

Extraction and Formulation of Macroalgal Phenolic Compounds for Cosmetic Application

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Thesis to obtain the Master of Science Degree in

Chemical Engineering

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November 2021

Acknowledgments

The journey to a master thesis is not simple and all the support I received helped me fulfill my objectives and finalize such important phase of my academic life. Hence, I leave here a few words in a very felt and thankful way.

Especially to my supervisor, Professor Dr. Isabel Marrucho, I am thankful for the orientation, support and permanent interest, always led by a high and rigorous scientific level, which has contributed to all the stages underlying this project and also for giving me the opportunity to pursue my interest in academical research in other projects.

To my co-advisor, Professor Dr. Alice Martins, I would like to show my gratitude for the support and the circumstances that led to the collaboration of IST Lisboa and MARE - IPEiria in creating this project for my thesis, as well as for welcoming me in the research center of MARE – IPEiria, in Peniche for three weeks.

To the professors Dr. Helena Margarida Ribeiro and Dr. Joana Marques Marto of FFUL, I am grateful for the opportunity of working on the pharmaceutical aspect of this project and for helping me to guide this project in a better way.

To Dr. Celso Alves, Joana Silva and Patrícia Susano, from MARE - IPEiria, for introducing me into the research area of biotechnology, for the meaningful the scientific discussions and orientation.

To my colleagues, Bruna Soares and Sara Bom, I would like to thank you for all the assistance, collaboration and companionship shown over the last few months.

To Dr. Maria Conceição Oliveira for providing the equipment and assistance much needed.

To my long-time friends I want to thank for walking this journey by my side and for making it easier.

To my family, who have always encouraged and supported me, I specially thank for all understanding, love, unconditional support, and strength.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Abstract

The aim of this work was to obtain antioxidant-enriched extracts from three seaweeds found in the Portuguese shore, *Codium tomentosum*, *Gelidium corneum* and *Sargassum muticum*, using natural deep eutectic solvents (NADES) for further dermatological applications. Extracts were obtained by maceration of powdered seaweeds with different NADES. Resulting extracts were evaluated regarding their total phenolic content (TPC) using Folin-Ciocalteu method, and antioxidant capacity by the DPPH and FRAP methods.

NADES showed better extraction performance than the conventional solvents and lactic acid-based NADES were generally the most efficient for phenolic compounds extraction. *S. muticum* extracts presented the highest phenolic content (2099 mg GAE/L) and the best antioxidant activities (DPPH reduction of 27%, FRAP 30 μ M FeSO₄), probably due to their high content in phlorotannins, confirmed by HPLC-DAD and LC-MS/MS. Antimicrobial activity over three microorganisms of the skin microbiota, the bacteria *Staphylococcus epidermidis* and *Cutibacterium acnes* and the fungus *Malassezia furfur*, was also assessed. No relevant antimicrobial activity was observed, suggesting that extracts can contribute to skin microbiota homeostasis. The sample with the best cosmeceutical interest, the *S. muticum* extract made with lactic acid:fructose, was able to maintain cell viability of HaCat cells and so, it was incorporated into a topical W/O emulsion. The obtained physical and rheology data of the cosmetic formulation assured its quality and safety.

Results suggest that NADES are a promising replacement of conventional solvents in the extraction of antioxidants from seaweeds, and that their application in topical formulations is safe.

Keywords

Seaweeds | Phenolic compounds | Natural Deep Eutectic Solvents (NADES) | Solid-liquid extraction | Cosmetic formulations

Resumo

O objetivo deste trabalho foi obter extratos antioxidantes de três algas marinhas da costa portuguesa, *Codium tomentosum*, *Gelidium corneum* e *Sargassum muticum*, com solventes eutéticos naturais (NADES) para aplicações dermatológicas. Os extratos foram obtidos por maceração de algas em pó com diferentes NADES sendo avaliados quanto ao seu conteúdo fenólico total (TPC) usando o método Folin-Ciocalteu e a sua capacidade antioxidante pelos métodos DPPH e FRAP.

Os NADES apresentaram melhor desempenho na extração de compostos fenólicos do que os solventes convencionais e os NADES com ácido láctico foram geralmente os mais eficientes. Os extratos de *S. muticum* apresentaram o maior teor fenólico (2099 mg GAE/L) e as melhores atividades antioxidantes (redução do DPPH de 27%, FRAP 30 μ M FeSO₄), provavelmente devido ao seu elevado teor em florotaninos, confirmado por HPLC-DAD e LC-MS/MS. A atividade antimicrobiana foi avaliada sobre três microrganismos da microbiota cutânea: bactérias *Staphylococcus epidermidis* e *Cutibacterium acnes* e o fungo *Malassezia furfur*. Não foi observada nenhuma atividade antimicrobiana relevante nos extratos, sugerindo a sua contribuição para a homeostasia da microbiota da pele.

A amostra de maior interesse cosmeceútico, extrato de *S. muticum* feito com ácido láctico:frutose, foi capaz de manter a viabilidade celular das células HaCat e, portanto, foi incorporado numa emulsão tópica A/O. A caracterização física e reológica da formulação cosmética garantiu a sua qualidade e segurança.

Os resultados sugerem que os NADES são substitutos promissores dos solventes convencionais na extração de antioxidantes de algas marinhas, e que sua aplicação em formulações tópicas é segura.

Palavras-chave

Macroalgas | Compostos fenólicos | Solventes eutéticos naturais profundos | Extração sólido-líquido | Formulações cosméticas

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Abbreviation List

[C₄C₁im][BF₄]	1-butyl-3-methylimidazolium tetrafluoroborate
CPE	Centrifugal partition extraction
DES	Deep eutectic solvent
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
EAE	Enzyme-assisted extraction
GAE	Gallic Acid Equivalent
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
IL	Ionic liquid
LA:Fru	lactic acid:fructose
LA:Glu	Lactic acid:glucose
LA:SA	Lactic acid:sodium acetate
MAAs	Mycosporine-like Amino Acids
MAE	Microwave-assisted extraction
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
NADES	Natural deep eutectic solvent
NIS	Non-indigenous species
PEF	Pulsed electric field-assisted extraction
PGE	Phloroglucinol Equivalents
PLE	Pressurized liquid extraction
SFE	Supercritical fluid extraction
SLE	Solid-liquid extraction
SLR	Solid-liquid ratio
SWE	Subcritical water extraction
TPC	Total phenolic content
TPTZ	(2,4,6-Tri(2-pyridyl)-s-triazine
UAE	Ultrasound-assisted extraction

1. Introduction

1.1. Context

Over the years the global beauty-industry market has been consistently resilient with an annual growth rate of 4.75%, for the last ten years. It accounts for millions of jobs generating \$438 billion in 2020 and it is predicted to exceed \$716 billion by 2025 and over \$780 billion by 2027 [1]. Geographically, the market is dominated by developed regions with Asia Pacific (43%), North America (24%) and Western Europe (16%) dominating the global market and accounting for more than 80% of the total market combined [2].

If divided, the beauty-industry can be categorized by segments: skincare, haircare, makeup, fragrances, or hygiene products. According to L'Oréal's 2020 Annual Report [2], the skincare segment accounts for 42% share of the business revenue, followed by haircare products, with 22%, and makeup with 16%. Figure 1 shows the evolution of the skincare market with higher growth rates than the global beauty market in the last four years [2].

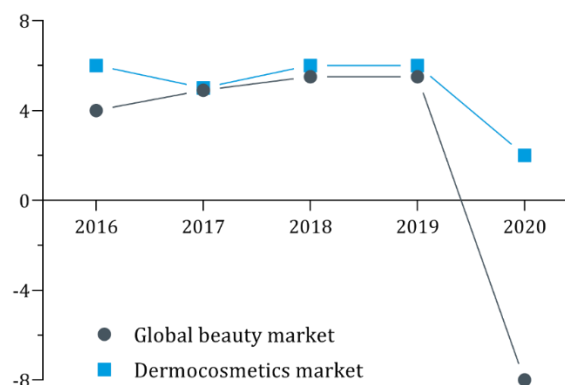


Figure 1: Evolution of the cosmetics market compared with the global beauty market, in %. Adapted from L'Oréal, 2020 [2].

Furthermore, the cosmetics market was the only market to record growth in 2020, with a +2% growth rate, a very positive statistic, considering the pandemic situation in the world and the -8% decrease of the global beauty market [2]. Due to the economic development and the rising living standards, consumers are more aware of both product effectiveness and ingredients rather than price. Hence, retailers are more focused on advertising superior quality over undercutting prices [1].

The increased interest in high quality and sustainable products is reflected in the huge demand for natural cosmetics. In fact, the cosmetic industry has the biggest share of consumers that prefer to shop organic. In 2020, the natural cosmetics market was at \$38.2 billion and is expected to reach roughly \$54.5 billion for the year 2027 — proof of the growing importance of the natural and organic cosmetics market [1].

Skin care products are focused on providing protection against degenerative skin conditions through contact application with the external parts of the human body manifesting beneficial topical actions. Before the use of synthetic substances, natural ingredients were the main source of all medicinal preparations, including skin care products. Now, modern formulations aim to replace synthetic substances for ones from natural origin, but aiming at keeping the increased performance of synthetic substances [3]. Plant extracts are added to skincare formulations for their varied properties such as antioxidant capacity, pigmentation inhibition, and antimicrobial activity, which are beneficial in attenuating and preventing various skin conditions [3]. Natural extracts help improve skin tone, texture, and appearance by delivering nutrients necessary for a healthy skin. In this context, marine seaweed have gained significant attention as extract sources for skin care. They represent one of the richest marine resources considered safe with negligible cytotoxicity and many beneficial effects on humans [4]. Regarding sustainability, seaweeds are considered a viable alternative feedstock of natural bioactive compounds. A wide range of primary and secondary metabolites biosynthesized by seaweeds like polysaccharides, carotenoids, phenolics, amino acids, and others, are reported for their beneficial functions as antioxidant, anti-inflammatory, and photoprotective agents and have been investigated for cosmeceutical preparations [5].

Solvent selection represents an important part of cosmetics development, as substances used throughout the process in extractions, separations, formulations, or synthesis processes. Commonly, organic solvents are the most used despite having recognized environmental and health concerns. The reduction of their use by the industry is one of the main solutions to improve sustainability, as well as selection of green alternatives [6]. One interesting alternative is the use of natural deep eutectic solvents (NADES), seen as a promising, green alternative to synthetic organic solvents to produce natural extracts. These solvents bring new challenges and opportunities to produce plant extracts with novel phytochemical compositions and biological activities. These challenges stem from the fact that despite the reasonable amount of known NADES, only a few are approved for cosmetic applications, due to safety and regulatory issues [7]. This study is focused on the extraction of phenolic compounds, well known antioxidant agents, using novel green solvents (NADES) for further incorporation into a cosmetic formulation.

1.2. Objectives

The main goal of this experimental work was the screening of NADES for the extraction of phenolics from the seaweeds, *Codium tomentosum*, *Gelidium corneum* and *Sargassum muticum*, as well as the use of the best extract in a topical formulation. For that purpose, the following objectives should be met:

1. Replication and comparison of conventional methods for phenolic extraction;
2. Selection and screening of NADES for phenolic extraction and cosmetic use;
3. Evaluation of the biological activities of the most promising seaweed extracts;
4. Characterization of the phenolic profile of the best extract;
5. Preparation and characterization of topical formulation with the best seaweed extract.

2. State of Art

2.1. Biorefinery Concept

In the last decades, society has become aware of the increasing environmental threats regarding the planet's sustainability. The overuse of Earth's natural resources forces us to look for sustainable solutions to meet the high energy demand of current and future populations. The way we currently produce and consume energy is unsustainable, only around one-fifth of energy supply worldwide is delivered by clean energy sources [8].

One of the major challenges ahead of us is climate change, mainly caused by the release of greenhouse gases such as CO₂. The consumption of fossil fuels, for energy production either from stationary or non-stationary point sources, together with cement plants, production of commodities, is responsible for around two-thirds of global greenhouse gas and the bulk of CO₂ emissions [8]. Although the tackle on climate change begins in the energy sector, through the development of renewable energy, with improved energy production and the diversification of primary energy sources [9], similar actions need to be taken in other industrial sectors.

Biomass, readily available, inexpensive, and low in carbon, is seen as one of future's key renewable feedstocks, capable of producing sustainable and green energy systems and simultaneously affording single, platform molecules, that can be processed into commodities and materials. The conversion of biomass in energy carriers and beneficial co-products can be carried out in biorefineries. The International Energy Agency defines biorefinery as "the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power and/or heat)" [10]. Other entities may define biorefinery differently, for instance, according to the National Renewable Energy Laboratory "a biorefinery is a facility that integrates conversion processes and equipment to produce fuels, power, and chemicals from biomass" [11].

Despite the different definitions, an integrated biorefinery should cover the upstream, midstream, and downstream processing of biomass into a range of products. This can occur in different systems as a facility, a process, a plant, or even a cluster of facilities. It's important to assess the entire value chain for environmental, economic, and social sustainability of all biorefineries [10].

Initially, the classification of biorefineries was based on technological status, type of raw materials or main type of conversion processes applied. However, Task 42 of the International Energy Agency has developed a more appropriate classification system related to the involved platforms, products, feedstocks and, if necessary, the processes. This approach offers a clearer 'biomass to end product' view defining the various feedstocks utilized and conversions within any given system [12]. Major feedstocks for biorefineries include dedicated wood and agricultural crops, organic residues (both plant and animal derived, and industrial and municipal wastes) and aquatic biomass (e.g. microalgae, seaweeds) [12]. Within the bio-

based economy, biorefineries can make a significant contribution to sustainable development by adding value to the use of biomass. This should be realized by maximizing biomass conversion efficiency and bringing together different market sectors into multi-disciplinary partnerships [12].

2.2. Seaweeds and the blue biorefinery

As a part of the aquatic biomass, seaweeds integrate the blue bioeconomy. With the objective of valorizing the aquatic biomass, from marine wild catch as well as aquaculture, the blue bioeconomy looks to complement the soil production of food, feed, nonfood (materials and chemicals) and bioenergy. The end goal of blue biorefinery is to convert seaweed biomass into a wide range of different products to seize its full potential. A series of sustainable and clean mechanical, chemical, and biological processes are required to develop the blue biorefinery without minimum impact in the ecosystems [13].

If blue refinery is organized in a cascade of products with descending price level, firstly would be components for health-promoting products, like fucoidans (a complex series of sulfated polysaccharides with health benefits). There are also cheaper molecules, with small molecular weight, secondary metabolites with bioactivities, like antioxidant or antifungal, useful in skin care products, cosmetics, and health-promoting food ingredients. Secondly, a range of functional food and feed ingredients, including both sugar polymers and proteins. Following, a set of chemical building blocks based on enzymatically produced monomer sugars derived from the seaweed polymers. Lastly, processing water and biorefinery residual fractions can be used with stimulatory effect on plant growth [13].

Of about 12,000 known species of seaweeds, only about 221 have been used commercially. The 10 most intensively cultivated species include the brown seaweeds: *Saccharina japonica*, *Undaria pinnatifida*, *Sargassum fusiforme*; the red seaweeds: *Porphyra/Pyropia* spp., *Eucheuma* spp., *Kappaphycus alvarezii*, *Glacilaria* spp. and the green seaweeds: *Ulva clathrata*, *Cauleurpa* spp. and *Monostroma nitidum*. This shows the huge potential of seaweeds yet to achieve [13].

