340 and 3420 cm$^{-1}$ that were interpreted as being due to O–H stretching (data not shown). The IR spectra between 1800 and 500 cm$^{-1}$ (Figure 1a,b) revealed small but distinct bands for both the samples at 1720 cm$^{-1}$ which indicated the presence of O-acetyl groups [21], whereas the absorption bands at ~1610 to 1620 cm$^{-1}$ (Figure 1a,b), most pronounced for the FSAR sample, indicated uronic acid [20]. The FSAR sample showed an intense IR band at around 1400–1470 cm$^{-1}$ which could be attributable to scissoring vibration of CH$_3$ (galactose, mannose) and asymmetric bending vibration of CH$_2$ (fucose, O-acetyl) as suggested previously for absorption around 1455 cm$^{-1}$ by Synytsya et al. [22].

2. Results

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absorption band at 1240 cm\(^{-1}\) observed for both samples, but being particularly prevalent for the FVES sample, was assigned as S=O stretching vibration, indicating the presence of esterified sulfate [20]. A similar absorption pattern around 820-840 cm\(^{-1}\) was observed for both FCSPs. The FSAR infrared spectrum showed an absorption band at 817 cm\(^{-1}\) (Figure 1a) whereas the FVES infrared spectrum displayed a broader absorption band at 838 cm\(^{-1}\) and a small shoulder of absorption at 822 cm\(^{-1}\) (Figure 1b). Since IR absorption at 840 cm\(^{-1}\) has been reported to be due to sulfate groups at the axial C-4 position whereas sulfate groups at the equatorial C-2 and/or C-3 positions have been reported to give a small absorption at 820 cm\(^{-1}\) [2], the observed absorption bands at 820-840 cm\(^{-1}\) were interpreted as being indicative of sulfate groups.

The proton NMR spectra (\(^2\)H\(_2\)O) of the FSAR and the FVES samples were complex with broad signals and with several signals in the chemical shift of the envelope of anomeric signals at 5.0-5.5 ppm (Figure 2a,b). The presence of the signals at 5.0-5.5 ppm is consistent with the presence of 3,4-linked L-fucopyranose residues for fucoidan from Sargassum fusiformis [21]. The independent signal, 4.61 ppm (Figure 2a) was assigned to 3-linked \(\beta\)-sulfated L-fucopyranose residues [21]. Moreover the intense signals at 5.10 and 5.18 ppm in the chemical shift of the envelope of the anomeric proton of FSAR (Figure 2a) were reported earlier and assigned to \(\alpha\)-linked and \(\alpha,\beta\)-linked L-fucopyranose residues for fucoidan from Hizikia fusiformis a.k.a Sargassum fusiformis [24]. The high field region signals 1.24 and 1.20 ppm (Figure 2a) were assigned to \(\alpha\)-linked 2-mono-O-sulfated and \(\alpha\)-linked unsulfated L-fucopyranose residues [21]. Moreover the intense signals at 4.57 and 3.99 ppm (Figure 2a) were assigned to the presence of 4-linked 2-mono-O-sulfated L-fucopyranose residues [21]. The independent signal, 4.61 ppm (Figure 2a) was assigned to a 3-linked \(\beta\)-galactopyranosyl residue when compared with the data of Farias et al. [25].

Figure 1. Infrared analysis of fucose-containing sulfated polysaccharides (FCSPs) from (a) Sargassum henslowianum C. Agardh (FSAR) and (b) Fucus vesiculosus (FVES) scanned between 1800 and 550 cm\(^{-1}\).

The proton NMR spectra (\(^2\)H\(_2\)O) of the FSAR and the FVES samples were complex with broad signals and with several signals in the chemical shift of the envelope of anomeric signals at 5.0-5.5 ppm (Figure 2a,b). The presence of the signals at 5.0-5.5 ppm is consistent with the presence of 3,4-linked L-fucopyranose residues [21]. The 1H-NMR spectra also both contained peaks at 1.1–1.3 ppm, with the signals for the FVES sample being particularly strong (Figure 2a,b). Previously, such high-field region signals at 1.1–1.3 ppm have been assigned to a C6 methyl proton group of L-fucopyranose [22] whereas several intense and narrow signals at 2.14–2.21 ppm have been attributed to CH\(_3\) protons of \(\alpha\)-acetyl groups [5]. The narrow and intense signals at 5.10 and 5.18 ppm in the chemical shift of the envelope of the anomeric proton of FSAR (Figure 2a) were reported earlier and assigned to \(\alpha\)-linked and \(\alpha,\beta\)-linked L-fucopyranose residues for fucoidan from Hizikia fusiformis a.k.a Sargassum fusiformis [24]. The high field region signals 1.24 and 1.20 ppm (Figure 2a) were assigned to \(\alpha\)-linked 2-mono-O-sulfated and \(\alpha\)-linked unsulfated L-fucopyranose residues [21]. Moreover the intense signals at 4.57 and 3.99 ppm (Figure 2a) were assigned to the presence of 4-linked 2-mono-O-sulfated L-fucopyranose residues [21]. The independent signal, 4.61 ppm (Figure 2a) was assigned to a 3-linked \(\beta\)-galactopyranosyl residue when compared with the data of Farias et al. [25].
FVES had an intense signal at 5.45 ppm (Figure 2b), which was assigned to α3-linked 2-mono-O-sulfated L-fucopyranose residues, whereas the signals at 5.40, 4.58 and 4.39 ppm (Figure 2b) were assigned to be due to di-sulfated residues, i.e., α3-linked 2,4-di-O-sulfated L-fucopyranose residues [26]. Hence in general, the 1H NMR confirmed the anticipated FCSPs structures of the two samples.

Figure 2. One-dimensional 1H NMR spectra of crude FCSPs from (a) Sargassum henslowianum C. Agardh (FSAR) and (b) Fucus vesiculosus (FVES) in D2O obtained using an INOVA 600 NMR spectrometer (Agilent Technologies, Tokyo, Japan).

2.3. Anti-Proliferative Effects of the FCSPs

The viability of melanoma B16 cells treated with the FSAR and FVES products, respectively, was determined via measurement of cell proliferation using an MTT based colorimetric assay. Both FCSPs products (FSAR and FVES) decreased the viability of melanoma B16 cells in a dose-dependent fashion, after 24 h of incubation of 6 × 10^5 cells density per well (Figure 3). In particular, a pronounced cell viability reduction was noticed after the addition of low levels, 0.1 mg/mL, of FSAR, producing a cell viability of ~80% of the control, and cell proliferation was halted gradually as the FCSPs dosage level increased (Figure 3) indicating moderate cytotoxicity. The FVES treated cells showed the same trend, but the FVES product generally induced a lower anti-proliferative effect than the FSAR product at the lower FCSP addition levels (P < 0.05), but a significantly higher effect than FSAR (P < 0.05) at the higher addition level, producing a drastic reduction of the proliferation of melanoma B16 cells leaving only ~6% of the cells viable at an FCSPs addition level of 1 mg/mL (Figure 3). The viability
previously published data [13].

The reduction pattern induced by the two FCSPs on the melanoma cells were in complete accord with previously published data [13].

Figure 3. MTT based colorimetric assay of cell viability of melanoma B16 cells after treatment for 24 h with different dosage levels of crude fucose-containing sulfated polysaccharides from Sargassum henslowianum C. Agardh (FSAR) and Fucus vesiculosus (FVES), respectively. Cell density was $6 \times 10^4$ cells per well. $ab$ indicate statistically significantly different ($P \leq 0.05$) cell viability levels after treatment with the two FCSP products at the same dosage level (mg/mL) ($n=4$).

2.4. Apoptosis of Melanoma Cells by FCSPs

Programmed cell death or apoptosis is characterized by certain morphological cell changes such as loss of plasma membrane integrity in addition to internucleosomal DNA cleavage. One of the earliest apparent changes in cells undergoing apoptosis is the translocation of the cell membrane phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane. This change in the cell membrane is now recognized as an early, essential feature of apoptosis. The translocation exposes the phosphatidylserine to the external cellular environment and this is a feature which can be measured by exposing the cells to fluorochrome-conjugated phospholipid-binding proteins such as phycoerythin (PE)-labelled Annexin V (PE-Annexin V). Such staining with Annexin V is typically used in conjunction with a vital dye such as 7-amino-acticomycin (7-AAD) to identify early stages of apoptotic cells (Annexin V$^+$, 7-AAD$^+$) which accompany the later apoptosis stages (both Annexin V and 7-AAD are positive). Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells undergoing apoptosis are permeable to 7-AAD. Figure 4 shows the number of melanoma cells undergoing apoptosis (% relative to a control not exposed to FCSPs) and the flow cytometric scan data of Annexin V staining induced by exposure of the melanoma cells to 0.2 mg/mL of the seaweed FCSPs samples from S. henslowianum (FSAR) and F. vesiculosus (FVES), respectively. Both FCSPs products induced significant apoptosis of the melanoma cells: The FSAR product appeared to induce a more potent apoptotic effect than the FVES product (Figure 4a) since the relative number of melanoma cells undergoing apoptosis (% relative to control sample not exposed to FCSPs) induced by the FSAR sample was significantly higher (41 ± 3%) than the apoptotic effect of
the FVES exposure (30 ± 5%). The data corresponded to the fluorescence-activated cell sorting (FACS) scan showing the accumulation of intense dots-color in cells that underwent the latest apoptosis stage (Figure 4b,c: both Annexin V and 7-AAD positive). The FVES sample induced more early apoptosis (Figure 4c than the FSAR, as characterized by the build-up of disperse dots-color (Annexin V’ and 7-AAD’) indicating loss of plasma membrane asymmetry. The data were in accordance with the anti-proliferative effects of the FCSPs treatments (Figure 3).