When comparing with agricultural production, blue biomass has the benefit of not occupying any land area and neither requiring resources such as freshwater, fertilizers, or pesticides. Furthermore, seaweeds have a significant role in ecosystems: intensifying the seaweed global production also contributes to the fight against climate change since they can recycle atmospheric carbon dioxide and thus minimizing environmental impacts. When produced in shallow water, seaweeds growth will impact the local ecosystem by removing excess nutrients in the waters. This will improve the quality of water, while simultaneously stimulating the marine biodiversity. Besides the ecosystems benefits, the production, farming, harvesting, and pre-treatment of seaweed have the potential to bring both social and economic development in local and coastal regions, generating jobs in new start-ups, SMEs, and larger industries [13].

In addition to seaweed biomass, aquatic biomass also comprises fish-processing residues, microalgae, and invertebrates. Nevertheless, the aim of this dissertation is to study the potential of seaweed

biomass, particularly *Codium tomentosum*, *Gelidium corneum* and *Sargassum muticum*, as a source of bioactive ingredients extracted by NADES for further cosmetic applications.

2.3. Portuguese coast: Valorization and use of seaweeds

The extensive Portuguese shoreline is responsible for a very interesting and diverse seaweed community. Latitudinal gradients present along the Portuguese continental coast, and its two archipelagos (Azores and Madeira) boosts the seaweed biodiversity, which is a valuable resource yet under explored, particularly economically.

Along the Portuguese shore, various factors influence the distribution of seaweed communities: sea surface temperature, photosynthetically active radiation (available light), wave-exposure and nutrient regimes (Figure 2). Different combinations of these factors enable the distribution of northern-cold and southern-warm species creating a transition zone where these species coexist. The coastal biodiversity in the Azores archipelago is dominated by warm-temperature species, while in the subtropical Madeira archipelago the seaweed communities present a mixture of species with colder and warmer affinities [14].



Figure 2: Shoreline of continental Portugal – featuring main wave-exposures, main sea surface temperatures and main currents – and its two archipelagos (Azores and Madeira). W, Winter; S, summer; Usual study sites, black dots [14].

The first data regarding seaweed collecting for soil fertilization in Portugal appear in the 14th century and is still, today, one of the end-uses for seaweed in the north of the country. However, this activity

decreased with the introduction of chemical fertilizers. Traditionally, there are two mixtures of seaweeds used as fertilizer: *moliço* and *sargaço*. The first, collected in the lagoon of Ria de Aveiro is composed by the following marine algae genus: *Ulva*, *Rhizoclonium*, *Gracilaria* and the marine angiosperms *Zostera*, *Ruppia* and *Potamogeton*. Nowadays, the collection of *moliço* is only done for cultural purposes. *Sargaço* is a mixture of mainly *Saccorhiza polyschides*, *Laminaria*, *Fucus*, *Codium*, *Palmaria palmata* and *Chondrus crispus*. The collecting of *sargaço* is made ashore from free-floating masses at beaches during summer. After collected, the *sargaço* is dried in large areas before it is sold directly to farmers. This activity is traditional in northern Portugal and can still occur in Viana do Castelo and Póvoa do Varzim districts [14].

Throughout the last century, the Portuguese agar industry gained worldwide recognition. During World War II, agar from Asia became scarce giving room for the development of the Portuguese phycocolloid industry. By 1971, there were six Portuguese agar factories and Portugal was one of the world's major agar producers with an estimated capacity of producing 1620 tons of agar per year, although this capacity was never reached. Since that time, capacity has been decreasing with only one factory, the IBERAGAR S.A., still open [14].

The main economic seaweeds in agar production that are currently harvested in Portugal include the agarophytes *Gelidium corneum*, on the continental coast of Portugal, and *Pterocladia*, harvested on the Azores Islands. The harvest of these seaweeds peaked by 1990, supplying almost half of the agarophyte industry, but then decreased, following the closure of the Portuguese agar factories. In Azores, new legislation was recently implemented (Portarias nº1 and 44/2014) creating interest in this activity and increasing harvest. The harvesting of *Gelidium corneum* only occurs at São Martinho do Porto, Alcobça, between 15 July and 15 November. At days of calm sea, divers go 4-10 m deep with expandable mesh bags, *xalavares*, attached to their waist. The harvested seaweeds are afterwards spread on sun-exposed ground to dry naturally and delivered to the agar-extraction factory [14].

For the last 20 years, seaweed cultivation has been a topic of research supporting the start of seaweed cultivation in Portugal. In Ílhavo, Aveiro, the company ALGApplus is using the Integrated Multi-Trophic Aquaculture concept to cultivate seaweeds at a fish farm. ALGApplus started in 2012 and have developed an interesting group of seaweed related brands: ALGA + (bulk seaweed), Tok de Mar (food products), SeaOriginals (well-being products) and Algaessence (nutritional products) [15]. This company produces and commercializes well known seaweed species such as *Ulva* sp., *Porphyra* spp., *Chondrus crispus* or *Gracilaria* sp., among others, including the one acquired for this project: *Codium tomentosum*.

There is also Aquazor – Aquicultura e Biotecnologias Marinhas dos Açores – an Azorean company licensed to cultivate seaweeds around different islands of the archipelago. Despite the abundance of edible seaweed species along the Portuguese coasts, using them as food is not traditional in Portugal, except for some Azores communities where there is the tradition of consuming seaweeds as a nutritional food supplement. For example, *Porphyra* spp. is collected and consumed fried or added to soups or omelets;

Osmundea pinnatifida and *Laurencia viridis* are pickled in vinegar with onions and eaten with fried fish; *Fucus spiralis* a local delicacy, the swollen receptacles are picked and eaten fresh [14].

Research on Portuguese seaweeds applications has been very active, regarding both cultivated and harvested. It started with research on agarophytes, and it has expanded to a wide range of pharmaceutical, cosmetics and biotechnology applications such as for example fish feed, bioactive extracts, and algal food products. Researchers are aware that human activities are harming biodiversity and natural diversity, as well as the establishment of non-indigenous species (NIS) also affecting the functioning of ecosystems around Portugal. NIS are defined as “species whose introduction or spread threaten biodiversity” - Convention on Biological Diversity [16], for instance, invasion by canopy-forming macroalgae (e.g. *Sargassum muticum*, *Undaria pinnatifida*) may affect ecosystems by modifying the levels of light, sedimentation or water movement. These developments led to the start of several companies like the aforementioned ones and many others, as well as the increase of research units and projects regarding seaweed resources, all over Portuguese higher education institutes, which is a way towards innovative and sustainable practices.

2.4. Seaweed background

In this work, the incorporation of novel extracts from the three aforementioned seaweeds, *Codium tomentosum*, *Gelidium corneum* and *Sargassum muticum*, in cosmetic formulations is assessed. Studies on the three seaweeds selected for this work are not abundant, yet some articles can be found regarding their chemical characterization, novel applications, and environmental impacts.

Codium tomentosum is a green macroalgae from the Codiaceae family, Bryopsidales order and, as a green seaweed it belongs to the Chlorophyta *phylum*, Figure 3 [17]. This marine species native to the north east Atlantic coast is found in intertidal zones, rock pools and lower seashores and persists throughout the year [18, 19]. In general, green macroalgae have a high content of polysaccharides, which in addition to the common cellulose and starch, include large amounts of unique sulfated polysaccharides [20]. *C. tomentosum* presents in its composition lipids with nutritional and health benefits, and organic acids and volatile compounds with antioxidant properties. Its organoleptic properties and composition make this macroalgae appreciated in the food and cosmetic industry, with some extracts being used as a skin protecting agent in commercial formulations [19]. Their extracts have shown the capacity of regulating water distribution in the skin, and thus giving protection against skin dryness [5].



Figure 3: *Codium tomentosum* [15].

Gelidium corneum, red seaweed (Gelidiaceae, Gelidiales, Rhodophyta), specimens grow in both intertidal and subtidal areas, Figure 4 [21]. Although its protein content is variable, the highest protein contents are generally found in green and red seaweeds (10–30% of DW) in comparison to brown seaweeds (5–15% of DW) [22]. In red seaweeds, the phenolic content is mainly composed of bromophenols, flavonoids, phenolics acids, phenolic terpenoids, and mycosporine-like amino acids [23]. This family of seaweeds, Gelidiaceae, is known for being one of the major agar producers in the world and produce agars with relatively high sulphate content [22, 24]. Agar is widely used in the food industry (mainly as texture modifying and thickening agent) and in the microbiology field [25].

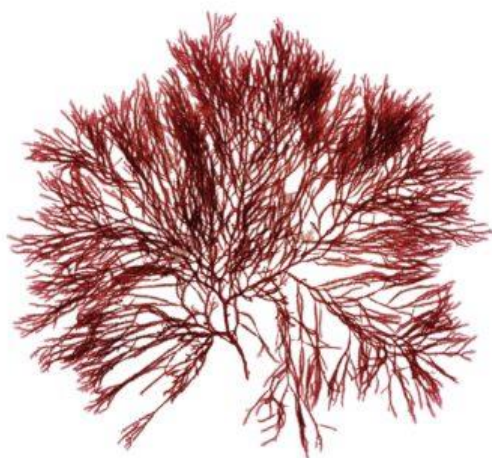


Figure 4: *Gelidium corneum* [26].

Sargassum muticum, Figure 5, a brown macroalgae (Sargassaceae, Fucales, Phaeophyta), is an invasive species in Europe since it is original from Japan and can be found on rocky shores. It is one of the most largely available Sargassaceae species on European shores, and therefore its sustainable biomass could represent an interesting asset in European resource development, as it is known for a high content of phlorotannins. Regarding phenolic content, Tanniou *et al.* studied its variability on phenolic content in several countries along European Atlantic Coast from Southern Portugal to South Coast of Norway. The highest phenolic content found was reported in *S. muticum* collected in Portugal in comparison to those

collected in Norway, Ireland, France or Spain [27]. The impact of this invasive seaweed on macroalgal assemblages has also been assessed by Sánchez *et al.* [28].



Figure 5: *Sargassum muticum* [29].

Brown seaweeds are constituted several biologically active substances such as polysaccharides, carotenoids, proteins, lipids, and ω -3 fatty acids and also by secondary metabolites, such as terpenes and polyphenols, like phlorotannins an important group of phenolic compounds almost exclusively biosynthesized by brown seaweeds. Their unique composition granted them usage in many different industries such as food, cosmetics, biotechnology, and pharmaceutical [30]. They are also known for naturally producing antioxidant compounds in great quantities [27].

Rodrigues *et al.* [22] studied the chemical composition of six representative edible seaweeds from Buarcos bay in Central West Coast of Portugal, among which two were *Codium tomentosum* and *Sargassum muticum*. In this study, *C. tomentosum* was associated with the highest total phenolic content of all seaweeds analyzed. Results were expressed in micrograms of catechol equivalents per gram of dry weight: 920 $\mu\text{g CE/g DW}$ for *C. tomentosum*, while *S. muticum* showed little phenolic content 499 $\mu\text{g CE/g DW}$.

The chemical and antioxidant potential of the green *C. tomentosum* and red *Plocamium cartilagineum* seaweeds was evaluated by Valentão *et al.* [18] and *C. tomentosum* exhibited the highest content of organic acids, with oxalic acid being the main compound present.

Another study assessed the antioxidant and photoprotective properties of several cyanobacteria and red macroalgae, including *G. corneum*, using compatible solvents with natural cosmetics. The highest carbon content found in red algae belonged to *G. corneum* and *P. umbilicalis* (351 and 340 mg/g DW , respectively). At the same time, *G. corneum* exhibited one of the lowest contents of phenolics compounds observed and ethanol was not an effective solvent for the extraction of phenolics from all samples. *G. corneum* also revealed the highest value of antioxidant activity [31].

2.5. Phenolic compounds from seaweeds

Phenolic compounds are an important group of chemical compounds present in seaweeds varying quantitatively and qualitatively for each specimen of red, brown or green seaweeds [22]. Polyphenols are one of the most important groups of seaweed phytochemicals and are specially looked up for their pharmacological activity and diverse health-promoting benefits, related to the high variety of seaweed biological activities. These compounds contain hydroxylated aromatic rings and can show a wide variety of structures, from simple monomers to high molecular weight polymers. The main biological activities associated with phenolic compounds are anti-diabetic, anti-inflammatory, anti-microbial, anti-viral, anti-allergic, antioxidant, anti-photoaging, and anticancer properties [23].

In the next subsections, the different phenolic compounds that can be found in seaweeds are introduced.

2.5.1. Phenolic Acids

Phenolic acids are characterized by a single phenolic ring and at least one carboxylic acid group as a substituent, as represented in Figure 6. They are also classified according to the length of their adjacent carbon chain. Phenolic acids with a carbon chain of only one carbon are designated as hydroxybenzoic acids and include some very used phenols as gallic acid (one of the reference phenolic compounds used in quantification assays), *p*-hydroxybenzoic, vanillic, and syringic acids. Derivatives of phenylacetic acids have two-carbon chain attached and hydroxycinnamic acids have a three-carbon chain, which are derived from the non-phenolic cinnamic acid. These include caffeic, ferulic, sinapic and *p*-coumaric acids [23]. As other phenolics, phenolic acids have revealed beneficial bioactivities as antioxidant, antimicrobial and antiviral being important in protective functions of algal cells [32].

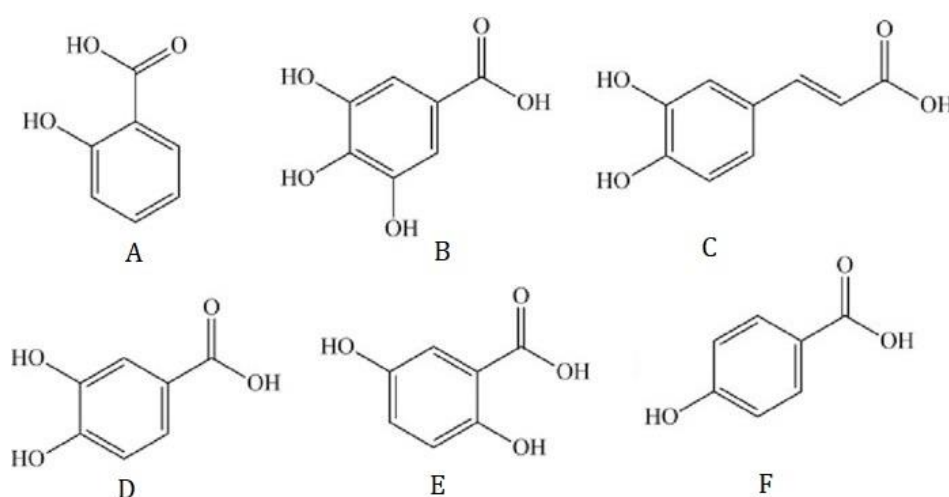


Figure 6: Structures of some phenolic acids found in seaweeds: (A) salicylic acid, (B) gallic acid, (C) caffeic acid, (D) protocatechuic acid, (E) gentisic acid, (F) *p*-hydroxybenzoic acid. Adapted from [33].