Figure 4. Flow cytometric analysis by Annexin V staining of Melanoma B16 cells treated for 24 h with 0.2 mg/mL crude fucose-containing sulfated polysaccharide (FCSP) products extracted from S. henslowianum C. Agardh (FSAR) and F. vesiculosus (FVES), respectively. (a) Apoptosis induced by FSAR (41 ± 3%, s = 2), FVES (30 ± 5%, s = 2); control 11.66 % (data not shown) (b) FSAR data and (e) FVES data for FACS scans of FCSP treated Melanoma 16 cells that were viable and not undergoing apoptosis (Annexin V’ and 7-AAD’); undergoing early apoptosis, with membrane integrity intact (Annexin V’ and 7-AAD’), in the latest stage apoptosis and dead (Annexin V’ and 7-AAD’), respectively.

2.5. FCSPs Activation of Caspase-3

In general, activation of caspase-3 initiates apoptosis in mammalian cells. The caspase-3 colorimetric assay employed in the present study is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage of the pNA-labeled substrate DEVD-pNA. The activity of caspase-3 was augmented after treatment of the melanoma cells for 24 h with the FCSPs from S. henslowianum (FSAR) and F. vesiculosus (FVES) (Figure 5). The recorded caspase-3 activity increased significantly in a dose-dependent manner in response to the FCSPs treatment dosage (0–0.8 mg/mL), i.e., from 100% of control at 0, to ~180% of the control response at 0.8 mg/mL (P ≤ 0.05) (Figure 5). No significant differences in the responses induced by the two types of FCSPs were recorded within the individual dosages of the FSAR and FVES treatments (Figure 5). The same trend of caspase-3 activity was observed in a 48 h treatment of melanoma cells with the FSAR and FVES samples (data not shown).
Fucose-containing sulfated polysaccharides (FCSPs) derived from naturally grown brown seaweeds by aqueous extraction have been shown to exert potentially beneficial bioactivities, including immuno-modulatory, anti-inflammatory and anti-tumorigenic effects. In keeping its natural properties, FCSPs must be extracted from brown seaweeds by use of a mild processing treatment and a minimal number of extraction steps.

Brown seaweeds constitute a part of the conventional diet in several Asian countries, especially in Japan, and in a Japanese cohort study the intake of seaweeds has been associated with lower mortality from all chronic diseases including cancer [28]. It has recently been demonstrated that FCSPs from brown seaweeds exert growth inhibitory activity on certain cancer cell lines in vitro [13,17,18]. Incorporation of brown seaweeds into animal diets has also revealed cancer inhibitory effects with no direct lethal consequences [29,30]. Natural FCSPs from brown seaweeds may therefore have significant potential as protective agents to control or prevent skin cancer provided that the FCSPs do indeed exert cancer-preventive effects.

In this study, in accordance with previous data [13], we found that unfractionated FCSPs, i.e., FSAR and FVES, extracted from the brown seaweeds S. henslowianum and F. vesiculosus, respectively, were composed of fucose, galactose, xylose, mannose and glucuronic acid, and showed that the fucose, galactose and glucuronic acid contents differed significantly among the two FCSPs products, but that their sulfate contents were similar (Table 1).

We also found both distinct differences and several similarities in the structural make-up of these FCSPs by use of FTIR and 1H NMR spectroscopy. The FT-IR analyses thus corroborated the presence...
of sulfate groups in both the FSAR and the FVES sample (Figure 1). The IR spectra indicated that the sulfate substitutions of the FCSPs extracted from the Sargassum sp. (FSAR) were located in the equatorial C-2 and/or C-3 positions as depicted by absorption bands at 817 cm\(^{-1}\). This finding was in agreement with data reported for fucoidan fractions isolated from Sargassum stenophyllum [4]. However, Daarte et al. [4] also reported that two other saccharide fractions from S. stenophyllum had an absorption band at 837 cm\(^{-1}\) indicating sulfate groups at the C-4 positions of the structural monosaccharides [4]. The spectra of the FCSPs from F. vesiculosus (FVES) displayed an absorption band at 838 cm\(^{-1}\) with a small shoulder at ~822 cm\(^{-1}\) indicating sulfate groups at both the C-4 and the C-2 position (Figure 1). This finding corresponds to previously reported \(^1\)H NMR data of FCSPs from F. vesiculosus that have indicated a typical structure of algal fucoidan consisting of \(\alpha_3\)-linked 2-mono-O-sulfated L-fucopyranose residues, and/or \(\alpha_3\)-linked 2,4-di-O-sulfated L-fucopyranose residues [2,26]. Small disparities in the IR spectra from different published reports can be due to factors such as sample handling and the FCSPs extraction procedure employed.

The present study also aimed at establishing whether crude FCSPs extracted from Sargassum hendersoni C. Agardh (FSAR) contained fucoidan-like structures composed of \(\alpha_3\)-linked or/and \(\alpha_3\)-linked L-fucopyranose residues. Even though signals consistent with the presence of \(\alpha_3\)-fucopyranose entities were recorded (with \(^1\)H NMR signals at 5.10 and 5.18 ppm, Figure 2a), the probability that the FSAR may contain a cocktail of polysaccharides is likely. Hence, the \(^1\)H NMR spectra also showed that the FSAR sample contained 3-linked \(\beta\)-galactopyranose residues as indicated by an independent signal at 4.61 ppm (Figure 2a). \(\beta\)-1-(\(\alpha_3\))-linked galactopyranose residues are known to be a typical structural feature of seaweed polysaccharides, from e.g., Laminaria angustata var. longissima, Botryocladia occidentalis [25,31]. However, another possibility might be that the FSAR sample was not composed of a mixture of different types of polysaccharides but rather, that the sample consisted of one type of a highly complex hetero-polysaccharide as suggested by Daarte et al. [4] for the fucoidans from Sargassum stenophyllum. It can safely be said that the \(^1\)H NMR spectra of the FCSPs samples were complex and overlapping. It is therefore difficult to draw any definite conclusions about the detailed structural features and differences among the two FCSPs; the detailed elucidations of the definite structural details were also beyond the scope of this present study, but clearly deserve further investigation. Nonetheless, the data confirmed that the diversity, i.e., the compositional and structural complexity of (potentially bioactive) algal fucose-containing sulfated polysaccharides, is much wider than originally believed.

The biological activities of the FCSPs against skin cancer cells were investigated \emph{in vitro}, and the results revealed that both FSAR and FVES can exert anti-proliferative effects on melanoma B16 cells \emph{in vitro}. The FSAR sample induced more significant reductions of the cell viability of melanoma cells than the FVES sample at low dosage levels (Figure 3). At higher dosages, the FSAR treatment still induced gradually more loss of cell viability, but the FVES had more potent anti-proliferative effects at higher dosages than FSAR which could indicate direct cell toxicity. The bioactivities of these FCSPs may be attributable to their distinct structural features, notably the level of sulfation (charge density), the distribution (e.g., random versus clustered) and binding of the sulfate substitutions, as well as other specific structural features of the sulfated fucans and the sulfated galactofucan complexes. The sulfate groups of FVES were substituted at the C-2 and C-4 position of the fucose substituents, typical for fucoidan from F. vesiculosus, and consistent with previously published data indicating that the sulfate
groups were substituted at C-2 of α-L-fucopyranose residues in fucoidan from e.g., *Fucus* spp. [3,21], but is also in agreement with the proposition that the sulfate groups were substituted at C-2 and/or C-3 positions of the monosaccharides according to the IR spectra (Figure 1); the observation of C-2 linked sulfate groups agreed with the data mentioned above for fucoidan from *Fucus* spp. [3,21], but is also in agreement with the proposition that the sulfate groups were substituted at C-2 on the 3-linked galactopyranose residues [27]. The possible presence of sulfated, 3-linked galactan in the structure of FSAR may contribute to the efficacy of FSAR to induce anti-proliferative effects as it has been reported that 2-O-sulfated 3-linked galactan is more bioactive than 2-O-sulfated 3-linked fucan and 3-O-sulfated 4-linked galactan [32–34].

In contrast, the sulfate substitutions in the FSAR were interpreted to be mainly at the C-2 and/or C-3 positions of the monosaccharides according to the IR spectra (Figure 1); the observation of C-2 linked sulfate groups agreed with the data mentioned above for fucoidan from *Fucus* spp. [3,21], but is also in agreement with the proposition that the sulfate groups were substituted at C-2 and/or C-3 positions of the monosaccharides according to the IR spectra (Figure 1); the observation of C-2 linked sulfate groups agreed with the data mentioned above for fucoidan from *Fucus* spp. [3,21], but is also in agreement with the proposition that the sulfate groups were substituted at C-2 on the 3-linked galactopyranose residues [27]. The possible presence of sulfated, 3-linked galactan in the structure of FSAR may contribute to the efficacy of FSAR to induce anti-proliferative effects as it has been reported that 2-O-sulfated 3-linked galactan is more bioactive than 2-O-sulfated 3-linked fucan and 3-O-sulfated 4-linked galactan [32–34].

The principal objective of this study was to assess whether any structural features of the FCSPs might be crucial for bioactivity, and the data suggest that the sulfate substitutions, and not necessarily only the fucose-backbone structure itself, confer this decisive bioactivity. It is however important to investigate whether other differently structured FCSPs may exert similar growth inhibitory and apoptosis inducing effects on cancer cells.

In this work we noted that both FCSPs activate caspase-3 in a dosage-response fashion (Figure 5). These findings affirmed the results reported previously which have shown that FCSPs (“fucosan”) from *F. vesiculosus* induce apoptosis in human lymphoma HS-Sultan cell lines and in HT-29 and HCT116 human colon cancer cells in *vivo*, and moreover that the exposure of these cells to the *F. vesiculosus* FCSPs appear to activate caspase-3 [17,18]. The *F. vesiculosus* FCSPs treatment was also shown to enhance mitochondrial membrane permeability of human colon cancer cells in *vivo*, and to induce cytochrome c and Smac/Diablo release from the mitochondria [17]. It has also been reported that pretreatment of HT-29 and HCT116 colon cancer cells with individual caspase-8 or caspase-9 inhibitors (Z-IETD-FMK and Z-LEHD-FMK, respectively) prior to fucoidan exposure reduced the levels of caspases, including caspase-3 [17]. It has likewise been shown that pretreatment of human lymphoma HS Sultan cells with a pan-caspase inhibitor, z-VAD-FMK, reduced fucoidan-induced apoptosis [18]. Hence, the available data support the proposition that fucoidan-induced apoptosis occurs through caspase activation pathways. The cascade mechanism by which the caspase-activation is presumed to take place via the fucose-mediated apoptotic pathway is illustrated in Figure 6.

Loss of plasma membrane is one of the earliest features of apoptosis and Annexin V staining can identify apoptosis at an early stage. However this assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway, because in either case the dead cells will stain with both Annexin V and 7-AAD. Both early and late apoptosis stages were observed by the FACS scanning indicating that the FCSPs had a direct apoptotic effect on
the melanoma B16 cells (in vitro) (Figure 4). The direct apoptotic action of the FCSPs was probably due to the interaction of the highly negative charge density of the FCSPs with the melanoma B16 cells (as a result of the sulfation). Recently, we reported that crude fucoidan from *Sargassum* sp. could trigger apoptosis indirectly by enhancing the activity of natural killer (NK) cells activity *in vivo* [13]. NK cells produce immunologically important cytokines, notably IFN-γ, which can promote the activation of T-cells to produce interleukin-2 and -12 that in turn further enhance the NK cell activation [14,36].

**Figure 6.** Proposed mechanism for inhibition of the proliferation of melanoma cells by FCSPs: Activation of macrophages via membrane receptors, which leads to the production of cytokines that enhance NK cell activation. Activated NK cells release Granzyme B and perforin through granule exocytosis into the space between NK cells and melanoma cells to initiate caspase cascades in melanoma cells. Assimilation of Granzyme B by the tumor cells is facilitated by perforin. Granzyme B then initiates apoptosis by triggering the release of mitochondrial cytochrome c and apoptosome formation leading to caspase-3 activation, which in turn translocates the nucleus causing DNA fragmentation—the distinct morphological change of cells by apoptosis [36,37].

The apoptosis induced by FCSPs via the activation of caspase-3 was reported previously to be mediated through a mitochondrial pathway [17–19,38]. However, it remains to be determined whether
differences in FCSPs structures will influence the apoptotic mechanism, including the mitochondrial pathway apoptosis cascade. The route of the mitochondrion-dependent apoptotic pathway is the release of apoptosis-inducing factor (AIF) and cytochrome c from the inner mitochondrial membrane into the cytosol. Cytochrome c interacts with Apaf-1 (apoptotic protease activating factor 1) and procaspase-9 to form the active apoptosis. The apoptosis then initiates the cleavage of procaspase-3, producing active caspase-3, which initiates the execution phase of apoptosis by proteolysis of substances whose cleavage commits the cell to apoptosis [39] (Figure 6). The influence of the different FCSPs structures on the mitochondrial membrane permeability and electric potential requires further study. We hope in the future to investigate the bioactivity and mechanism of FCSPs on certain degenerative diseases in vivo and to further elucidate specific molecular targets of FCSPs for inhibition of cancer cells.

4. Experimental Section

4.1. Chemicals

Dried S. henslowianum C. Agardh was obtained from Viet Delta Ltd. (Ho Chi Minh, Vietnam) and the Fucose-containing sulfated polysaccharides (FCSPs) from the S. henslowianum (FSAR) were extracted in our laboratory (see below). Crude fucoidan from F. vesiculosus (FVES) was obtained from Sigma-Aldrich (Steinheim, Germany); according to the product description the FVES had been prepared from F. vesiculosus via the extraction method described by Black and Dewar [40]. Hydrochloric acid (37%), D-glucose and D-xylose were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (99%, TFA), trichloroacetic acid (99%, TCA), CaCl₂, Na₂SO₄, BaCl₂, arabinose, rhamnose, D-galactose and L-fucose were from Sigma-Aldrich Co. (Steinheim, Germany). Agarose D-2 was obtained from Hispanagar (Burgos, Spain). Caspase-3 colorimetric assay kit was obtained from Biovision, Inc. (Mountain View, CA, USA). Minimal essential medium eagle (MEM-eagle) cell culture media was purchased from Sigma-Aldrich Co. (Steinheim, Germany); foetal bovine serum (FBS) was from Flow Laboratories (North Ryde, N.S.W., Australia); streptomycin-penicillin and Trypan Blue were from Gibco (Canada). Cell Proliferation Kit 1 was obtained from Roche Applied Science, Germany. The PE Annexin V Apoptosis Detection Kit 1 was obtained from BD Biosciences (Franklin Lakes, NJ, USA). All chemicals used were analytical grade.

4.2. Extraction of FCSPs from S. henslowianum C. Agardh

The Sargassum FCSP product (FSAR) used was extracted from S. henslowianum C. Agardh by use of an optimized single-step extraction procedure described previously [6]. Briefly, the dried S. henslowianum seaweed was ground and sieved to pass through a 500 μm sieve and 100 g of dried ground seaweed was extracted in 2 L of 0.03 M HCl with continuous stirring at 200 rpm for 4 h at 90 °C water bath (Julabo, Germany). The suspended seaweed was filtered, and the extract was precipitated using 60% ethanol, the precipitate collected after centrifugation at 10,600 rpm for 10 min (Sigma Laboratory Centrifuge 4K15, VWR, Denmark), and the resulting pellet was freeze dried. This freeze dried pellet constituted the fucose-containing sulfated polysaccharides (FSAR).
4.3. Acid Hydrolysis and FCSPs Composition Analysis

The freeze dried FSAR and FVES samples (20 mg) were hydrolyzed separately in 2 M TFA (final concentration) at 121 °C for 2 h, then the hydrolyzed mixture were freeze dried at –57 °C (Heto Lyolab 3000, England). Each dried powder sample was resolubilized in doubly distilled water and centrifuged at 10,000 rpm for 10 min to collect the supernatant (Sigma Laboratory Centrifuge 4K15, VWR, Denmark). Each supernatant was filtered through a 0.2 μm syringe tip filter (SUN-Sri, Rockwood, TN) prior to injection into the HPAEC-PAD for monosaccharide analysis [41]. Analysis of sulfate content was done according to the method described by Jackson and McCandless [42].