In seaweeds, phenolic acids have been reportedly found in different species from the red, brown and green groups. Onofrejev *et al.* [32] have extracted several bioactive phenolic acids (protocatechuic, *p*-hydroxybenzoic, 2,3-dihydroxybenzoic, chlorogenic, vanillic, caffeic, *p*-coumaric, salicylic acid) from three different seaweeds (*Porphyra tenera* - red, *Undaria pinnatifida* – brown, *Sargassum polyceratum* - green) using the combination of pressurized-liquid and solid-phase extraction (PLE–SPE) using acetone/hexane (50:50 v/v) mixture as solvent for a first cycle and methanol/water (80:20 v/v) mixture as solvent for a second cycle.

2.5.2. Bromophenols

Bromophenols are amongst the most present phenolic compounds in red and green seaweeds [23], but have been reported as common to all major algal groups [34]. They are secondary metabolites and possess interesting bioactivities for pharmaceutical purposes such as antioxidant, anticancer, antimicrobial and anti-diabetic [35]. In their structure, at least one hydroxylated benzene ring is present, just like in all phenols, and also bromide substituents in varying degrees, Figure 7 [23].

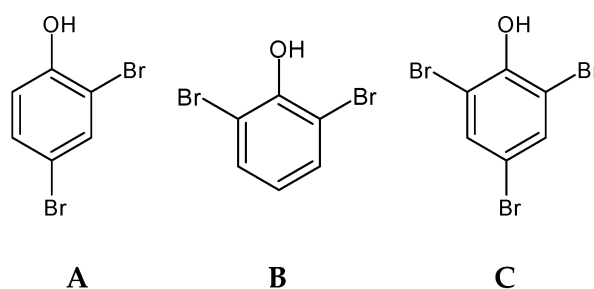


Figure 7: Bromophenols: (A) 2,4-bromophenol; (B) 2,6-bromophenol; (C) 2,4,6-tribromophenol [23].

The biosynthesis of bromophenols is not still established, with some authors suggesting their formation from tyrosine [23], while others indicate their synthesis in the presence of bromoperoxidases, hydrogen peroxide, and bromide [35]. Bromophenols have been firstly identified in red algae [36] and thereafter many bromophenols were identified in diverse species of seaweed. They are mainly detected in red seaweeds from the Ceramiales order, but can also be found in green and brown seaweeds [34, 37].

Seaweed and seafood flavor is strongly influenced by the presence of bromophenols, specifically 2-bromophenol, 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol, and 2,4,6-tribromophenol [23].

2.5.3. Phenolic Terpenoids

Phenolic terpenoids, Figure 8, have been mainly found in red and brown seaweed [38] and have been considered for use in cosmetics, food and pharmaceutical industries due to their compelling bioactivities, antibacterial, antioxidant, anti-inflammatory and antitumor [39]. Brown seaweed contain meroditerpenoids, divided in plastoquinones, chromanols, chromenes, and are mainly found in the

Sargassaceae family, where the studied species *Sargassum muticum* is included [40]. In Rhodomelaceae, diterpenes and sesquiterpenes have been identified and isolated from these red seaweeds [38]. Phenolic terpenoids have already been identified in the green seaweed *Codium tomentosum*, used in this work [18].

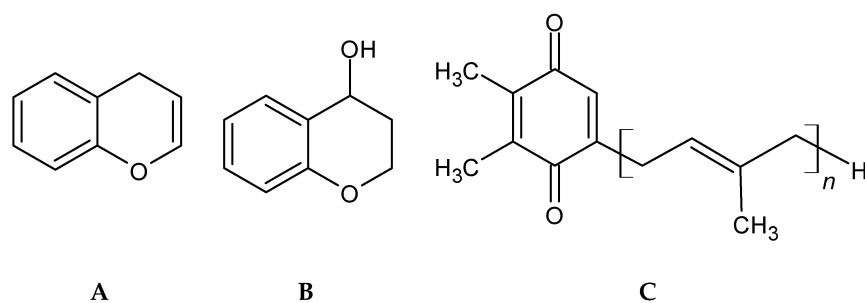


Figure 8: Main phenolic terpenoids found in seaweed: (A) Chromene; (B) Chromanol; (C) Plastoquinone [23].

There is considerable interest in brown seaweed related to their phenolic terpenoid content. Studies have used *Sargassum siliquastrum* and *Cystoseira baccata* to isolate and identify dozens of meroditerpenoids, some of them for the first time [41, 42]. For example, tetraprenyltoluquinol chromane meroterpenoid, obtained from the brown macroalgae *Sargassum muticum*, was used to protect human dermal fibroblasts from reactive oxygen species damage [39], improving the antioxidant activity of this compound.

2.5.4. Mycosporine-like Amino Acids

Mycosporine-like Amino Acids (MAAs) are low molecular weight molecules, usually lower than 400 Da, water-soluble that strongly absorb UVA and UVB rays [43]. As for molecular structure, MAAs consist of cyclohexenimine ring conjugated with two amino acid, amino alcohol or amino group substituents, Figure 9 [43].

In marine algae, these compounds are located free in the intracellular space and concentrated around organelles sensitive to UV rays [44]. They have been discovered in many different marine and fresh-water species, mainly detected in Cyanobacteria and Rhodophyta yet also found in some microalgae or brown and green algae [40].

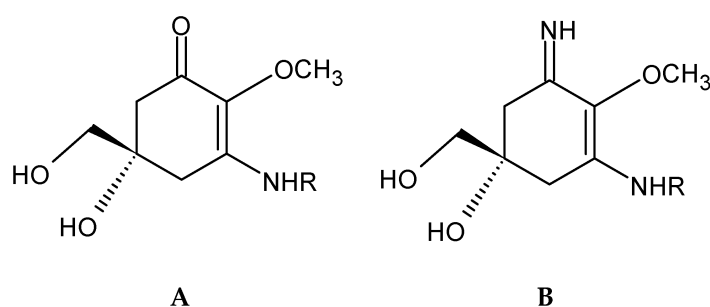


Figure 9: Mycosporine-like amino acids (MAAs); (A) Aminocyclohexenone; (B) Aminocyclohexeniminone [23].

Sun *et al.* [45], in 2020, summarized and analyzed MAAs related studies over 30 years (1990–2019), mainly focusing on their distribution, relative content, and type. They confirmed that MAAs are present in 572 species marine macroalgae, namely 45 species of green seaweed (Chlorophytes), 41 species of brown seaweed (Phaeophytes) and 486 species of Rhodophytes (red seaweed), proving a strong presence of MAAs in red seaweed.

Seaweed-derived MAAs have showed health beneficial properties through UV-irradiated HaCaT cells including anti-photoaging, protecting macromolecules damage, antioxidant capacity and anti-inflammatory [43]. Torres *et al.* [46] studied the most common MAAs (asterina-330, shinorine, palythine, palythanol, and porphyra-334) in red algae regarding their *in vitro* antioxidant capacities, comparing them with other synthetic (BHT and Trolox) and natural (ascorbic acid, gallic acid, *p*-coumaric acid, quercetin, and rutin) antioxidants. A good example of MAAs benefits is also its use in sunscreen cream containing 0.005% MAAs extracted from *Porphyra umbilicalis* (nori), which neutralized photodamage caused by UVA radiation as efficiently as cream containing 1% synthetic UVA and 4% UVB filter. Furthermore, formulations of Porphyra-334 (one of the most studied seaweed-derived MAAs) increased the photoprotective activity of sunscreen formula [43].

2.5.5. Phlorotannins

As mentioned before, phlorotannins are the most studied group of all seaweed phenolic metabolites due to their interesting bioactivity. They are exclusively produced by macroalgae, principally by brown macroalgae, like *Ecklonia cava*, which biosynthesize phlorotannins, such as eckstolonol [39]. The relatively high content of phlorotannins (5%–30% DW) have stimulated interest in these compounds, although in 2006 a group of researchers screened the phlorotannin content of 25 Japanese marine algae and analyzed the content of the most promising species, *Sargassum ringgoldianum* [47].

Despite being secondary metabolites, phlorotannins are exceptionally involved in the development and growth of the cell walls as well as the secondary defense mechanisms [44]. Phlorotannins are oligomers of the simple phenol phloroglucinol (1,3,5-trihydroxybenzene).

Phlorotannins can be subdivided into six groups, depending on the structural linkage: phlorethols (ether bonds), fuhalols (ether bonds and hydroxyl groups), fucols (they contain phenyl bonds), fucophloroethols (they have ether and phenyl bonds), and eckols and carmalols (with dibenzodioxin bonds), Figure 10 [44]. Within these classes, the formation of structural isomers, as well as conformational isomers, occurs. Increasing complexity leads to other criteria for their classification as linear or branched phlorotannins. Like other tannins, phlorotannins can often be covalently bound to algal proteins or other cell wall polysaccharides.

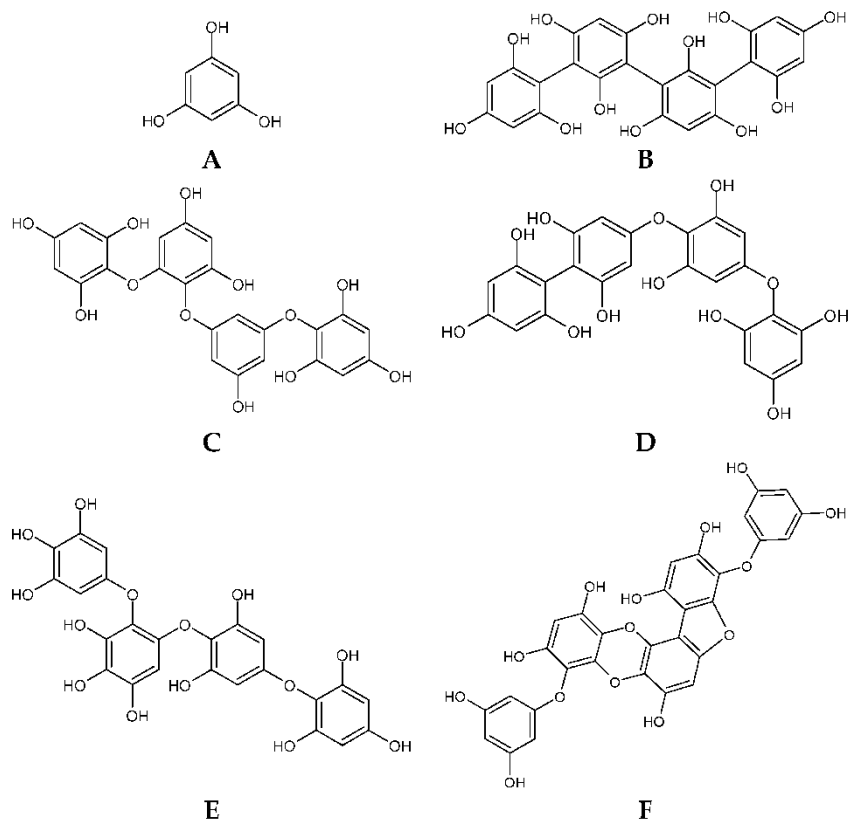


Figure 10: Chemical structures of phlorotannins: (A) Phloroglucinol; (B) Tetrafulcol A; (C) Tetraphlorethol B; (D) Fucodiphlorethol A; (E) Tetrafulhalol A; and (F) Phlorofucofuroeckol [23]

Regarding phlorotannin uses, these compounds have been associated with different properties of interest for diverse applications. They display a broad range of promising health benefits, some of them common between all phenolic compounds as antioxidant, anti-microbial, anti-inflammatory, and cytotoxic effects some of the bioactivities more studied. Therefore, they are seen as relevant for uses in cosmetics, drugs, and also in the food industry [23]. Their antioxidant capacity is up to 10 times higher than other antioxidant compounds such as ascorbic acid or tocopherol, indicating a possible use in the treatment of inflammatory diseases. A good example of health beneficial phlorotannins are the phenolics dioxinodehydroeckol, dieckol, and phlorofucofuroeckol, isolated from *Ecklonia cava* that demonstrated anti-proliferative, anti-tumor, anti-inflammatory, anti-adipogenic (obesity), and anti-tumorigenic activities. Some studies also reported anti-diabetic properties from brown seaweed phenolics originated from the species *Ascophyllum nodosum*, *Fucus distichus*, and *Padina pavonica*. Anti-allergenic properties have also been detected in dioxinodehydroeckol and phlorofucofuroeckol A extracted from *E. stolonifera* [23]. Bactericidal effects have been found in *Ecklonia kurome* extracts tested against several pathogenic microorganisms, such as, methicillin-resistant *S. aureus*, *Bacillus cereus*, *Campylobacter jejuni*, *E. coli*, *Salmonella enteritidis*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus* [44].

Yuan *et al.* [48] studied the antioxidant properties of four brown macroalgae (*Ascophyllum nodosum*, *Laminaria japonica*, *Lessonia trabeculate* and *Lessonia nigrecens*) and detected the presence of phlorotannin hexamer and dimer derivatives through HPLC-DAD-ESI-MS.

2.5.6. Flavonoids

Flavonoids, Figure 11, constitute the largest group of plant phenolics. They are well known and studied regarding terrestrial plants, although information on the flavonoid content of seaweeds is still scarce [40]. Yoshie-Stark *et al.* [49] studied the flavonoid composition of 27 Japanese seaweeds and concluded that red algae had larger amounts of these compounds than brown and green algae.

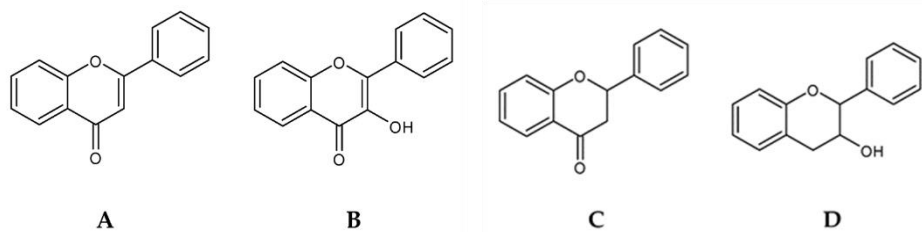


Figure 11: Main classes of flavonoids found in algae: (A) Flavones; (B) Flavonols; (C) Flavanones; (D) Flavan-3-ol [23].

The extraction of these compounds using DES have already been reviewed by Skarpalezos and Detsi [50] but only from various plants substrates. They summarized a set of factors affecting the extraction of flavonoids using DES, namely, temperature, molecular structure and composition of the DES, extraction time, water content, the use of additives, solvent/sample ratio, and pH, compromising the efficiency and yield of the process. Overall, the use of DES in the extractions of flavonoids from biomass yielded overwhelmingly promising results, with reported success in isolation and extraction of the target compounds compared with conventional solvents.

2.6. Seaweed extraction technologies

The process of obtaining natural compounds from raw materials starts with the extraction. Depending on the extraction principle, each method may include solvent extraction, distillation, pressing and sublimation. Within these, solvent extraction is the most widely used method. The various stages of solvent extraction methods can be understood as the following sequence of steps: (1) the solvent penetration into the solid matrix; (2) the solute dissolution in the solvents; (3) the diffusion of the solute out of the solid matrix; (4) the collection of the extracted solutes [51].