4.4. 1H NMR and FTIR Spectroscopy

The 1H NMR spectra were obtained using an INOVA 600 NMR spectrometer (Agilent Technologies Japan, Ltd., Tokyo Japan) equipped with a 1H[15N-31P] pulse field gradient indirect-detecting probe. Standard pulse sequences were used in all operations. The 1H chemical shift (δH) was referenced to HOD (δH 4.76 ppm, 2H2O). The 1H NMR spectrum was assigned through the 1H–1H decoupling technique. A NMR spectrum of 1-fucose was utilized as a reference for chemical shift assignment. The lyophilized FCSPs powders were dissolved in deuterium oxide (2H2O) and evaporated to exchange the unstable 1H with 2H. The evaporation and dissolution step was repeated five times, and the samples (10 mg) were finally dissolved in 0.75 ml 2H2O and then subjected to NMR spectroscopy. The IR spectra were obtained using a Spectrum One FT-IR spectrometer (Perkin Elmer, Waltham, MA, USA) equipped with universal attenuated total reflectance (UATR) accessories. Analysis of each of the FSAR and FVES powders, ~1 mg of each, was done using diffuse reflectance infrared transform spectroscopy (DRIFTS) and the spectrum was evaluated by Perkin Elmer Spectrum software version 5 (Perkin Elmer, Waltham, MA, USA).

4.5. Cell Culture and Anti-Proliferative Assay

Melanoma B16 cells (MC) were grown in MEM eagle medium supplemented with 10% (v/v) heat inactivated FBS, 1% (w/v) streptomycin-penicillin and 1% (v/v) of 200 mM L-glutamine at 37 °C under 5% CO2. Monolayer cultivation was carried out by adding 100 μL of the cell-MEM-FBS mixture into separate wells in 96-flat well plates at a density of 6 × 10^4 cells per well followed by incubation for 24 h in 5% CO2 at 37 °C. For the anti-proliferation assay the medium was removed after the 24 h of monolayer cell cultivation and replaced with 100 μL of MEM medium containing 2% FBS and varying concentrations (0.1–1.0 mg/mL) of the crude FCSPs, i.e., FSAR and FVES, respectively, and the mixtures were then incubated for 24 h. Quantification of cell proliferation was carried out using a tetrazolium salt (MTT (3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide)) based colorimetric assay following the protocol supplied with the Cell Proliferation Kit 1 (Roche Applied Science, Germany). Briefly, 20 μL MTT solution (5 mg/mL) was added to the cell cultures after the 24 h of incubation with the FCSPs, and the cell cultures were then re-incubated for 4 h. Finally, 100 μL of stabilization solution was added to each well and the plates were incubated overnight at 37 °C under 5% CO2. Absorbance was measured using an Elisa reader at 550–690 nm.
4.6. Cell Culture and Caspase-3 Assay

Melanoma B16 cells (MC) were grown in MEM eagle culture medium supplemented with 10% (v/v) heat inactivated FBS, 1% (w/v) streptomycin-penicillin and 1% (v/v) of 200 mM L-glutamine maintained at 37 °C under 5% CO₂. For the caspase-3 assay, monolayer cultivation was carried out in a petri dish (60 × 15 mm) by adding 5 mL culture medium containing melanoma cells at a density of 1 × 10⁵ per mL and varying concentrations (0.2, 0.4 and 0.8 mg/mL) of the FSAR and FVES, respectively. The mixtures were then incubated for 24 and 48 h in 5% CO₂ at 37 °C. The caspase-3 assay was performed according to the protocol supplied with the assay kit (Biovision Inc., Mountain View, CA, USA) used to assay the activity of caspases that recognize the amino acid sequence DEVD. The assay was based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. Concisely, the melanoma B16 cells exposed to FSAR and FVES, respectively, were harvested and resuspended in 50 μL of cell lysis buffer and incubated on ice for 10 min. and the mixture centrifuged for 1 min (14,000 g, 4 °C). Each supernatant was then transferred to a fresh tube, and reaction buffer (50 μL) and 4 mM DEVD-pNA substrate (5 μL) were added, and this reaction mixture was then incubated at 37 °C for 1 h. Absorbance of pNA light emission was quantified using a microtiter plate reader at 405 nm.

4.7. Apoptosis Assay by Fluorescence-Activated Cell Sorting (FACS)

After 24 h of monolayer cultivation of melanoma B16 cells with 0.2 mg/mL of FSAR or FVES, and no FCSPs addition as control, the culture medium was removed, and the cells harvested by addition of 1 mL Trypsin-EDTA. The harvested cells were washed twice with 0.1 M PBS and then resuspended in binding buffer according to the protocol for the Annexin V Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA). 100 μL of this solution at 1 × 10⁵ cells was transferred to a culture tube and 5 μL of Annexin V and 5 μL of 7-amino-actinomycin (7-ADD) were added, and the mixture incubated at room temperature for 25 min. Then, 400 μL of binding buffer was added and the extent of apoptosis and staining pattern of the cells were tracked by flow cytometric analysis on a FACScan instrument (Becton Dickinson).

5. Conclusions

The tumor inhibitory bioactivity of fucose-containing sulfated polysaccharides (FCSPs) from Sargassum henslowianum C. Agardh (FSAR) and F. vesiculosus (FVES) was demonstrated through evaluation of inhibition of melanoma cell proliferation, activation of caspase-3, and apoptosis of melanoma B-16 cells in vitro. The structural traits of the FCSPs products were shown to be complex and to differ among the two FCSPs making it delicate to draw definite conclusions about structural effects and mechanisms. However, since the sulfate levels were relatively high as well as relatively similar among the two FCSPs, we propose that the bioactivity effects of the FSAR and FVES might be attributable to the sulfation (charge density), positioning and bonding of the sulfate substitutions in the FCSPs. The work clearly indicates that unrefined fucose-containing sulfated polysaccharides from both Sargassum henslowianum C. Agardh and Fucus vesiculosus may have therapeutic potential as skin-cancer preventive agents.
Acknowledgments

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18. through activation of

16. induces apoptosis of human HS-sultan cells accompanied by activation of caspase-3 and


10. M. Fucoidan induces apoptosis of human HS-sultan cells accompanied by activation of caspase-3 and


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**Samples Availability:** Available from the authors.

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5 Seaweed nutrient assimilation and growth response

Industrial sewage, fish farm effluents, and agricultural runoff are the major causes of eutrophication of aquatic systems. Eutrophication has adverse environmental effects including hypoxia; on the other hand, some aquatic organisms may experience increased population, promoted growth, and the production of algae, including fast-growing seaweed. The use of large-scale seaweed populations is thought to provide new and cost-effective technologies for sequestering and preventing the distribution of waterborne pollutants. Seaweeds can remove several pollutants through assimilation, bioaccumulation, and detoxification. Integration of seaweed cultivation with fish aquaculture has been proposed to reduce the nutrient burden of fish effluents (Chopin et al., 2001). Ulva sp. has been considered the integrated biofilter system and has shown reasonably high efficiency in the removal of waste inorganic nutrients (Chung et al., 2002). Seaweed accumulates a wide range of heavy metals regardless of radioactivity status. In highly polluted seawaters, bioaccumulation can be as high as 1 g per dry gram of seaweed tissue, while approximately 5–100 mg/g in normal seawaters (Burdin and Bird, 1994; Grüven et al., 1993).

Opportunistic green macroalgal such as U. lactuca can rapidly consume the available inorganic nutrients at a rate that depend on the availability of ammonium and nitrate (Pedersen and Borum, 1996) and, hence, influence their differential growth responses. U. lactuca has a high growth potential doubling time of approximately 2 days with a production potential of 45 T (total solids) ha^{-1} yr^{-1} (Christensen and Sand-Jensen, 1990; Bruhn et al., 2011). Nitrogen enrichment studies have shown uptake rates and growth responses that were more in favor of ammonium than nitrate since they require energy-demanding nitrate reductions (Pedersen and Borum, 1996; Solomonson and Barber, 1990). U. lactuca was known to have high affinity for dissolved inorganic carbon (DIC). Rapid photosynthesis in dense floating mats leads to DIC depletion and increases pH and oxygen levels; as a consequence, these changes may all inhibit carbon fixation and, thus, reduce growth. Photosynthetic experiments have shown that the growth response of Ulva species was weakly affected by oxygen levels, whereas pH approaching 10 was highly inhibitory (Christensen and Sand-Jensen, 1990).
5.1 Evaluation of growth and assimilation
Quantitative determination and accurate measurement of productivity responses are essential aspects for understanding the primary production and the response to different transient nutrients. The common method and technique to determine seaweed growths in culture systems are gravimetric, and direct contact to the algal tissue when removing of surface moisture (Misuya et al., 2008). Measurement of the surface area of circular disks from algal thalli has been used in some growth monitoring studies (Sand-Jensen, 1988; Christensen and Sand-Jensen, 1990). The traditional method for determining uptake rates has been to measure changes in nitrogen concentration in the incubation medium over time (Harlin and Wheeler, 1985). More recently, incorporation of the nitrogen-stable isotope $^{15}$N into algal tissue has been used to calculate uptake rates (Naldi and Wheeler, 2002). Specific growth rates were mostly expressed in percentage day$^{-1}$, while uptake rates were expressed as μM N g DW$^{-1}$ day$^{-1}$. The uptake rate was calculated using the changes in ammonium and nitrate concentration during each sampling interval according to $V =$ [(S$_{f}$ x vo$_{l}$) - (S$_{i}$ - vo$_{l}$)]/t x B), where S$_{f}$ is the ammonium concentration, vo$_{l}$ is the water volume at the beginning of a sampling interval, S$_{i}$ is the ammonium concentration, vo$_{l}$ is the water volume at the end of the sampling interval, t is the time elapsed between 2 successive samplings, and B is the amount of biomass DW (Pedersen, 1994). A specific growth rate (μ) as a percent increase in disc area expansion was calculated according to μ = $100 \times \ln (A_{f}/A_{i})/t$, where A$_{i}$ is the initial disc area and A$_{f}$ is the disc area at time t (DeBoer et al., 1978).

5.2 Growth response of *U. lactuca*
This section is an extended elucidation of Paper 1: differential growth response of *U. lactuca* to ammonium and nitrate assimilation.

5.2.1 Relevance
Increased fluctuating levels of nutrients in the estuarine ecosystem stimulate the abundance and production of fast-growing algae like the ephemeral macroalga *U. lactuca* (Twilley et al., 1985). The abundant growth of ephemeral macroalgae is so invasive that it could lead to oxygen depletion and shading among other marine habitats. Enormous quantities of this seaweed create environmental concerns (e.g., microbial waste and rancid odors) when cast away on the beach. *U. lactuca* a common ephemeral macroalgae from the tropical to polar climates that has been harvested from natural populations or cultivated in land-based systems and as part of integrated multi-trophic aquaculture systems. Nevertheless, most of today's *U. lactuca* biomass is unused,
Hence, utilization of *U. lactuca* is of ardent importance. Production of alternative biofuels from non-starch biomass has directed attention to the utilization of *U. lactuca* as primary substrates for anaerobic digestion to biogas. Moreover, Ulva species are rich in rare cell wall polysaccharides and vitamins A, B2, B12, and C, and they exert antioxidant, antimicrobial, and antiviral activities (Ivanova et al., 1993; Abd El-Baky et al., 2008; Ortiz et al., 2006). For this reason, cultivation of *U. lactuca* for either crude biomass production or for the production of biologically active compounds is currently receiving increased attention (Hiraksa and Oka, 2008). Nonetheless, a major prerequisite for the successful exploitation of cultivated *U. lactuca* for commercial applications is that both the growth rate and the yield are optimized. This in turn requires both an understanding of the influence of different nutrients on the growth response and a precise methodology to measure the growth.

5.2.2 Hypotheses and objectives

The utilization of ammonium and nitrate by seaweed varies among species, and the assimilation of these nutrients influences the growth. It has been observed that the presence of ammonium inhibits nitrate uptake (Thomas and Harrison, 1987). These interactions, however, were only discussed in relation to nitrogen uptake rates and the understanding of the influence of these interactions on seaweed growth responses, including those of *U. lactuca*, remains limited. The common method to determine growth rate and biomass yield in seaweed culture systems involves determination of the initial and final weight of seaweed samples. This requires removal of surface moisture either by wiping with a filter cloth or by centrifugation. The extent of remaining surface moisture induces varying degrees of measurement inaccuracy (Mouya and Neori, 2008; Vandermeulen and Gordin, 1990). In continuous culture monitoring, the harvested seaweeds are dehydrated prior to weighing, and then a stock strain is taken and used as the initial inoculum material for further culture. However, seaweed needs to recuperate from the harsh dehydration-hydration treatment before it can resume growth. Therefore, the interruption of cellular growth is almost unavoidable (Phillips et al., 2002).

Therefore, the objective of this work was to evaluate the growth response of *U. lactuca* cultured in artificial seawater exposed to different sources and levels of nitrogen (NH₄⁺ and NO₃⁻) using a more accurate growth monitoring technique. To achieve the precise evaluation, a photo-scanning technology was used to obtain digital images of the sizes of the frond discs, and in this way...
examined the growth kinetics by measuring the surface area expansion of the seaweed discs using digital image processing software.

5.2.3 Result highlights
The growth response of \textit{U. lactuca} exposed to different sources of nitrogen (NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{3}\textsuperscript{−}, and the combination NH\textsubscript{4}NO\textsubscript{3}) was examined using photo-scanning technology. Differential increases of the surface area of \textit{U. lactuca} discs with time in response to different N-nutrient enrichments were expected. The NH\textsubscript{4}Cl- and NaNO\textsubscript{3}-rich media (50 \textmu M of N) accelerated \textit{U. lactuca} growth to a maximum specific growth rate of 16.4 ± 0.18% d\textsuperscript{−1} and 9.4 ± 0.72% d\textsuperscript{−1}, respectively. The highest obtained biomass production was 22.6 ± 0.24 mg-DW m\textsuperscript{−2} d\textsuperscript{−1}. The \textit{U. lactuca} growth response favored ammonium as the nitrogen source, and its presence apparently discriminated nitrate uptake by \textit{U. lactuca} when exposed to NH\textsubscript{4}NO\textsubscript{3}.

Apart from showing a significant differential growth response of \textit{U. lactuca} to different nitrogen sources, the work exhibits the applicability of a photo-scanning approach for acquiring precise quantitative growth data for \textit{U. lactuca} as exemplified by assessment of the growth response to two different N-sources.

5.2.4 Consideration and justification
The exploitation of land-based resources for bioenergy production created global concern for its impact on the food production, availability, and price of commodities. Meanwhile, the utilization of marine-based resources, notably non-commercially important seaweed, is gaining attention. This leads macroalgae like \textit{U. lactuca} to be considered for the production of bioenergy because it exhibits production potential for either land-based culture or off-shore cultivation (Bruhn et al., 2011). Furthermore, other potential applications of \textit{U. lactuca} biomass (e.g., source of functional and bioactive compounds) must be established to add more value to the seaweed biomass.

Nevertheless, the growth rate, yields, and culture condition must primarily be optimized to successfully exploit \textit{U. lactuca} for commercial applications. Therefore, understanding the effect of different nutrients on the growth response is critical, and it requires precise methodology to measure the growth with the purpose of effectively evaluating nutrient assimilation.

Over the growth experiment duration, an artificial seawater medium was used without renewal, which may have influenced the growth kinetics of the algal frond. However, the results indicate that \textit{U. lactuca} can grow even without renewing the artificial seawater medium for a certain period. The maximum growth rate was close to that of a published study (Neori et al., 1991), examined the growth kinetics by measuring the surface area expansion of the seaweed discs using digital image processing software.

5.2.3 Result highlights
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Over the growth experiment duration, an artificial seawater medium was used without renewal, which may have influenced the growth kinetics of the algal frond. However, the results indicate that \textit{U. lactuca} can grow even without renewing the artificial seawater medium for a certain period. The maximum growth rate was close to that of a published study (Neori et al., 1991),
where they used fresh seawater medium and extensive medium renewal. However, the amount of biomass accumulated during this experiment was relatively low compared to those of other earlier works (Neori et al., 1991). This variation was probably attributed to different monitoring techniques and culture conditions, such as water flow velocity, temperature, density, and illumination intensity.