Solvent properties have a great effect not only on extraction efficiency, but also on the selectivity, solubility, cost, and safety of the process. Yet extraction experimental conditions will also play its part. High temperatures will increase solubility and diffusion but may cause solvent loss or the decomposition of thermolabile components. The duration will increase efficiency, until the equilibrium is reached. The greater the ratio between liquid and solid the higher the extraction yield is, but this can lead to solvent saturation and supersaturation and separation difficulties. Solute properties should also be considered since, generally finer particles result in better extraction although it may harm filtration [51].

2.6.1. Traditional extraction methods

Traditional methods, often known as solid-liquid extraction (SLE), are the most commonly and frequently used extraction techniques. The most used traditional methods include: soxhlet, maceration and percolation. Several solvents have been used as well as mixtures of solvents with wide polarity ranges, depending on the envisaged solute. These include methanol, ethanol, acetone, ethyl acetate, trichloromethane and water. Using a mixture of solvents, like water and ethanol, may improve the yield of phenolic compounds due to the presence of different compounds with different polarities. However, this extraction method has several drawbacks such as the usage of a large volume of solvents, long extraction time, low extraction yield, degradation of extracted compounds, separation difficulties. Additionally, upscaling this technology may bring difficulties of practicality, energy, economic, and environmental largely due to the large amounts of organic solvents used. Thus, in order improve the overall process several emerging technologies were developed and are being used to extract phenolics from marine biomass [52].

Soxhlet

This method is an automatic continuous extraction with high extraction efficiency that requires less time and solvent consumption than maceration or percolation [51]. This process has some advantages compared to other traditional methods such as less time consuming than maceration and percolation, there is no need for extract filtration, allows the treatment of parallel samples [53] and solvent recycling [23]. One the other hand, the high temperature and long extraction time will increase the possibilities of thermal degradation [51]. This method has been used by Bhuyar *et al.* [54] to study the antioxidant and antibacterial activity of red seaweed *Kappaphycus alvarezii* against pathogenic bacteria reporting a total phenolic content of 20.25 ± 0.03 mg GAE/g DW, for the ethanolic extract, and 19.10 ± 0.81 mg GAE/g DW for the hot water extract. A Soxhlet extractor is represented in Figure 12.

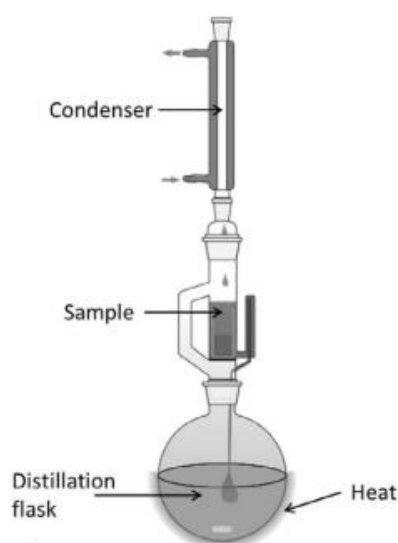


Figure 12: Schematic representation of a traditional Soxhlet extractor [53].

Maceration

In maceration the compounds are extracted by submerging the seaweed biomass in an appropriate solvent with or without stirring [52]. Is a very simple extraction method with the disadvantages of long extraction times, sometimes overnight, low extraction efficiencies and the need of a final filtration or centrifugation [51, 53]. This method has been used by Srikong *et al.* [55] to assess the antibacterial and antioxidant activities of differential solvent extractions from the green seaweed *Ulva intestinalis*, obtaining a result of 197 ± 16 mg GAE/g DW of TPC for the dichloromethane extract.

Percolation

The percolation is generally more efficient than maceration due to its a continuous mode, in which the saturated solvent is constantly being replaced by fresh solvent [51]. The major downsides of percolation are the same as maceration involving long extraction times, low efficiencies, and high amounts of solvents. There is little information regarding the use of percolation in seaweeds. However, its use in plants is widely reported. For example, Jamshidi *et al.* [56] studied the extraction of antioxidants from leaf and aerial parts of *Lythrum salicaria*, comparing it with ultrasonic assisted extraction and polyphenol fraction. Regarding seaweeds, this method has been used by Baliano *et al.* [57] to obtain bioactive methanolic extracts of *P. gymnospora* for wound-healing purposes.

2.6.2. Emerging technologies

Emerging extraction technologies take advantage of novel technological developments such as ultrasound-assisted extraction (UAE) or microwave-assisted extraction (MAE). In these methods biological agents, such as enzymes in enzyme-assisted extraction (EAE) and/or new types of solvents, like in pressurized liquid extraction (PLE) and/or supercritical fluid extraction (SFE) are also used. There are other, less frequently used technologies, such as pulsed electric field-assisted extraction (PEF), where heat is generated by passing an electrical current through the material, ohmic heating or centrifugal partition extraction (CPE), a liquid-liquid extraction technique managed under a centrifugal field. Technology also advanced in the development of new types of solvents used, including ILs and DES [58, 59].

Ultrasound-assisted extraction (UAE)

In UAE, ultrasound waves that travel through the medium and induce pressure variations are used. The acoustic cavitation disrupts the cell walls of the seaweed biomass, leading to the reduction of size particle and consequently enhancing the contact between the solvent and the target compounds. This method can be used together with other methods, such as MAE [23, 60]. Other advantages of this method include accelerated heat and mass transfers, reduced amount of solvent required, reduced extraction times, high reproducibility, reduced energy consumption [52]. On the other hand, extreme heat can be generated leading to the degradation of thermolabile compounds, and depolymerization of certain compounds into lower molar mass fragments [61]. This method has been used by Rodrigues *et al.* [62] assessing the impact

of EAE and UAE on the biological properties of red, brown, and green seaweeds from the central west coast of Portugal, namely *Osmundea pinnatifida*, *S. muticum* and *C. tomentosum*, respectively. This method was also used to compare the extraction phenolic compounds and the antioxidant capacity of the brown seaweed *Sargassum vestitum* with the microwave-assisted and solid-liquid methods.

Microwave-assisted extraction (MAE)

This method involves the utilization of microwave radiation to induce the vibration of water molecules within the cells of the seaweed biomass. Vibrations cause the increase of the temperature of the intracellular liquids and the water evaporation breaking down the cell walls and the releasing of the intracellular contents into the extraction medium. This method enables a high extraction yield, reduced extractions times and reduced use of solvents [63]. As drawbacks, it needs to include a separation procedure (filtration or centrifugation), the increased temperature can cause the aforementioned issues and, in this case, operational parameters may vary significantly depending on the sample [64].

Enzymatic-assisted extraction (EAE)

Currently applied in diverse industrial applications (energy, agriculture, feed, and food), the principal advantage of enzymatic-assisted extraction (EAE) is the extraction of valuable components without using denaturing conditions such as harsh solvents or high temperatures. In EAE, enzymes are used to disrupt the cell wall and cell membranes and reach the targeted compounds, aiming for extraction enhancement [61]. The degradation of structural and cell wall polysaccharides allows the release of the cell content, including the phenolic compounds into the extraction medium, increasing the extraction yield [52]. This method has proven to reduce extraction time and energy consumption, while preserving biological activity and also, offering the possibility of combination with other methods. Rodrigues *et al.* have studied the impact of EAE and UAE on red, brown and green seaweeds including *C. tomentosum* and *S. muticum*, reaching maximum extraction yields of 62% and 31% (g of extract / g DW), respectively. This group concluded that EAE is one of the best methods in terms of food compatible extraction with higher yields, stating that enzyme selection can favor some biological properties [62].

Pressurized liquid extraction (PLE)

Pressurized liquid extraction is also known as accelerated solvent extraction, enhanced solvent extraction or subcritical water extraction (SWE), when water is the only solvent [51]. This method applies high pressures keeping solvents liquid above their boiling temperature, resulting in high solubility and diffusion rates. This extraction technique requires a very short time and relatively small amounts of solvent and reduces phenolic degradation, since it occurs in the absence of light and oxygen. Otero *et al.* [65] applied PLE to *Laminaria ochroleuca* using four solvents of different polarities. Ethanol/water 1:1 (v/v) mixture showed the best results, with 52% of extraction yield and up to 173 mg GAE/g DW, with the Folin-Ciocalteu method. Zakaria *et al.* [66] also obtained higher yields of phenolic acids in *Chlorella sp.* extracts, comparing with the Soxhlet extraction.

Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE) uses supercritical fluid (any substance kept above its critical point and critical pressure) as extraction solvent. These conditions alter the physical properties of the solvent, giving it diffusion coefficients similar to the gases and solvation properties similar to the liquids, which is the main advantage of this method. The most widely used supercritical fluid in food, pharmaceutical, and cosmetic industries is carbon dioxide (ScCO_2), due to its low critical temperature and pressure (31 °C, 73.8 bar), affinity for non-polar solutes, inertness, low cost, non-toxicity, and potential to extract thermolabile compounds. *S. muticum* was subjected to SFE by Anaëlle *et al.* [67] using CO_2 and ethanol (88:12 v/v), but this method was shown to be less efficient method comparing to traditional extractions and CPE, regarding phenolic content.

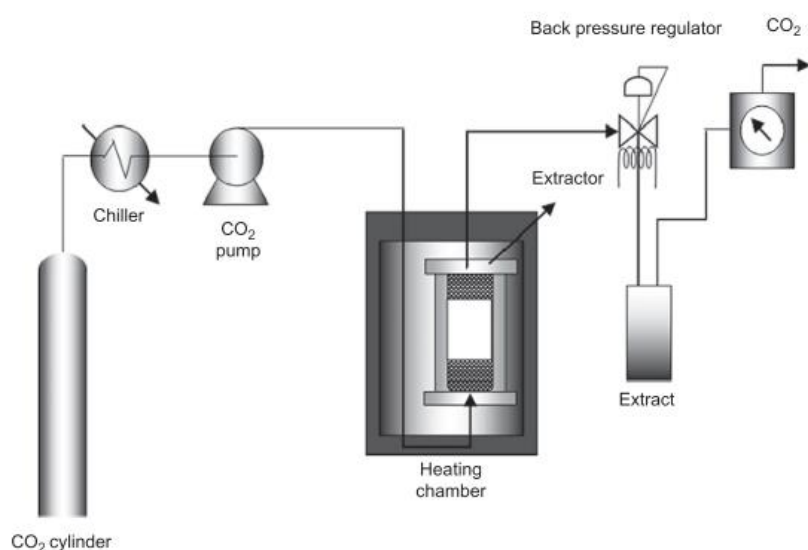


Figure 13: Typical basic scheme of supercritical CO_2 extraction in laboratory scale [68].

2.6.3. Novel solvents for phenolic extraction

Novel solvents have been a high interest research topic for quite some time. Ionic Liquids were once labeled as “solvents of the future”, Rogers and Seddon [69], due to their versatility as solvents and interesting chemical and biological properties. These solvents have been used to extract added-value compounds from natural sources [58, 70–74]. More recently, Deep Eutectic Solvents (DES) and Natural Deep Eutectic Solvents (NADES) are being used as solvents, replacing ILs due to toxicity and environmental concerns.

Deep Eutectic Solvents

Deep eutectic solvents were introduced as a cheap and greener alternative to ILs and quickly became widely used, with more than one thousand references today in ISI Web of Science. In comparison, ILs are generally expensive due to their difficult synthesis and purification steps, and thus not environmentally

friendly, while DES are usually inexpensive, easy to prepare just by heating and mixing, and generally more environmentally friendly than ILs, although this may depend on the starting materials used [75, 76]. Deep eutectic solvents should not be mistaken by a new family of low cost ILs, since they are mixtures and not pure compounds like the former, at the best a solution of ionic and molecular species (although neutral DES, composed just of molecular species were also proposed), while ILs are fluids constituted solely by ionic species.

Deep Eutectic Solvents have been proposed in 2003, when Abbott *et al.* [77] described mixtures of amides with quaternary ammonium salts that had melting points much lower than those of their pure compounds focusing their study on the formation of a hydrogen bonding complex between a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) at a well-defined stoichiometric proportion. Only in 2014, Abbott and his co-workers extended the definition of DES to all compositions of a non-ideal eutectic mixtures of Lewis or Brønsted acids and bases, which can contain a variety of anionic and/or cationic and/or neutral species [78]. A general solid-liquid phase diagram of DES is depicted in Figure 14 .

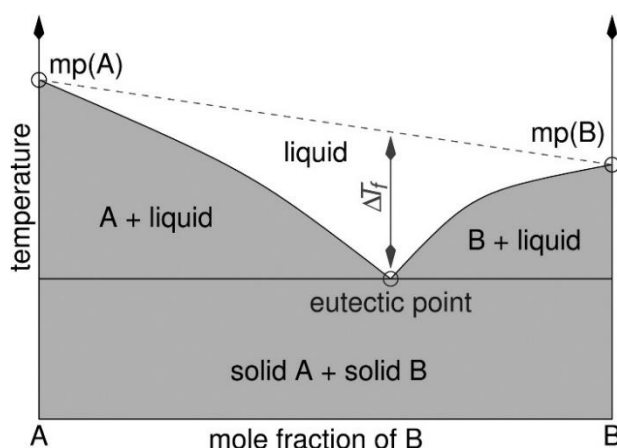


Figure 14: Schematic representation of a eutectic point on a two-component phase diagram [78].

If pure solid phase is assumed and influence of the temperature on the heat capacities is neglected, classical thermodynamics proposes Equation 2.1 to describe these melting curves,

$$\ln(x_i\gamma_i) = \frac{\Delta_m H}{R} \left(\frac{1}{T_m} - \frac{1}{T} \right) + \frac{\Delta_m C_p}{R} \left(\frac{T_m}{T} - \ln \frac{T_m}{T} - 1 \right) \quad 2.1$$

where γ_i is the activity coefficient of compound i at a molar liquid composition x_i ($\gamma_i = 1$, if ideality is assumed), T is the absolute temperature, T_m and $\Delta_m H$ are the melting temperature and enthalpy of the pure compound, respectively, R is the universal gas constant, and $\Delta_m C_p$ is the difference between the molar heat

capacity of compound i in the liquid and solid phases. In particular, if the equilibrium temperature and the melting temperature of the pure compound are similar, the last term of the equation has a negligible value when compared to melting enthalpy term and thus, can be neglected [75].

Definition wise, the term ‘deep eutectic solvent’ is often extended to mixtures with small deviations from ideality. So far, most works about DES target their applications more than the DES nature and what is their difference from conventional eutectic mixtures. Hence, the lack of thermodynamic characterization of DES (solid-liquid phase diagrams, understanding of interactions in the liquid phase, and even melting properties of the pure compound) sometimes leads to misconceptions [75]. Some authors [79–81] have recently been researching the nonideality of DES aiming for greater understanding of the solid-liquid phase boundary. Different thermodynamic models, statistical analyses based on molecular properties combined with experimental are being used to predict solid-liquid equilibria and clarify the relation between the liquid-phase structure and its thermodynamic nonideality.

The eutectic composition is the result of intersecting the melting curves of pure compounds, dominated by their fusion properties, more than the interactions between the HBD:HBA. Therefore, there is no stoichiometric meaning related to the eutectic composition. This is a variable composition that can assume any value depending on the relationship between the fusion properties and the non-ideality deviations expressed by the activity coefficient, as represented in Figure 14 [75]. If we are looking for a liquid solvent at the operating temperature of a target application, there is no need to focus only on the eutectic composition since any liquid mixture below the operating temperature could be used and defined as a DES, this refers to the compositions comprised between x_1 and x_2 on Figure 15.