5.3 Paper 5: Differential growth response of *Ulva lactuca* to ammonium and nitrate assimilation


Authors: Marcel Tutor Ale, Jørn Dalgaard Mikkelsen, Anne S. Meyer

Address: Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering Technical University of Denmark (DTU) Soeltofts Plads Bldg. 229, 2800 Kgs. Lyngby, Denmark

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Differential growth response of *Ulva lactuca* to ammonium and nitrate assimilation

Marcel T. Ale \* Jørn Dalgaard Mikkelsen - Anne S. Meyer

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Abstract Controlled cultivation of marine macroalgal biomass such as *Ulva* species, notably *Ulva lactuca*, is currently studied for production of biofuels or functional food ingredients. In a eutrophic environment, this macrophyte is exposed to different sources of nitrogen (NH₄⁺; NO₃⁻) and the combination NH₄NO₃ by using photo-scanning technology for monitoring the growth kinetics of *U. lactuca*. The images revealed differential increases of the surface area of *U. lactuca* does with time in response to different N-nutrient enrichments. The results showed a favorable growth response to ammonium as the nitrogen source. The NH₄Cl and NaNO₃ rich media showed a favorable growth response to ammonium as the nitrogen source. The NH₄Cl and NaNO₃ rich media (50 μM of N) accelerated *U. lactuca* growth to a maximum specific growth rate of 16.4±0.18% day⁻¹ and 9.4±0.12% day⁻¹, respectively. The highest biomass production rate obtained was 22.5±0.24 mg DW m⁻² day⁻¹. The presence of ammonium significantly discriminated the nitrate uptake by *U. lactuca* when exposed to NH₄NO₃. Apart from showing the significant differential growth response of *U. lactuca* to different nitrogen sources, the work exhibits the applicability of a photo-scanning approach for acquiring precise quantitative growth data for *U. lactuca* as exemplified by assessment of the growth response to two different N-sources.

Keywords Ammonium; Nitrate; Growth monitoring; Seaweed cultivation; *Ulva*; Nutrient uptake

Introduction

*Ulva lactuca* is an important macroalga in marine ecology. Its fronds are soft, sheet-like structures that are two cells thick, and this morphology is the reason for its common name “sea lettuce”. Recently, production of alternative fuels from non-starch biomass has also directed the attention to utilization of marine algae, including seaweed or macroalgae, as sources of biomass for biofuel production (Knut and Menimarian 2004). The rapid growth of *U. lactuca* is attributed to its high photosynthetic rates and high C and N-nutrient uptake capacity (Magmann et al. 1996; Naiti and Wheeler 2002; Sand-Jensen 1988). Scourfield has also been proposed as a biomass source for production of functional food ingredients, pharmaceuticals, and cosmetics (Bodin-Dubignon et al. 1997; Cumashi et al. 2007; Bixler 1996; De Rock-Holzthauer 1995). *Ulva lactuca* species are particularly rich in true cell-wall polysaccharides and have been proposed as being an important source of dietary fiber, mainly soluble fiber (Lahaye 1999; Lahaye and Axelson 1993). *Ulva lactuca* is also a good source of Vitamin A, B₂, B₁₂, and C and is rich in γ-tocopherol and *U. lactuca* extracts have been shown to exert antioxidant, anti-microbial, and anti-viral activities in various in vitro assays (El-Baky et al. 2008; Ortiz et al. 2006). It has been shown previously, that *U. lactuca* is suitable for propagation under controlled conditions (Vermaat and Sand-Jensen 1987; Luo 2004; Sato et al. 2008a, b). For this reason, cultivation of *U. lactuca* in tanks for either crude biomass production or for production of biologically active compounds is currently receiving increased attention.

M. T. Ale, J. D. Mikkelsen - A. S. Meyer
Center for Bioprocess Engineering, Department of Chemical and Biotechnical Engineering, Technical University of Denmark, Søltofts Plads, Building 229, 2800 Kgs. Lyngby, Denmark
e-mail: mta@kt.dtu.dk

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The chemicals used for nitrogen source were NH4Cl (99.5%), available digital image processing software. NH4NO3 (99.5%), and NaNO3 (99.0%), purchased from (Thomas and Harrison 1987). However, these interactions were discussed only in relation to nitrogen uptake rates and the understanding of the influence of these interactions on seaweed growth responses, including growth responses of $U$. lactuca, is still limited. The common method to determine growth rate and biomass yield in seaweed culture systems involves determination of the initial and final weight of seaweed samples. This requires removal of surface moisture either by wiping with filter cloth or by contribution. The extent of remaining surface moisture induces varying degrees of inaccuracy to the measurement (Miyazaki and Nozue 2008, Vandermeulen and Gordin 1990). In this present work, the objective is to evaluate the growth response of $U$. lactuca exposed to different sources of nitrogen (NH4 and NO3). We cultured $U$. lactuca frond disks for 10 days inside a beaker containing artificial seawater (ASW) enriched with equimolar levels of nitrogen (50 μM) of two different N-nitrates, NaCl and NaNO3, to assess the growth responses. Another experiment was conducted in the same set-up for 4 days to evaluate the ammonium and nitrate uptake rates when $U$. lactuca was exposed to 50 μM NH4NO3. We used photo-scanning to obtain digital images of the fronds, and in this way examined the growth kinetics by measuring the surface area expansion of the seaweed disks daily using commercially available digital image processing software.

**Materials and methods**

The chemicals used for nitrogen source were NH4Cl (99.5%), NH4NO3 (99.5%), and NaNO3 (99.0%), purchased from Sigma-Aldrich, (Bornem, Belgium). Commercial marine sea salt (Sera Marine Basic Salt—Heinsberg, Germany) was used to prepare artificial seawater (ASW) principally as described by Sato et al. (2004a, b). For nitrate determination, a low-range lab nitrate test kit supplied with nitrate reductase was used (The Nitrate Eliminator Co., Inc. Michigan).

**Aquatume culture: condition and set-up**

Fresh $U$. lactuca fronds were obtained from the National Environmental Research Institute of Denmark (DIME), University of Aarhus (Silkborg, Denmark). The original seaweed material was cultured in ponds with natural seawater. Upon arrival to our laboratory, the seaweed fronds were transferred into a 2 L beaker containing 1.5 L ASW with a 35 g l−1 concentration of marine sea salt toubriciate. The ASW medium had a pH of 8.35 and 2.15 ppt salinity measured using a handheld conductivity meter (Con-11, Eutech Instruments—Singapore). The fronds were acclimatized in the beaker with aeration for 3 days prior to transfer into a 112-L aquarium tank for long term cultivation. The water at ambient temperature in the 112-L aquarium was led directly to a 45-L reservoir tank and the water was then pumped back to the aquarium at a rate of approximately 1.760 l h−1. The reservoir was aerated and the water (ASW) was renewed weekly.

**Fish culture set-up**

The growth monitoring of the $U$. lactuca disks during differential cultivation was carried out using a perfused flask (500 mL) positioned inside a rectangular canister that served as a condiment basin. The culture flask was perfused at a certain height to allow the medium to overflow after reaching the 300 mL level into the condiment basin. The medium was then recirculated at a speed of 1.5 l h−1 using a peristaltic pump. A small magnetic bar was placed at the bottom of the flask to mix the water twice daily and to prevent the contact of the magnetic bar and the $U$. lactuca disks, a 5-mm mesh was fitted inside in the middle of the flask. A schematic diagram is shown in Fig. 1. In the experiment, the individual fronds were exposed with 50 μM NH4Cl, 50 μM NaCl and NaNO3, to assess the growth responses. Another experiment was conducted in the same set-up for 4 days to evaluate the ammonium and nitrate uptake rates when $U$. lactuca was exposed to 50 μM NH4NO3. We used photo-scanning to obtain digital images of the fronds, and in this way examined the growth kinetics by measuring the surface area expansion of the seaweed disks daily using commercially available digital image processing software.

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**Nitrogen uptake measurement**

Ammonium concentration was determined with the endogenous-blue method (Kondolf 1976). Nitrate measurement was conducted using low-range lab nitrate test kit.
supplied with infinite redaction as described by Campbell et al. (2004). The uptake rate was calculated from changes in ammonium and nitrate concentration during each sampling interval according to $\Delta V = \frac{(N_i - N_0)}{t}$ where $N_i$ is the ammonium concentration, $N_0$ is the ammonia concentration, $t$ is the time elapsed in days between two successive sampling events and $B$ is the amount of biomass dry weight (DW) (Pedersen 2004). Therefore, the ammonium concentration ($\text{NH}_4$) and nitrate concentration ($\text{NO}_3$) were used as the standard for disk image calibration. After measuring the initial area of all the disks, three disks with known areas were randomly collected, weighed and subjected to drying in a 105°C oven for 4 h and then the disk dry weight was obtained. The measured dry weight (DW) was used to convert the disk area into dry weight, resulting in a conversion factor of 0.027 mg DW mm$^{-2}$ (Pedersen 2004). The specific growth rate ($G$) was calculated according to this equation $G = \frac{\ln(A_i/A_0)}{t}$ where $A_i$ is the disk area at the end of sampling interval, $A_0$ is the area at the beginning of the sampling interval, and $t$ is the time elapsed in days between two successive sampling events. Specific growth rate ($\mu$) as percent increase in disk area expansion was supplied with infinite redaction as described by Campbell et al. (2004). The uptake rate was calculated from changes in ammonium and nitrate concentration during each sampling interval according to $\Delta V = \frac{(N_i - N_0)}{t}$ where $N_i$ is the ammonium concentration, $N_0$ is the ammonia concentration, $t$ is the time elapsed in days between two successive sampling events and $B$ is the amount of biomass dry weight (DW) (Pedersen 2004). Therefore, the ammonium concentration ($\text{NH}_4$) and nitrate concentration ($\text{NO}_3$) were used as the standard for disk image calibration. After measuring the initial area of all the disks, three disks with known areas were randomly collected, weighed and subjected to drying in a 105°C oven for 4 h and then the disk dry weight was obtained. The measured dry weight (DW) was used to convert the disk area into dry weight, resulting in a conversion factor of 0.027 mg DW mm$^{-2}$ (Pedersen 2004). The specific growth rate ($G$) was calculated according to this equation $G = \frac{\ln(A_i/A_0)}{t}$ where $A_i$ is the disk area at the end of sampling interval, $A_0$ is the area at the beginning of the sampling interval, and $t$ is the time elapsed in days between two successive sampling events. Specific growth rate ($\mu$) as percent increase in disk area expansion was.
Results