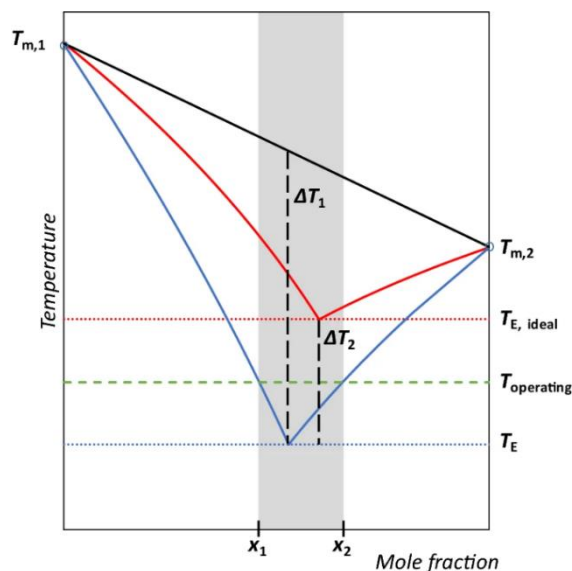


Figure 15: Representation of the SLE of a simple ideal eutectic mixture (red line) and a deep eutectic mixture (blue line)[75].

DES are, in fact, a mixture of two or more pure compounds for which the eutectic point temperature is below that of an ideal liquid mixture, presenting significant negative deviations from ideality as

represented in Figure 15. This temperature depression should enable the mixture to be liquid at the operating temperature of the target application for a composition range. For this reason, the SLE phase diagram should be required, and the melting properties of the pure compounds known to distinguish a simple and a deep eutectic mixture.

Deep eutectic solvents are easily prepared by heating and stirring the components together under an inert atmosphere to obtain a homogeneous liquid. Other methods of preparing DES include vacuum evaporation, or freeze-drying. With vacuum evaporation the components are dissolved in water before being evaporated under vacuum. In the freeze-drying method the components are dissolved in water and subjected to freeze-drying [76]. The simple preparation of DES is one of the advantages of these mixtures. No solvent is required neither are purification steps, leading DES to be an economically interesting alternative for conventional organic solvents and ILs [76]. Also, their possible use in biochemical processes have drawn attention for the use of DES as an alternative for common organic solvents [82].

Natural Deep Eutectic Solvents

An important type of DES have been introduced by Choi *et al.* [83], in 2011, combining natural compounds typically plant metabolites common in ILs such as choline, sugars and natural organic acids. Over 30 combinations were proposed, and these solvents were called NADES – Natural Deep Eutectic Solvents – since they are only constituted by natural compounds. These authors believed that NADES could play an important role in explaining several biological phenomena, such as the biosynthesis of small molecules and macromolecules not soluble in water.

Natural deep eutectic solvents have been showing very promising results in a broad range of fields in the last years. They have great potential for novel applications since they are more environmentally friendly, sustainable, and less toxic than ILs and DES. As shown in Figure 4, the increasing research on NADES highlights the development of green and sustainable processes and their great value to science with a wide range of potential applications in the fields of chemistry, biology, pharmacology, medicine, etc. A wide range of applications in biotechnology are being developed using these solvents in areas such as biocatalysis on enzymatic activity and stability, extraction of natural products of diverse polarity, in biomass pretreatment, clinical therapy, preparation of nutraceutical/pharmaceutical products and for electrochemical detection of bioactive materials. Several reviews can be found relative to the applications of NADES [50, 84–86].

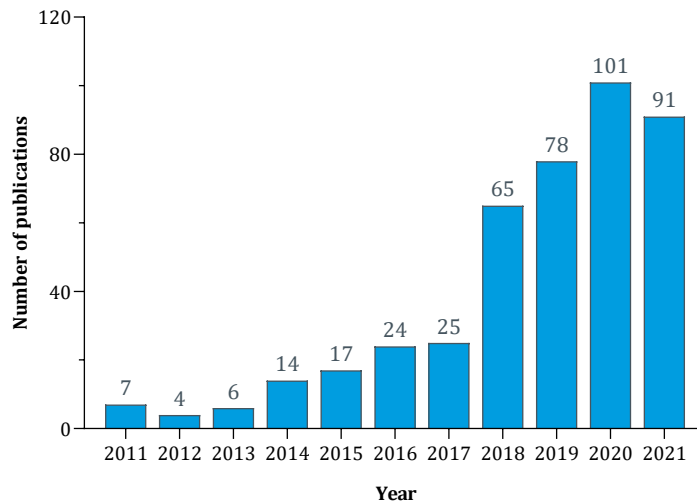


Figure 16: Number of publications about NADES in the last decade. ISI Web of Science, keyword: 'NADES'.

With special interest for the natural cosmetic industry, NADES can bring advantages to the extraction of natural bioactive compounds. Although there is still a lack of information regarding their toxicology, if biosafety can be assured with cytotoxicity studies, their use in pharmaceutical formulations would be of major importance. If so, purification steps between extraction and formulation could be reduced.

The different physical properties of the diverse NADES mixtures, including viscosity and polarity, have shown a great influence on the solubilization and extraction effectiveness [87] and thus on their use as extraction solvents. Therefore, a large number of NADES composed by salts (choline chloride, sodium acetate, ammonium acetate), acids (lactic, malic, oxalic and tartaric acid), polyalcohols (xylitol, glycerol, ethyleneglycol and 1,2-propanediol), sugars (fructose, glucose, maltose and sucrose), aminoacids (L-alanine, L-proline glycine) and many others were already used to study the extraction from natural substrates – Table 1.

Table 1: Selection of NADES already used for extractions with natural substrates.

NADES		Molar Ratio ($n_1:n_2$)	Water content (%, v/v)	Ref.
Compound 1	Compound 2			
Choline chlorine	Sucrose	4:1, 1:1	25	[87]
	Fructose	1.9:1	30	[88]
	Glucose	2:1	30	[88]
	Glycerol	1:1, 1:2	0, 30	[89], [88]
	Malic acid	1:1, 2:1	30, 2	[88], [90], [91]
	Tartaric acid	2:1	20	[92]
	Xylose	2:1	30	[88]
	1,2-propanediol	1:2	20	[93]
	Ethyleneglycol	1:1, 1:2	0, 20	[89], [93]
	Lactic Acid	1:1, 1:2, 1:3	0, 30, 40	[90], [89]
	Oxalic acid	1:1	20	[93]
	Urea	1:2	30	[93]
Citric acid	Fructose	1:1	0	[93], [94]
	Glucose	1:1	0, 14	[94]
	Maltose	4:1	12	[93]
Lactic Acid	Ammonium acetate	7:1	25	[95]
	Fructose	5:1	0, 4, 16, 30	[87], [96]
	Glucose	1:1, 5:1, 6:1	0, 15, 16	[87], [94], [97]
	Glycine	7:1	25	[95]
	L-alanine	7:1	25	[95]
	Nicotinamide	7:1	25	[95]
	Sodium Acetate	7:1	25	[95]
L-proline	Glycerol	1:2.5	10	[98]
	Xylitol	1:1.5	10	[98]
	Malic acid	1:1	10	[87]

NADES in cosmetic formulations

Despite the many possible combinations of natural compounds to form NADES, the use of some of these compounds in cosmetics is restricted by the European Commission. Hence, to create a selection of NADES for possible use as a solvent in the cosmetic industry it is necessary to evaluate if all the compounds are approved by the CosIng - database for information on cosmetic substances and ingredients. If any compound listed in Annex II of this regulation it needs to be discarded from cosmetic formulations [99].

The NADES presented in Table 1 are only a part of the hundredths of mixtures of compounds used to successfully extract different solutes with different properties. Choline chloride is by far the most widely used compound to prepare NADES. It's use on NADES have been used to leverage this class of solvents biological application, since choline chloride is part of complex B vitamin. Many examples can be found in the open literature regarding the application of choline-based NADES in biological applications like extractions solvents or catalysts [87–93, 100, 101]. However, choline chloride and its derivatives are presented in Annex II of the European Cosmetic Regulation EC No. 1223/2009 and so are prohibited in cosmetics. For this reason, the use of any solvent mixture containing choline chloride envisaging cosmetic plant extracts should be discarded. This fact highlights the need of designing and testing new NADES mixtures compatible with cosmetic applications, and to evaluate their plant extraction performance in comparison with conventional solvents [99].

In Table 2 are listed the NADES approved by the European Commission to be used in cosmetic industry that have already been used as extraction solvents of solutes from natural substrates.

Table 2: NADES approved by the European Commission for the preparation of cosmetic formulations.

NADES		Molar Ratio (n ₁ :n ₂)	Water content (%, v/v)	Ref.
Compound 1	Compound 2			
Lactic Acid	Ammonium acetate	7:1	25	[95]
	Fructose	5:1	0, 4, 16, 30	[87], [96]
	Glucose	1:1, 5:1, 6:1	0, 15, 16	[87], [94], [97]
	Glycine	7:1	25	[95]
	L-alanine	7:1	25	[95]
	Sodium Acetate	7:1	25	[95]
L-proline	Glycerol	1:2.5	10	[98]
	Xylitol	1:1.5	10	[98]
	Malic acid	1:1	10	[87]

With cosmetic applications envisaged, some other requirements are also imposed: NADES should be bio-based and available at a reasonable cost at industrial scale, they should have a low or tunable viscosity at room temperature to allow homogeneous dispersion of the bio-material and easy solid/liquid separation, they should also demonstrate long-term stability, including microbial stability, and lastly the solvents should be compatible with classical cosmetic formulations such as emulsions or gels and have a safe profile.

2.6.4. Seaweed phenolics extractions

First of all, it should be mentioned that as seaweeds are a natural biomass, different compositions are possible, supporting the fact that the same method can yield different results for different samples. Thus, sample homogenization prior to extraction is a very important step that should not be neglected, since it greatly reduces the scattering of the results, leading to more confident conclusions. In general, emerging technologies are greener than conventional methods and also perform better, in terms of maximizing the extraction yield. Regarding phenolics extraction, MAE and PLE are more used than SFE, since the latter is typically used to extract non-polar compounds [63].

Comparison between the emerging technique MAE and the traditional maceration method has been carried by Yuan *et al.* [48] using four different brown seaweeds. Using the same solvent for both methods, improved TPC yields were obtained for all MAE extracts comparing with traditional maceration. This is particularly true for *A. nodosum*, with a yield of 139.80 mg GAE/g DW, exceeding the maceration extraction yield of 51.47 mg GAE / g DW.

Ultimately, the combination of different methods was suggested to improve extraction yield of phenolic compounds from seaweeds. Dinh *et al.* used a combined method of subcritical water assisted with ionic liquids (SWE+IL) to extract different phenolic acids from the brown seaweed *Saccharina japonica*, reporting a phenolic content of 39.5 mg PGE/g DW, at 175°C, with subcritical water extraction. When SWE+IL was used the TPC was enhanced to values of 58.9 mg PGE/g DW, using 0.25 M [C₄C₁im][BF₄], 1-butyl-3-methylimidazolium tetrafluoroborate [58].

A summary of the results reported in literature for the extraction of phenolics from seaweeds using conventional solvents is presented in Table 3, as well as the respective extraction conditions: time, temperature, solid-liquid ratio (SLR) and specific conditions of the emerging technologies. In general, the TPC results are reported in terms of grams of gallic acid per gram of dry seaweed (mg GAE/g DW). Nevertheless, other compounds can be used as standards for this assays, as is the case of phloroglucinol and catechol, used in the studies of Anaëlle *et al.* [67] and Rodrigues *et al.* [22], respectively.

Since almost every study presented in Table 3 use different seaweeds, direct comparison is not feasible, but individual results confirm the promising value of emerging technologies. The works of Yuan *et al.* [48] and Dang *et al.* [102], show the beneficial improvements of the MAE over the traditional maceration.

MAE is becoming a very used emerging technology with confirmed improvements as: increased extraction efficiency of phenolic compounds, minimized extraction time and extracts of improved quality.

The extraction enhancement of high pressure methods have also been confirmed by Dinh *et al.* [58] which used SWE to improve SLE extraction, using only water as solvent. This work also combined SWE with ILs, taking a step further in phenolic extraction and proving that combining different methods could bring interesting synergetic effects.

Although emerging technologies are proving to be more effective in phenolic extraction there are also challenges associated with these methods, especially in terms of industry applications. EAE extraction reported by Rodrigues *et al.* [62] resulted in higher phenolic extraction over UAE, however there upscaling issues regarding EAE, since the most commonly available enzymes are nonspecific enzymes like carbohydrases and proteases and could lead to co-extraction of other compounds [52]. Upscaling problems are also verified for other emerging technologies, as PLE, SFE or MAE, by requiring state-of-the art equipment with high capital expenditures.

Notwithstanding some of the disadvantages of maceration, this method has sometimes proved itself, as in the case of the work of Anaëlle *et al.* [67] where it presented higher extraction efficiencies than both emerging techniques, SPE and PLE.

Unfortunately, information on seaweed phenolic extractions with NADES is scarce, with only one article found regarding this issue. Obluchinskaya *et al.* [90] in 2018 studied the use of NADES on phenolic extraction from two brown seaweeds: *Fucus vesiculosus* and *Ascophyllum nodosum*. Results reported values of 174.8 mg PG/g DW from *F. vesiculosus* and 148.4 mg PG/g DW from *A. nodosum*, using exhaustive extraction with conventional solvents (acetone 70%, v/v). They further studied the effect of water content on the extractions. In this study, aqueous solutions of choline-chloride and lactic acid NADES (60-70% H₂O, v/v) were shown to be the most efficient for phenolic extraction, with a 10-fold increase in phenolics extraction yield compared to the pure NADES, and comparable to those of traditional extractants acetone and ethanol (96% v/v). This group demonstrated that the efficiency of aqueous NADES solutions could vary depending on the amount of H₂O, which affects NADES polarity and viscosity. They also pointed out that H₂O content should be optimized, as well as the ratios between NADES components, since they could considerably affect extraction capacity.

These results show that although some studies have been carried regarding phenolic extractions from seaweeds, there is still space for improvement. The use of NADES is still very recent in the extraction of seaweed phenolics but the ultimate goal would be to take advantage of synergetic effects of combining methods reaching for high extraction efficiencies and if possible low operation costs.

Table 3: Summary of the literature reports for the extraction of phenolic compounds from seaweeds.

Seaweed	Method	Solvent	TPC (mg GAE/g DW)	Conditions Time, Temp. (°C), SLR (g/ml)	Ref.
<i>K. alvarezii</i>	Soxhlet	Hot water	6.3	3 h, 70, 1:15	[54]
		Ethanol 70%	3.2	30 min, r.t., 1:15	
<i>U. intestinalis</i>	Maceration	Methanol	54	7 d, r.t., 1:10 (mg GAE/g extract)	[55]
		Ethanol	88		
		Dichloromethane	197		
		Hexane	150		
<i>S. vestitum</i>	Maceration		40	12 h, 30, 1:50	[102]
	UAE	Ethanol 70%	48	60 min, 30, 1:50, 50 kHz*	
	MAE		58	75 s, ---, 1:50 2.45 GHz*	
<i>E. cava</i>	UAE	Water	48	12h, 30, 1:100, 40 kHz*	[103]
		Methanol 50%	64		
		Methanol	58		
<i>S. muticum</i>	Maceration	Ethyl acet. 50%	186 mg PG/g DW	3h, 40, 1:75	[67]
	PLE	Ethanol 75%	102 mg PG/g DW	90 min, 60, 1.5, 10.3 MPa*	
	SFE	CO ₂ -EtOH (88:12)	35 mg PG/g DW	90 min, 60, ---, 15.2 Pa*	
<i>S. japonica</i>	SLE	Water	2.4	24h, r.t., 1:32	[58]
	SWE	Water	39	5 min, 175, 1:32, 5 MPa*	
	SWE+IL	0.5 M [C ₄ C ₁ im][BF ₄]	59	(mg PG/g extract)	
<i>L. trabeculate</i>	Maceration		50	4h, r.t., 1:10	[48]
	MAE	Methanol 70%	74	15 min, 110, 1:10, 2.45 GHz*	
<i>L. nigrecens</i>	Maceration		78	4h, r.t., 1:10	[48]
	MAE	Methanol 70%	107	15 min, 110, 1:10, 2.45 GHz*	
<i>A. nodosum</i>	Maceration		51	4h, r.t., 1/10	[48]
	MAE	Methanol 70%	140	15 min, 110, 1:10, 2.45 GHz*	
<i>S. japonica</i>	Maceration		38	4h, r.t., 1:10	[48]
	MAE	Methanol 70%	73	15 min, 110, 1:10, 2.45 GHz*	
<i>S. muticum</i>	EAE	Viscozyme	84 µg CE/g DW	24h, 50, 1:25, 50 kHz*	[62]
		Cellulase	81 µg CE/g DW		
	UAE	Hot water	54 µg CE/g DW		
<i>C. tomentosum</i>	EAE	Viscozyme	65 µg CE/g DW	24h, 50, 1:25, 50 kHz*	[62]
		Cellulase	79 µg CE/g DW		
	UAE	Hot water	69 µg CE/g DW		

CE—catechol equivalent; PG—phloroglucinol equivalent

* Method specific condition: Pressure (Pa) / Frequency (Hz)

3. Aim of Studies

This project aims at the development of sustainable extracts using marine biomass from the Portuguese Atlantic coast, namely *Codium tomentosum*, *Gelidium corneum* and *Sargassum muticum*. Although blue biorefinery concepts have been unravelling the potential of marine biomass as a source of important biomolecules with chemical and biological properties, it remains still underexplored.

This project focuses on the valorization of invasive macroalgae from the Portuguese coast and aims to use novel sustainable solvents in extraction of valuable compounds such as phenolics, leveraging lower yields of extraction. Conventional extraction techniques usually require a large volume of organic solvents and long extraction time. The development of novel strategies looks for minimizing the generation of hazardous waste and their associated impact on the environment. Previously, NADES were used successfully to extract phenolic compounds from aerial plant material. However, there is still a lack of information regarding their use in marine biomass. Therefore, the development of new NADES composed of inexpensive nontoxic natural compounds, such as lactic acid and others, for maximum extraction of biologically active compounds from algae with variable phenolic compositions is necessary.

Sustainability is also a present challenge in cosmetic and dermocosmetic areas. The replacement of unsustainable synthetic ingredients for green alternatives is one of the main challenges of the cosmetic industry. Although several studies reveal the potential of sustainable ingredients, the inclusion of natural, organic, or green chemistry derived ingredients in formulations require further work. The functionality and safety of each ingredient should be assayed to assure formulation stability and performance regarding consumer aesthetic preferences [6]. Incorporating natural ingredients in creams, an increasing tendency, may have an impact in their leading properties, such as physicochemical and rheological [104]. Therefore, this project also aims to evaluate the impact of incorporating novel seaweed extracts in skin care oil-in-water (O/W) creams, their characterization, and comparison with a control cream.

4. Materials and Methods

4.1. Seaweed collection and preparation

4.1.1. *Codium* sp.

The green seaweed *C. tomentosum*, known as Velvet Fingers, was produced in an Integrated Multi-Trophic Aquaculture system and grounded to a powder (ALGApplus, Ílhavo, Portugal) Portions of *C. Vermilara* were identified in the sample, therefore the name *Codium* sp. was given to the whole mixture.

4.1.2. *Gelidium corneum*

The red seaweed *G. corneum* was collected in Baleal, Ferrel, Peniche, Portugal (39° 22' 35.8" N, 9° 22' 23.7" W) and identified by Dra. Teresa Mouga (Polytechnic of Leiria). After collection the seaweed was immediately transported to the laboratory and washed, firstly with sea water to remove unwanted organisms and detritus, then with distilled water. *G. corneum* was dried at 70 °C in a perforated board, ground (Krupps, Solingen, Germany) and stored at room temperature. Afterwards, *G. corneum* was ground with liquid nitrogen to get a powder.

4.1.3. *Sargassum muticum*

The brown seaweed *S. muticum* was collected at Praia Norte beach, Viana do Castelo, Portugal (41° 41' 44.2" N 8° 51' 8.1" W) and immediately transported to the laboratory. After cleaned and washed, firstly with sea water to remove invertebrate organisms, epiphytes, and detritus, then with distilled water. *S. muticum* was frozen at -20°C and freeze-dried (Scanvac Cool Safe, LaboGene, Lyngø, Denmark). The dried algal material was ground into a powder in a grinder which and stored protected from light, at room temperature.

4.2. Chemicals and reagents

In Table 4 is the information regarding the different chemicals and solvents used along this work. Purified water was obtained by reverse osmosis (Elix 3, Millipore, Massachusetts, USA).

Table 4: Chemicals and Solvents used.

Name	Linear Formula	Purity (%)	Supplier
NADES preparation			
D-(+)-Glucose	C ₆ H ₁₂ O ₆	≥ 99.5	Sigma-Aldrich, Steinheim, Germany
D-Fructose	C ₆ H ₁₂ O ₆	≥ 99	Alfa Aesar, Germany
Glycerol	C ₃ H ₈ O ₃	≥ 99	Sigma-Aldrich, Steinheim, Germany
L-(-)-Malic Acid	C ₄ H ₆ O ₅	≥ 97	Sigma-Aldrich, Steinheim, Germany
L-(+)-Lactic Acid	C ₃ H ₆ O ₃	88-92	Sigma-Aldrich, Steinheim, Germany
L-Proline	C ₅ H ₉ O ₂	≥ 99	Alfa Aesar, Kandel, Germany
Sodium Acetate	C ₂ H ₃ NaO ₂	≥ 99	Sigma-Aldrich, Steinheim, Germany
Total Phenolic Content (TPC) - macroscale			
Folin-Ciocalteu's Reagent			AppliChem, Darmstadt, Germany
Gallic Acid	C ₇ H ₆ O ₅	≥ 97.5	Sigma-Aldrich, Steinheim, Germany
Sodium Carbonate	Na ₂ CO ₃	≥ 99.5	Labkem, Barcelona, Spain
Total Phenolic Content (TPC) - microscale			
Folin-Ciocalteu's Reagent			Sigma-Aldrich, Steinheim, Germany
Gallic Acid	C ₇ H ₆ O ₅	≥ 97.5	Sigma-Aldrich, Steinheim, Germany
Sodium Carbonate	Na ₂ CO ₃	≥ 99.5	VWR Chemicals, Pennsylvania, USA
DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity			
DPPH	C ₁₈ H ₁₂ N ₅ O ₆		Sigma-Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	(CH ₃) ₂ SO	≥ 99.5	Sigma-Aldrich, Steinheim, Germany
Ferric Reducing Antioxidant Power (FRAP) Assay			
Iron (III) Chloride	FeCl ₃	≥ 97	Sigma-Aldrich, Steinheim, Germany
Iron (II) Sulfate	FeSO ₄ ·7H ₂ O	≥ 99	Sigma-Aldrich, Steinheim, Germany
TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine)			Sigma-Aldrich, Steinheim, Germany
Buffer Acetate			

Biological Activity		
Name	Supplier	
MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)	Sigma-Aldrich, Steinheim, Germany	
DMEM - high glucose	Sigma-Aldrich, Steinheim, Germany	
FBS (fetal bovine serum)	Sigma-Aldrich, Steinheim, Germany	
EDTA (ethylene diamine tetraacetic acid)	Sigma-Aldrich, Steinheim, Germany	
Antimicrobial Evaluation		
Trypticase Soy Broth	Merck KGaA, Darmstadt, Germany	
Tryptic Soy Broth	Merck KGaA, Darmstadt, Germany	
Leeming-Notman	Merck KGaA, Darmstadt, Germany	
Formulation		
Commercial Name	INCI Name	Supplier
Tego Care® PSC3	Polyglyceryl-3 Dicitrate/Stearate	Evonik, Essen, Germany
Tego Alkanol® 1618	Cetearyl Alcohol	Evonik, Essen, Germany
Liquid Paraffin		António M. S. Cruz, Lisbon, Portugal
CETIOL® V	Decyl Oleate	BASF, Rhein, Germany
Almond Oil		António M. S. Cruz, Lisbon, Portugal
Glycerin		António M. S. Cruz, Lisbon, Portugal
Nipagin®	Methylparaben	Fagron Iberica, Barcelona, Spain
Nipazol®	Propylparaben	Fagron Iberica, Barcelona, Spain
Sodium hydroxide		Merck KGaA, Darmstadt, Germany
Hydrochloric acid		Merck KGaA, Darmstadt, Germany

4.3. NADES screening

4.3.1. NADES preparation

NADES were prepared using a heating method where both compounds were weighted in an analytic scale Adventurer, (OHAUS, New Jersey, USA) and mixed in a vial at around 80 °C, with a heat plate MR Hei-Tec (Heidolph, Schwabach, Germany), until a homogeneous liquid was formed. Additionally, 25 to 50% (% v/v) of water was added after cooling so that viscosity issues are overcome. The chemicals used to synthesize all NADES are summarized in Table 4.

4.3.2. SLE extraction

Seaweed material was weighted and mixed with the correct amount of solvent in a closed vial for 120 min, at room temperature, at around 500 rpm in a multi stirrer Poly 15, Variomag, USA. The obtained liquid extract was collected with a syringe, centrifuged at 6000 rpm for 15 min in a IKA mini G centrifuge (IKA, Staufen, Germany), and the supernatant was collected.

Initially, the separation of the liquid and solid fractions was done by vacuum filtration but, to improve the separation, this step was replaced by the centrifugation and collection with a syringe.

After the initial NADES screening, all extractions were performed in triplicate.

4.3.3. Quantification of Total Phenolic Content (TPC) – macroscale

To evaluate each of the selected NADES regarding the extraction of phenolic compounds from the three seaweeds the Folin-Ciocalteu method, which assesses the Total Phenolic Content (TPC), was used.

The quantification of the TPC of each extract was determined according to the Folin-Ciocalteu procedure proposed by Singleton *et al.* [105], using gallic acid as standard. For the initial NADES screening this procedure was adapted for a final volume of 10 mL and afterwards it was adapted to microplate when assessing the biological activities of most promising extracts, in section 4.4.1- Antioxidant Activity.

Standard solutions of gallic acid of different concentrations were prepared and used to obtain the calibration curve. The concentrations used were: 0, 50, 100, 150, 200, 250, 300, 500, 1000 mg/L. The blank solution was pure water, the same as the 0 mg/L sample. The calibration curve used for this assay is represented in Figure 44, in Appendix A.

Initially, 0.1 mL of sample, blank or standard, 7.9 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent were added to a vial, mixed, and rested for 8 minutes. Next, 1.5 mL of 20% (w/v) sodium carbonate solution was added, and the mixture was stored, in the dark, for 1 hour at room temperature. At last, the absorbance was measured at 750 nm in a 10 mm cell with a spectrophotometer (UV-1800 Shimadzu, Kyoto, Japan).

During the initial NADES screening it was realized only one replicate for each NADES/seaweed combination aiming for an overall estimation of extraction efficiency and phenolic content of each extract.

4.3.4. Extracts optimization

Parametric tests aiming at finding the best fitting NADES parameters and extraction conditions for major phenolic extraction were carried out. Different experimental parameters influencing the extraction, namely NADES' component ratio (from 1:1 to 7:1), NADES' water content (from 25 to 50%, v/v), extraction

temperature (room temperature and 40 °C) and solid-liquid ratio, SLR, (1:5, 1:10 and 1:20 g/mL) were tested. Finally, UAE was also considered.

The referred factors were evaluated through successive tests, keeping the best fitting parameter of the previous test to the subsequent test. All parameters were evaluated with triplicates.

4.4. Evaluation of the biological activities

4.4.1. Antioxidant Activity

The antioxidant potential of the most promising seaweed extracts was evaluated by two different methods, namely: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power (FRAP). Additionally, the total phenolic content (TPC) of each sample was also quantified aiming to establish a relationship of this parameter with the antioxidant capacity.

I. Quantification of Total Phenolic Content (TPC) – microscale

Briefly, 2 µL of extract was added to 158 µL of distilled water and 10 µL of Folin–Ciocalteu reagent, vortexed, and then 30 µL of 20% Na₂CO₃ (w/v) were added. After one hour of reaction in the dark, the absorbance was measured at 750 nm (Epoch Microplate Reader, BioTek Instruments, Vermont, USA) against a blank solution. A calibration curve was used with standard solutions of concentrations between 0.01 and 1 mg of gallic acid per liter (mg GA/L). Results are expressed in milligrams of gallic acid equivalents per liter of extract, mg GAE/L.

Every sample was evaluated with triplicates.

II. DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Radical Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical method is based on the measurement of the scavenging activity of antioxidants towards the stable free radical of DPPH. This radical does not dimerize due to the delocalization of the spare electron over the molecule as a whole and producing a deep violet solution in ethanol with an absorption around 517 nm. In the presence of an antioxidant a hydrogen atom is donated, the radical is neutralized giving rise to the reduced form with the loss of the violet color. The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry [106].

The DPPH radical scavenging activity was performed according to Brand-Williams and co-workers adapted for microplate [107]. The reaction occurred in the dark with 2 µL of each sample and 198 µL of the DPPH solution (0.1 mM in ethanol). After 30 min incubation, the absorbance was measured at 517 nm (Epoch Microplate Reader, BioTek Instruments, Vermont, USA). Dimethyl sulfoxide (DMSO) was used as a standard. The scavenge potential was calculated in percentage of control. For the extracts that scavenged

the DPPH radical in more than 50%, IC₅₀ values (mg/mL) were determined, defining the concentration of sample extract that produces a 50% reduction of the DPPH radical absorbance.

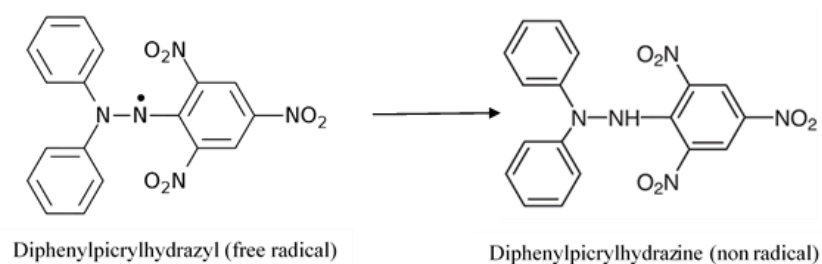


Figure 17: DPPH radical and its stable form. Adapted from [106].