Cultivation, imaging, and growth measurement

Disks prepared from _U. lactuca_ fronds grew steadily and reproducibly. The difference in _U. lactuca_ disk area growth induced by the different N-enrichment (no N- addition, 50 μM NH₄Cl or 50 μM NaNO₃) was monitored daily throughout the cultivation period. Images of the disk fronds presented in Fig. 2 exhibit the increase in the surface area of _U. lactuca_ disks over time with the different nitrogen sources. The fronds that were enriched with NH₄Cl and NaNO₃ produced an olive-green color, which may be a result of the accumulation of chlorophyll in the fronds. The color was slightly more yellow-green but also darker in the case of ASW-grown fronds (Fig. 2). The color differences may result from differences of nutrients.

The ammonium-enriched medium induced a significantly higher increase (P<0.05) in surface area expansion (148.8 mm² day⁻¹) than the nitrate-enriched medium (98.6 mm² day⁻¹), while the disks incubated in the ASW without N-enrichment grew only slowly with no significant area expansion during the 10 days of cultivation (Fig. 2). Nitrate availability in the medium contributed little to the growth but nevertheless the disk area expansion after 10 days was significantly higher (P<0.05) with the nitrate-enriched medium than that obtained with ASW only.

Nitrogen uptake and interaction

Further analysis of the nutrient uptake response of _U. lactuca_ in relation to different nitrogen sources showed that ammonium was favorably assimilated corresponding to a high uptake rate of ammonium (Fig. 4a, b). Both ammonium and nitrate were assimilated as indicated by the decrease of concentration in the medium (Fig. 4a). Significant differences (P<0.05) were observed for both the concentration and uptake rate of ammonium and nitrate over time (Fig. 4a, b). The abrupt assimilation of ammonium with high uptake rate from 0 to 1 day of cultivation was probably due to starvation of the seaweed frond. Nitrate in the medium was slowly assimilated by _U. lactuca_ until an increased uptake rate was observed between 3 and 4 days of cultivation (Fig. 4b). The _U. lactuca_ disks were also exposed to 50 μM NaNO₃ in order to evaluate the uptake response of _U. lactuca_ to the combination of the two nitrogen sources. This combination of ammonium and nitrate in the medium demonstrated the interactive response of _U. lactuca_ to these nutrients (Fig. 4c). This interaction showed that the simultaneous presence of ammonium and nitrate resulted in an NH₄⁺ uptake rate which was similar to that of the NH₄Cl uptake rate, when exposed to ammonium alone, but a relatively higher uptake rate of NO₃⁻ as compared to when exposed to NaNO₃ alone (compare Fig. 4b and c). However, the ammonium uptake rate was still significantly higher than calculated according to μ = 100 ln(A(t)/A₀) where A₀ is the initial disk area and A(t) is the disk area at time t (DeBoer et al. 1978). Statistical analysis was performed using Proc GLM and Proc Mixed of SAS Statistical Software version 9.1 (SAS Institutes, Inc., USA) and graphs were made using Sigma Plot 10.0 (Systat Software Inc., USA).

Biomass yield and specific growth rate

Both ammonium- and nitrate-enrichment elicited a normal, hyperbolic growth response as depicted in the accumulation of biomass (Fig. 3). On the NH₄Cl-enriched medium the biomass yield showed a steep increase during days 2-5 whereas the yield varied much less during the first days for the NO₃⁻ growth. The difference in the disk growth in response to the type of N-source was noticed from the fifth day of cultivation onward (Fig. 3). The growth increment (specific growth rate) stalled after about 8 days of cultivation, presumably as a result of nitrogen limitation in the media. The specific growth rate increased with substantial amount of NH₄Cl and stabilized after the nutrient was depleted. The maximum specific growth rate of _U. lactuca_ cultured on NH₄Cl was 10.4 ± 0.18% day⁻¹ and 9.4 ± 0.72% day⁻¹ with NO₃⁻ during 10 days of cultivation. The growth kinetics on nitrate illustrated a modest growth response as exemplified by the biomass yield of _U. lactuca_ on the nitrate nutrient source relative to that on the NH₄Cl (Fig. 3). Ammonium increased the biomass yield significantly (P<0.05) relative to the NO₃⁻ nutrient. The maximum biomass yield was thus 22.5 ± 0.24 mg m⁻² day⁻¹ by ammonium and only 13.0 ± 0.40 mg m⁻² day⁻¹ by nitrate enrichment.

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Fig. 3. Biomass yield (Y biomass to the left) and specific growth rate (SGR, Y rate scale to the right) of U. lactuca during 10-days of cultivation. The maximum specific growth rate was 16.4 ± 0.18%·day−1 (day 0) by ammonium and 15.6 ± 0.08%·day−1 by nitrate (day 6) by intrinsic attachment.

The maximum specific growth rate was 16.4 ± 0.18%·day−1 assimilated from NH4 and 5.4 ± 0.72%·day−1 from NO3. Initial ammonium and nitrate concentrations were 50 μM NH4Cl and 50 μM NaNO3. Maximum biomass yield were 22.5 ± 0.24 mg DW·m−2·day−1 by ammonium and 13.0 ± 0.40 mg DW·m−2·day−1 by nitrate enrichment.

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renewing the medium for a certain period. The maximum results indicate that the effect of the water renewal is unclear. Nevertheless, our experiment was relatively low as compared to other studies (Neori et al. 1991; Msuya and Neori 2008). Growth kinetics. The common experimental practice in the benthic zone, which should be beneficial for the bioconversion process. Thus, U. lactuca growth responses can grow even without renewing the medium for a certain period. The maximum growth rate (\( \mu_{\text{max}} \)) in this condition was 16.39 ± 0.13% day\(^{-1} \). This is close to the \( \mu_{\text{max}} \) reported in a previously published study (Neori et al. 1991), where they used fresh seawater medium and extensive renewal of medium.

The amount of biomass accumulated during this experiment was relatively low as compared to other earlier works where they harvested considerable amounts of biomass (Neori et al. 1991; Msuya and Nnoma 2008). The water flow velocity may be the cause of the relatively lower biomass production. In this work, the water circulation was controlled to be 1.5 L h\(^{-1} \), this circulation was almost equivalent to the water flow in the shallow benthic zone, which should be beneficial for U. lactuca growth. This assumption is supported by the previous works of Doty (1971) and Parker (1981) that showed the application of simulated current consistently enhanced U. lactuca growth responses. This assumption is supported by the previous works of Doty (1971) and Parker (1981) that showed the application of simulated current consistently enhanced biomass yields and biofiltration performance of U. lactuca under low nutrient concentrations in laboratory experiments.

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References


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6 Conclusions and perspectives

As already stated, seaweeds are the subject of a wide range of interesting research topics; therefore, it entails crossing beyond another border of academic discipline to elucidate the problems and accomplish specific objectives. The present study provided information about the most recent developments in the chemistry of fucoidan/FCSPs, emphasizing the significance of different extraction techniques for the structural composition and biological activity using an interdisciplinary approach. The utilization of marine seaweeds that have washed up on the coastline as a source of bioactive compounds like FCSPs and the growth response of U. lactuca to nutrient assimilation were thoroughly investigated.