The equation used to calculate the scavenging activity percentage is shown below:

Scavenging activity percentage:

$$AA\% = 100 - \left[\frac{(Abs_{sample} - Abs_{blank}) \times 100}{Abs_{control}} \right] \quad 4.1$$

where Abs_{sample} means absorbance of the sample extract, Abs_{blank} is the absorbance of the blank and Abs_{control} means absorbance of control.

III. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed accordingly with Benzie and Strain with slight modifications [108]. This method measures the ability of the antioxidants to reduce ferric-tripyridyl-triazine (Fe³⁺-TPTZ) complex to the blue colored ferrous form (Fe²⁺) which absorbs light at 593 nm. Briefly, standard or sample extract (10 µL) were mixed with FRAP reagent (300 mM acetate buffer, pH 3.6; 10 mM TPTZ solution (2,4,6-Tri(2-pyridyl)-s-triazine); 20 mM Iron (III) Chloride, FeCl₃, 10:1:1 (v/v/v)) and poured into microplate wells. The plate was incubated in the dark for 30 min and the absorbance readings were taken at 593 nm using an Epoch Microplate Reader (BioTek Instruments, Vermont, USA).

Iron (II) Sulfate, FeSO₄·7H₂O, was used as the control (0 – 10 µM) and the results are expressed in µM FeSO₄.

4.5. Evaluation of the biological activities on *in vitro* cellular models

4.5.1. Cell culture maintenance

HaCaT (300493) cells were acquired from the Cell Lines Services Germany (CLS) biobanks. The HaCaT cells were cultured in DMEM – high glucose medium supplemented with 10% FBS, and 1% EDTA. Cells were kept in a 95% moisture and 5% CO₂ atmosphere (Unitherm, Planegg, Germany), at 37 °C.

Subculture was performed according to biobank instructions whenever cultures reached 80–85% confluence.

4.5.2. Cytotoxicity evaluation

The cytotoxic activities of seaweed extracts were evaluated on HaCaT cells (4×10^4 cells/well) after seeding in 96-well plates (Thermo Fisher Scientific, Seoul, Korea) and incubated until they reached total confluence. Cells were then treated with the selected extracts (2 μ L) for 24 h. Untreated cells were used as control. The effects were estimated using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) colorimetric assay, as described by Mosmann [109]. A volume of 100 μ L of MTT solution was added to all wells, and the microplates incubated at 37°C for 1 h. The intracellular formazan crystals were then extracted and solubilized with DMSO, and the absorbance was measured at 570 nm using an Epoch Microplate Reader (BioTek Instruments, Vermont, USA). The results were expressed as percentage of control untreated cells.

4.5.1. Antimicrobial activity

Antimicrobial activity of the extracts was evaluated against three different microorganisms. Two Gram-positive bacteria, *Staphylococcus epidermidis* (DSM 1798) and *Cutibacterium acnes* (DSM 1897), and one fungus, *Malassezia furfur* (DSM 6170) were acquired from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ) biobank. Briefly, *S. epidermidis* was grown at 37 °C, on Trypticase Soy Broth, *C. acnes* at 37 °C, on Tryptic Soy Broth with anaerobic conditions media, and *M. furfur* at 30 °C on Leeming-Notman medium. The antimicrobial activity of each extract (2 μ L) was determined during the exponential growth, at 600 nm. Results were expressed as percentage of control.

4.5.2. Statistical analyses

Non-parametric analyses were performed using the Kruskal–Wallis test, followed by multiple comparisons using Dunn’s tests. Results are presented as the mean and standard error of the mean (SEM). All data analyses were done with GraphPad program (GraphPad PRISM 8.0.2 software, La Jolla, California, USA).

4.6. HPLC-DAD and LC-MS/MS Characterization of phenolic composition

Aliquots of 10 μ L of the extract SLFE (*S. muticum*, NADES - lactic acid:fructose, 7:1, 50% H₂O v/v, 1:3 g/mL) was analysed on a LC-MS system, constituted by an HPLC Dionex Ultimate 3000SD with a Dyode Array Detector coupled on line to a LCQFleet ion trap mass spectrometer, with an ESI source (Thermo Scientific, Massachusetts, USA). Chromatographic separations were achieved with a C18 Luna column 100 Å (150 x 4.6 mm, 5 μ m particular size, Phenomenex), using a flow rate of 0.3 mL/min, and at controlled temperature of 35 °C. The mobile phase was 0.1% of acid formic in water (v/v, eluent A) and acetonitrile

(eluent B), and the elution gradient was as follows: 0-2 min linear gradient 2% B; 4-18 min linear gradient to 50 % B, 18-22 linear gradient to 100 % B, 22-27 isocratic 100% B; 27-29 min linear gradient to 0% B, and then the column was re-equilibrated with 0% B for 7-min. The mass spectrometer was operated in the ESI positive and negative ion modes, with the following optimized parameters: ion spray voltage, ± 4.5 kV; capillary voltage, 16/-18 V; tube lens offset, -70/58 V; sheath gas (N₂), 40 arbitrary units; auxiliary gas (N₂), 20 arbitrary units; capillary temperature, 300 °C. MS² spectra were obtained by Collision Induced Dissociation (CID) experiments with an isolation window of 2 Da, a collision energy varying between 25 and 32%, and with an activation time of 30 ms. Spectra typically correspond to an average of 20–35 scans and were recorded in the range between 100-1000 Da. Acquisition and data processing Data acquisition and processing were performed using the Xcalibur 2.2 SP1.48 software (Thermo Scientific, Massachusetts, USA).

4.7. Topical formulation

4.7.1. Preparation of an O/W emulsion

The preparation of the O/W formulation was performed as described by Marques *et al.* [110]. The emulsion was prepared through the combination of different ingredients, accordingly with the formulation presented in Table 5.

Table 5: Percentage of ingredients in *Sargassum muticum* extract formulation.

Ingredients	Quantitative Composition (%, w/w)
Polyglyceryl-3 Dicitrate/Stearate (Tego Care® PSC3)	3.0
Cetearyl Alcohol (Tego Alkanol®1618)	7.0
Liquid Paraffin	2.5
Decyl Oleate (Cetiol® V)	4.5
Sweet Almond Oil	5.0
Glycerin	5.0
Purified Water	71.8
Methylparaben (Nipagin®)	0.18
Propylparaben (Nipasol®)	0.02
Sargassum Liquid Extract	1.0

The preparation of the emulsion involved the following steps:

- I. Adding the oily compounds (polyglyceryl-3 dicitrate/stearate, cetearyl alcohol, liquid paraffin, decyl oleate and almond oil) to a bowl and the non-oily compounds (glycerin and 70% of the purified water) to another bowl and place both bowls in a water bath at 70–80°C until total homogenization;
- II. Adding the propylparaben and methylparaben in the oily and non-oily bowls, respectively;
- III. Verting the oily ingredients into the non-oily ingredients bowl;
- IV. Taking the mix to Ultra-Turrax T 25 for 2 minutes;
- V. Manual stirring the mix until it is cold;
- VI. Adding the extract and then agitating the mix until it is homogeneous.

Along with the extract formulation a blank formulation was prepared to be used as control standard in the assays using the emulsion. As well, a formulation using the selected NADES, lactic acid:fructose, 7:1, 50% (v/v) water, was prepared.

To prepare all formulations the following equipment were used: a Thermostatic water bath 5 L by Nahita (Auxilab S.L., Navarre, Spain) and a IKA T 25 Ultra-Turrax (Staufen, Germany).

All the formulations were subjected to pH adjustments with the addition of Sodium hydroxide (40%) or Hydrochloric acid (10%) until a pH of 5 was achieved.

4.7.2. Physical characterization of the formulations

I. Appearance, pH, physical stability

The macroscopic appearance of each formulation was visually analyzed and used as first stability indicator. The pH of each formulation was measured with the pH-Meter, SevenEasy™ (Mettler Toledo, Greifensee, Switzerland), at room temperature. There were performed at least three measurements for each emulsion and the data was collected after stabilization of value on the equipment. In order to evaluate the physical stability of emulsions, 2 g of each formulation was subjected to three centrifugal cycles at 4000 rpm for 5 min in each cycle, using a Medifuge small benchtop centrifuge by Heraeus (Thermo Scientific, Massachusetts, USA). This assay was performed at 25°C, 5 days after preparation of the emulsions (t = 120 hours).

II. Optical microscopy

Each formulation was visualized by optical microscopy, using an Eclipse Ci microscope (Nikon, Tokyo, Japan). Images were acquired with the software ToupView (ToupTek, Zhejiang, China).

III. Droplet size analysis

Droplet size distribution was obtained by light scattering using a Malvern Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) combined with a Hydro S accessory. Simply, for a correct turbidity, about 0.5 g of formulation was added in the sample chamber containing 120-150 mL of water at 1750 rpm stirring, corresponding to an obscuration between 10% and 20%. The data was expressed in terms of relative volume distribution and given as diameter values corresponding to percentiles of 10%, 50%, and 90% (mean \pm SD; n = 5). Measurements were performed 8 days after preparation of the emulsions (t = 192 hours).

IV. Rheology analyses

Rheology analyses were performed using a controlled stress Kinexus Rheometer (Malvern Instruments, Worcestershire, UK).

Dynamic viscosity measurement

Dynamic viscosity was measured using a cone-and-plate geometry (truncated angle 4° and radius 40 nm), the analysis was carried out between 1 and 1000 Pa on a logarithmic increment, ranging from 0.1 to 100 s⁻¹. All measurements were performed 5 days after preparation of the emulsions (t = 120 hours), at 25°C.

Representative mathematical models (Bingham, Casson, Herschel-Bulkley) were fitted to viscosity measurements, and the best fitting was based on the correlation coefficient.

Oscillation frequency test

Oscillation frequency sweep tests were performed for all formulations, using a cone-and-plate geometry (truncated angle 4° and radius 40 nm), at frequencies ranging between 0.01 and 1 Hz. All measurements were performed at 25°C, at t = 72 hours.

5. Results and Discussion

5.1. NADES preparation

From the listed NADES approved by the European Commission, presented in Table 2, some were selected to be synthesized and tested in extractions of seaweeds. NADES synthesis is concluded when a homogeneous liquid is obtained at room temperature. To be mentioned that this was not achieved for every NADES, and in fact some combinations revealed preparation difficulties as it can be seen in Figure 18, samples D and E.

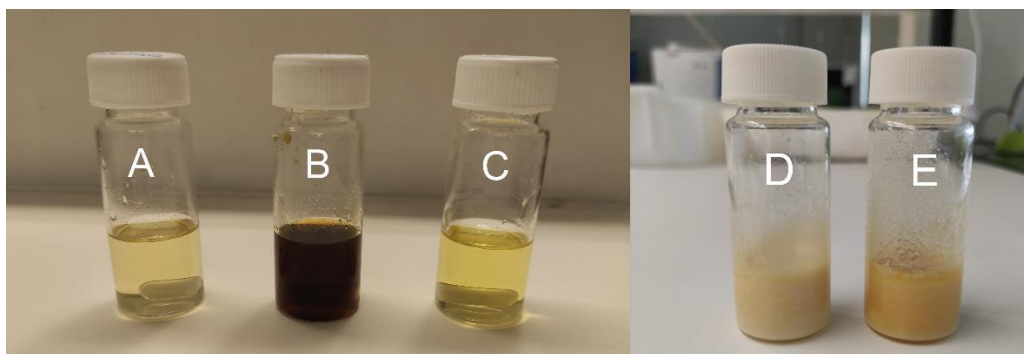


Figure 18: Example of NADES preparation. A - lactic acid:proline (1:1), B -glycerol:proline (1:1), C - lactic acid:fructose (5:1), D -malic acid:proline (1:1), E - malic acid:glucose (1:1); all NADES have 25% (v/v) water in their composition.

In total five NADES were selected to be tested in phenolic seaweed extractions. Four NADES were selected from previous projects concerning extraction of phenolics from seaweeds: lactic acid:fructose (5:1), lactic acid:glucose (5:1), lactic acid:sodium acetate (7:1), and glycerol:proline (1:1), tested in the molar ratios found in the literature to ensure feasibility [87, 95]. The NADES lactic acid:proline (1:1) was also tested due to the strong presence of both compounds in other NADES from literature, and since lactic acid is an HBD and Proline an HBA, this combination should be employed [111]. Initially, all NADES were tested in the concentrations reported originally and afterwards the composition effect on phenolic extraction was assayed.

At first, a water content of 25% (v/v) was also employed, to enable NADES use as extractants, by lowering their viscosity and increasing solubility. This factor was also assessed in NADES optimization section.

5.2. NADES screening

In order to evaluate the extraction potential of NADES, different extractions were carried out with each of the three seaweeds: *C. tomentosum*, *G. corneum*, and *S. muticum*. Additionally, conventional solvent water/ethanol (70:30, v/v) was used for comparison purposes. Results of extract concentration (mg GAE/L) and TPC (mg GAE/g DW) were obtained with the Quantification of Total Phenolic Content (TPC) – macroscale, and are presented below, Table 6 to 8.

Equation 5.1 was used to convert extract phenolic concentration in phenolic extraction yield from raw seaweed.

$$TPC (mgGA EQ/g DW) = \frac{Conc. (mgGA EQ/L)}{SLR (g DW/ml) \cdot 1000} \quad 5.1$$

Table 6: NADES screening TPC results using *Codium tomentosum*.

Solvent	Molar Ratio	SLR (g/ml)	TPC (mg GAE/g DW)	Conc. (mg GAE/L)
HBD : HBA (for NADES)				
H ₂ O/EtOH	-----	1:20	1.18	59.1
H ₂ O/EtOH (Overnight)	-----	1:20	1.18	59.0
H ₂ O/EtOH	-----	1:5	0.638	127.5
lactic acid:fructose	5:1	1:5	0.679	135.9
lactic acid:glucose	5:1	1:5	0.451	90.2
lactic acid:sodium acetate	7:1	1:5	0.674	134.7
lactic acid:proline	1:1	1:5	0.726	145.1
glycerol:proline	1:1	1:5	0.164	32.8

Table 7: NADES screening TPC results using *Gelidium corneum*.

Solvent	Molar Ratio	SLR (g/ml)	TPC (mg GAE/g DW)	Conc. (mg GAE/L)
HBD : HBA (for NADES)				
H ₂ O/EtOH	-----	1:20	1.30	65.1
H ₂ O/EtOH (Overnight)	-----	1:20	1.35	67.4
H ₂ O/EtOH	-----	1:5	0.84	168.8
lactic acid:fructose	5:1	1:5	1.91	383.7
lactic acid:glucose	5:1	1:5	1.50	299.0
lactic acid:sodium acetate	7:1	1:5	1.27	253.0
lactic acid:proline	1:1	1:5	0.30	57.3
glycerol:proline	1:1	1:5	--- *	--- *

* Inconclusive results due to interference

Table 8: NADES screening TPC results using *Sargassum muticum*.