The use of different extraction and purification techniques appears to have contributed to the confusion about the nature and composition of FCSPs ever since it was first described by Kylin early in the 20th century. As detailed in the following, we now know that fucoidan is built of 1→3-linked α-L-fucopyranosyl or of alternating 1→3- and 1→4-linked α-L-fucopyranosyl residues that may be sulfate-substituted, and that some fucoidans isolated from certain brown algae have completely different structures composed of sulfated galactofucans with backbones of (1→6)-linked β-D-galacto- and/or (1→2)-β-D-mannopyranosyl units with (1→3) and/or (1→4)-α-L-fucopyranosyl branching. The available data thus show that the term fucoidan has been used for several different chemical structures and vice versa, that fucoidan is a term that covers a diverse family of FCSPs (Paper 1). It is, therefore, more correct to use the term FCSPs, rather than fucoidan, as a collective term for these polysaccharides.

The varied chemical composition and diverse structures of FCSPs from brown seaweed may have hindered the development of an in-depth understanding of the precise properties of significance for structure-function correlations. Nevertheless, important structural bioactivity issues appear to include the degree of sulfation of the FCSP molecules. Ovulsulfated FCSPs have thus been found to be excellent potent inhibitors of tumor cell invasion compared to desulfated native FCSPs. Loss of anticoagulant activity has been observed with decreasing degrees of sulfation, although antiproliferative effects on fibroblast cell lines were retained (Paper 1).

A simple and practical method for recovering a suite of complex FCSPs from Sargassum sp. and the effect of different treatment parameters on the integrity of the polysaccharide have been

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A simple and practical method for recovering a suite of complex FCSPs from Sargassum sp. and the effect of different treatment parameters on the integrity of the polysaccharide have been
established (Paper 2). The preservation of the structural integrity of the FCSP molecules appears to be crucial for maintaining its biological properties, and it has been clearly shown that the extraction treatment employed affects the composition and thus the structural features of the FCSP substances (Paper 2). Evidently, the chemical composition and yield of the isolated products are strongly influenced by the method of extraction, as was expected. The data presented (Paper 2) showed that the polysaccharide obtained from a single-step extraction method may be heterogeneous, and that the composition varies with the length of the extraction process. Fucose, sulfate, and glucuronic acid were the important components of the polysaccharide mixture and this is typical for a fucoidan compound (Paper 2). The results (Paper 2) also demonstrated that FCSPs were vulnerable to harsh extraction conditions. Hence, we confirmed that extraction condition significantly influenced FCSP composition, and structural alteration may have occurred. Undoubtedly, the presence of impurities could influence the biological properties of FCSPs and, therefore, may currently hinder our complete understanding of the biological activity of fucoidan or FCSPs. Hence, the development of standard extraction procedures for FCSPs including hydrolysis treatment, purification, and fractionation methodology, preferably with specific steps adapted to the particular botanical order of the seaweed, will generate a better common basis for the analysis and understanding of bioactivities and the mechanisms determining FCSP bioactivities. On this basis, it may even be possible to target specific structural features and, in turn, tune the extraction procedure to obtain specific bioactivities via the use of targeted extraction methodologies.

The bioactivity of the isolated FCSP products from Sargassum sp. against LLC and MC was investigated (Paper 3). The study showed that FCSPs induce apoptosis of MC cells (Papers 3 and 4) and exert anti-tumor activity through the inhibition of the growth of LLC and MC, which was probably due to the enhancement of NK cell activity as the principal effector mediating tumor cell death. We showed that both FCSPs samples from Sargassum sp. and F. vesiculosus induces apoptosis by activating caspase-3 and exerts anti-tumor activity by inhibiting the growth of cells (Paper 4). FCSPs from Sargassum sp. and Fucus vesiculosus thus appear to be potent against lung and skin cancer cell lines, and its mode of action is associated with the immune response (Paper 3).

Furthermore, the bioactivity of crude fucoidan toward these 2 types of cancer cell lines was possibly augmented by the sulfate groups in the fucoidan structure. Nevertheless, further examination about these findings is needed to elucidate the underlying factors of FCSP bioactivity.

The unfractionated FCSP structures from Sargassum sp. could probably be heterogeneous and branched as expected; however, another possibility could be that these FCSPs were not mixtures
of different types of polysaccharides but members of the same polysaccharide family. The structural investigation presented by $^1$H NMR spectra indicate that in both Sargassum sp. and F. vesiculosus, FSCPs samples contained fucoidan-like structures, but definite structural information about whether the structure is heterogeneous or members of the same polysaccharide family should further be investigated (Paper 4). The quantitative variation of its components and distribution patterns as well as the differences in its structural details was probably not due to sample heterogeneity but rather to extreme compositional and structural dispersion. At any rate, the crucial bioactive effectiveness of these unfractinated FSCPs may be attributed to their distinct structural features, such as level of sulfation (charge density) and the position and bonding of the sulfate substitutions or sulfated fucans (i.e., C-2 and/or C-4 of α3-linked 1-fucopyranosyl residues) and sulfated galactans (i.e., C-2 of 3-linked galactopyranosyl residues) complexes (Paper 4). Nonetheless, we now understand that the type and variety of algal FSCPs is much wider than originally believed.

In addition to the investigation of the potential of the brown seaweeds Sargassum sp. and F. vesiculosus as natural sources of bioactive compounds, the washed up nuisance green seaweed U. lactuca was also examined for its growth and nutrient assimilation potential. This study was performed to illustrate the need for a precise monitoring method of the growth of U. lactuca in order to successfully exploit it for commercial application. Our understanding of the growth kinetics of U. lactuca is highly dependent on the precise monitoring of its growth and measurement of the uptake response to different nutrient sources. This includes minimizing inaccuracies during the growth measurement of seaweed fronds. This work exhibited the adaptability of the photo-scanning approach for attaining accurate quantitative growth data of U. lactuca as demonstrated by evaluation of the growth response to ammonium and nitrate (Paper 5). The experimental set-up was not designed to assess the exact cell growth physiology, which is why no firm conclusion can be drawn as to whether the U. lactuca disc growth was caused by cell division or cell extension. Nevertheless, because of the accuracy of the photo-scanning measurements, the increase in biomass yield could easily be calculated from the increase of seaweed disc area (Paper 5). This result showed that U. lactuca grew faster with ammonium than with nitrate as the nitrogen source (Paper 5). This corresponded well with the finding that the ammonium uptake was faster than the nitrate uptake. This difference was probably due to the ammonium being presented in a reduced state that can be easily assimilated and directly utilized by algae for the synthesis of amino acids and proteins resulting in cell and tissue growth. Nitrate, however, must first be reduced to nitrite and then to ammonium for the algae to utilize this
6.1. Future perspectives

Marine algal cultivation is a promising source of bioactive compounds and natural products. The study of the extraction of fucoidan and fucose from seaweed holds potential for the development of new drugs and functional food ingredients.

6.2. Conclusion

In conclusion, the study of the extraction of fucoidan and fucose from seaweed is crucial for the development of new drugs and functional food ingredients. The use of fucoidan and fucose in the treatment of diseases and in the development of functional food products is expected to increase in the future.

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References


utilization of seaweed by using the entire seaweed frond for the production of chemicals and/or biochemicals and energy. Thus, a concept of seaweed biorefinery should be developed.

The first phase of this concept includes isolation of commercially important chemicals/biochemicals such as hydrocolloids and the bioactive sulfated polysaccharide compounds. In the second phase, the remaining residues are collected as bulk biomass and then converted into chemicals and energy carriers (heat, liquid, gas, and electricity) by catalytic and enzymatic conversions and/or by digestion and pyrolysis. The residue-containing minerals are targeted for use in fertilizer applications in the final phase of the biorefinery cycle.

Many potential applications exist for the products acquired from biorefineries. Seaweed components derived thereof can be used as hydrocolloids for food and feed applications or can be formulated into natural health supplements; in contrast, mineral-containing seaweed residues are incorporated into fertilizer products.

The detailed processes, procedures, and specific working conditions of this seaweed biorefinery concept are yet to be determined.
7 References


7 References


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In this Doctoral thesis

Fucose containing sulfated polysaccharides (FCSPs) derived from brown seaweed exhibited crucial biological activities including anti-proliferative effects on lung and skin cancer cells in vitro. FCSPs also demonstrated immunomodulating effect by enhancing natural killer cells activity.

Conventional extraction technology for FCSPs involves treatment that is detrimental to its structural properties, yield and compositional attributes. Thus it hinders our understanding on the exact structure-function relations of FCSPs, as a consequence it limits commercial application possibilities for this valuable compound.

This PhD thesis delivers new understanding about the influences of extraction treatment parameters on the chemical nature of FCSPs and showed the most recent investigation of FCSPs as potential therapeutic agent for certain type of cancers.

The author of this PhD thesis is an agricultural engineer with MSc in agricultural development specialized in production and analysis of hydrocolloid from seaweed biomass for food and other commercial applications. He was involved in developing sustainable technology for algae bioremediation of wastewater and algae biomass production for bioenergy.