Solvent	Molar Ratio	SLR (g/ml)	TPC (mg GAE/g DW)	Conc. (mg GAE/L)
HBD : HBA (for NADES)				
H ₂ O/EtOH	-----	1:20	2.61	130.4
H ₂ O/EtOH (Overnight)	-----	1:20	2.54	127.2
H ₂ O/EtOH	-----	1:5	2.23	446.1
lactic acid:fructose	5:1	1:5	7.33	1465.9
lactic acid:glucose	5:1	1:5	5.77	1153.2
lactic acid:sodium acetate	7:1	1:5	5.97	1193.1
lactic acid:proline	1:1	1:5	2.65	528.9
glycerol:proline	1:1	1:5	2.12	424.6

Overnight extractions with conventional solvents, water/ethanol (70:30, v/v), resulted in similar phenolic extractions when compared with the equivalent two-hour assay (59.1 and 59.0 mg GAE/L, for *C. tomentosum*; 65.1 and 67.4 mg GAE/L for *G. corneum* and 130 and 127 mg GAE/L for *S. muticum*) indicating that two hours are appropriate for these extractions.

In general, conventional extractions using 1:20 g/mL SLR, resulted in higher TPC than the 1:5 g/mL SLR extraction for the 3 seaweeds used: 1.18 and 0.638 mg GAE/g DW, for *C. tomentosum*; 1.34 and 0.84 mg

GAE/g DW for *G. corneum* and 2.61 and 2.23 mg GAE/g DW for *S. muticum*. Traditional seaweed extractions usually employ high quantities of organic solvents (1:10, 1:20, 1:30 or as high as 1:100 g/mL) [25, 55, 112]. However, these extractions are commonly followed by solvent removal processes, such as evaporation, while one of the objectives of this work was the incorporation of NADES solvents in the final formulation. The lack of the solvent removal step favors the overall process economically and energetically but, on the other hand, it turns the overall extract phenolic concentration into the key factor for selection NADES.

From Tables 6 to 8, it can be seen that the most efficient extractions were carried out using lactic acid:sodium acetate (7:1) with 134.7 mg GAE/L, lactic acid:fructose (5:1) with 135.9 mg GAE/L, and lactic acid:proline (1:1) resulting in 145.1 mg GAE/L for *C. tomentosum*; for *G. corneum* lactic acid:glucose (5:1) and lactic acid:fructose (5:1) stood out with 299.0 and 383.7 mg GAE/L, respectively; while with *S. muticum* lactic acid:fructose (5:1) was the best performing NADES with 1465.9 mg GAE/L. Generally, and despite the difference in the phenolic compounds present in the 3 seaweeds under study, it can be concluded that the presence of lactic acid favors the extraction of phenolic compounds.

In order to avoid the effect of interfering compounds in the determination of phenolic content, the absorbance of every sample was read against a blank sample containing the NADES used for that extraction [113, 114]. However, extraction with glycerol:proline (1:1) for *G. corneum* resulted in faulty results due to possible interferences.

5.3. NADES optimization

The initial screening of the selected NADES enabled to start an optimization process aiming for the most suited NADES for each of the two most promising seaweeds: *Sargassum muticum* and *Gelidium Corneum*. The extraction efficiency of a SLE is typically affected by several experimental conditions. In this work, the following conditions were tested: water content, mole ratio between NADES' components and the conditions and extraction method. Hence, the optimization of the extraction process focused on these factors. Also, to be mentioned that for *S. muticum*, the performance of UAE was also evaluated

The optimization process started with the following extraction conditions: extraction time: 2 hours, SLR of 1:5 g/mL, water content 25 % (v/v) and room temperature. Firstly, the influence of the ratio between NADES's components was studied. NADES were synthesized with different molar ratios: 1:1, 3:1, 5:1 and 7:1.

The second factor optimized was the water content. Initially, NADES with 25% (v/v) of water were used to screen the different NADES performance and pre-select the best NADES. Extractions were then carried out using NADES with 35% and 50% (v/v) water, in order to find the most suitable water concentration for each one of the studied seaweeds.

It could be important to evaluate extraction efficiency based on the solid-liquid ratio that influences the solvent availability. This ratio is represented by grams of seaweed by milliliter of NADES (g/mL) and the studied ratios were 1:5, 1:10 and 1:20 g/mL. Although, since the solvent used for extraction will not be separated from extract, the most concentrated extracts, those with the higher SLR, will be favored even if

TPC values are lower, regarding the initial mass of seaweed. Extraction data of this parameter is presented in Table 20 and 21 of Appendix B.

The temperature of the extraction tested at room temperature, and also at 40 °C, for both seaweeds, since above this temperature the thermal degradation of some phenolic compounds might occur.

These experimental conditions were tested sequentially, and, after each test, the most suitable condition was maintained throughout the following tests.

5.3.1. Optimization of *Gelidium corneum* extraction

For the phenolic extraction of *G. corneum*, extractions employing lactic acid:fructose, lactic acid:glucose and lactic acid:sodium acetate were carried with different molar ratios as represented in Figure 19.

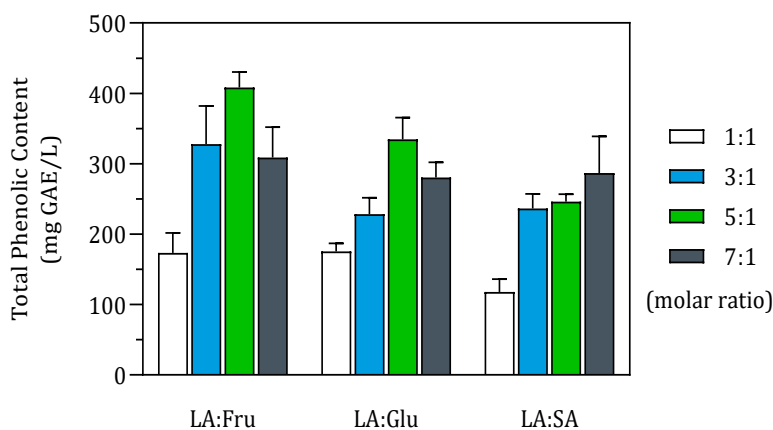


Figure 19: Effect of NADES component ratio on *Gelidium corneum* extractions. LA:Fru – lactic acid:fructose; LA:Glu – lactic acid:glucose; LA:SA – lactic acid:sodium acetate. All NADES were tested with 25% (v/v) water. Results are the mean \pm SD of three independent experiments.

Changing the molar ratio of the NADES resulted in higher phenolic extraction for lactic acid:fructose (408.4 mg GAE/L) and lactic acid:glucose (334.8 mg GAE/L), respectively, both with 5:1 molar ratio, while lactic acid:sodium acetate showed lower extraction performances and the best suited ratio was that with the higher content of lactic acid, 7:1 (286.6 mg GAE/L). The water content in the best suited NADES lactic acid:fructose (5:1) and lactic acid:glucose (5:1) was evaluated using 25%, 35% and 50% (v/v). Results regarding the effect of water content on *G. corneum* extractions are presented in Figure 20.

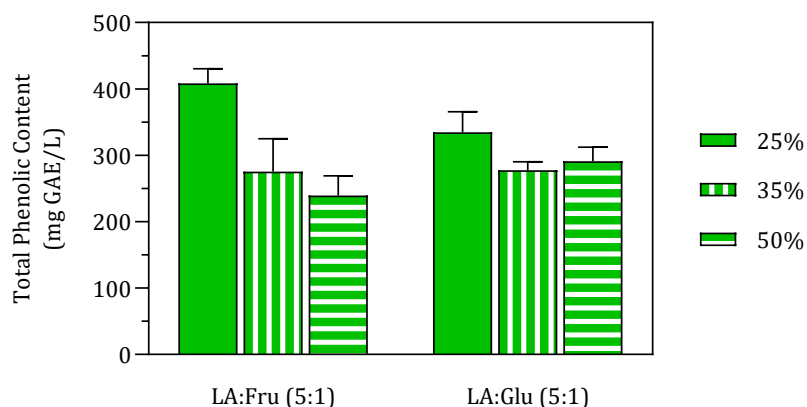


Figure 20: Effect of water content on *Gelidium corneum* extractions. LA:Fru (5:1) – lactic acid:fructose with 5:1 molar ratio; LA:Glu – lactic acid:glucose with 5:1 molar ratio. Results are the mean \pm SD of three independent experiments.

Both NADES showed better phenolic extraction performance with only 25% (v/v) water, lactic acid:fructose (5:1) 408.4 mg GAE/L and lactic acid:glucose (5:1) 334.8 mg GAE/L, than with higher water contents. Water is often added as a third component of NADES, in order to decrease viscosity and to promote the solubilization of solid organic compounds [7], allowing to use DES inside the solid-liquid equilibrium region.

At last, the temperature effect was evaluated and extractions at room temperature and 40 °C were carried out - Figure 21 - seeking for high phenolic extractions due to reduced viscosity and surface tension of the solvents [92].

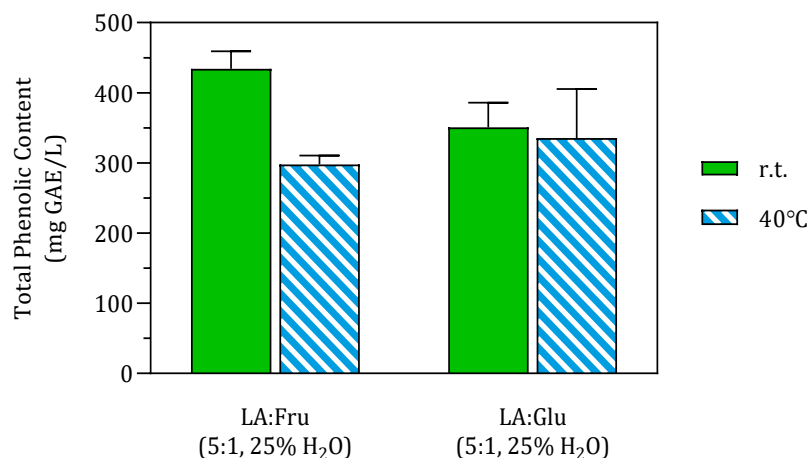


Figure 21: Effect of temperature on *Gelidium corneum* extractions. Results are the mean \pm SD of three independent experiments.

Yet, temperatures higher than room temperature did not improve the extraction efficiency of TPC. In fact, at room temperature extractions TPC results were the highest, lactic acid:fructose (5:1) 408.4 mg GAE/L and lactic acid:glucose (5:1) 334.8 mg GAE/L, both with 25% water content (v/v), for *G. corneum* phenolics extraction.

5.3.2. Optimization of *Sargassum muticum* extraction

The use of NADES for the extraction of phenolic compounds from *S. muticum* was also optimized according to the experimental conditions described above. The results regarding the effect of NADES composition in the TPC extraction efficiency are presented in Figure 22.

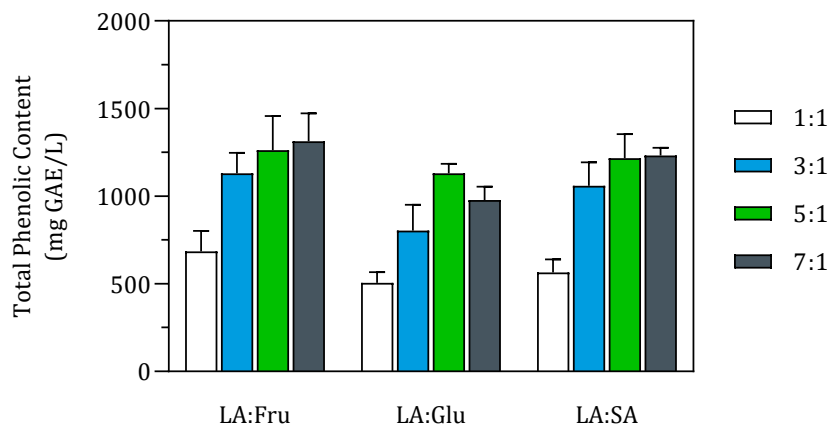


Figure 22: Effect of NADES composition on *Sargassum muticum* extractions. LA:Fru – lactic acid:fructose; LA:Glu – lactic acid:glucose; LA:SA – lactic acid:sodium acetate. All NADES were tested with 25% (v/v) water. Results are the mean \pm SD of three independent experiments.

It can be observed that high proportions of lactic acid also favored phenolic compounds extraction, more specifically, 1314.3 mg GAE/L using lactic acid:fructose (7:1), 1130.6 mg GAE/L for lactic acid:glucose (5:1), while lactic acid:sodium acetate (5:1) and (7:1) showed similar results, 1217.3 and 1231.9 mg GAE/L, respectively. Consequently, lactic acid:fructose (7:1) and lactic acid:sodium acetate (7:1) were selected for the study regarding the effect of the water content on the TPC extraction. The results can be found in Figure 23.

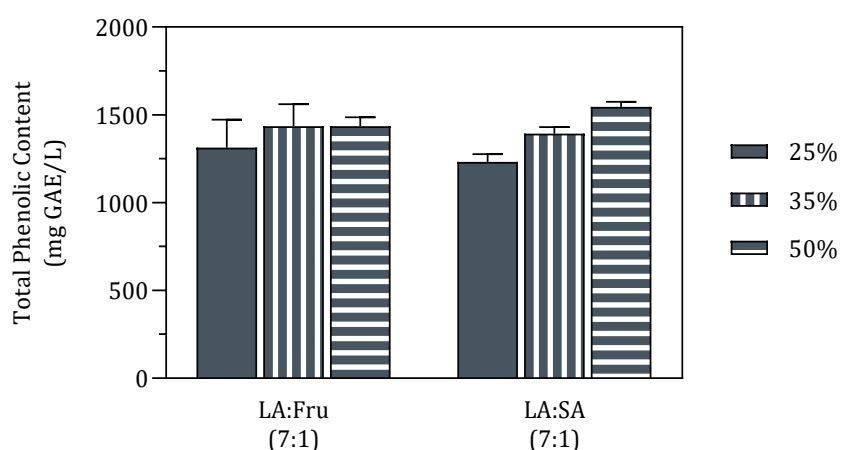


Figure 23: Effect of water content on *Sargassum muticum* extractions. LA:Fru (7:1) – lactic acid:fructose with 7:1 molar ratio; LA:Glu (7:1) – lactic acid:glucose with 7:1 molar ratio. Results are the mean \pm SD of three independent experiments.

Contrary to the results obtained for TPC extraction from *G. corneum*, in this case higher water content revealed higher phenolic extraction for both NADES used, with lactic acid:sodium acetate (7:1) extracting 1546 mg GAE/L for 50% (v/v) of water, while for lactic acid:fructose (7:1) similar values were obtained for NADES containing 35% and 50% (v/v) water, 1435 and 1436 mg GAE/L, respectively. This can suggest that the compounds extracted from *S. muticum* have different polarities than those extracted from *G. corneum*. Consequently, NADES with 50% (v/v) water content was selected to pursue the optimization studies since it brings economic and environmental benefits to the process.

Temperature effect on the extraction of TPC from *S. muticum* was also assessed, and the results are shown in Figure 24. The results from the use lactic acid:fructose (7:1) with 50% (v/v) of water with UAE are also depicted.

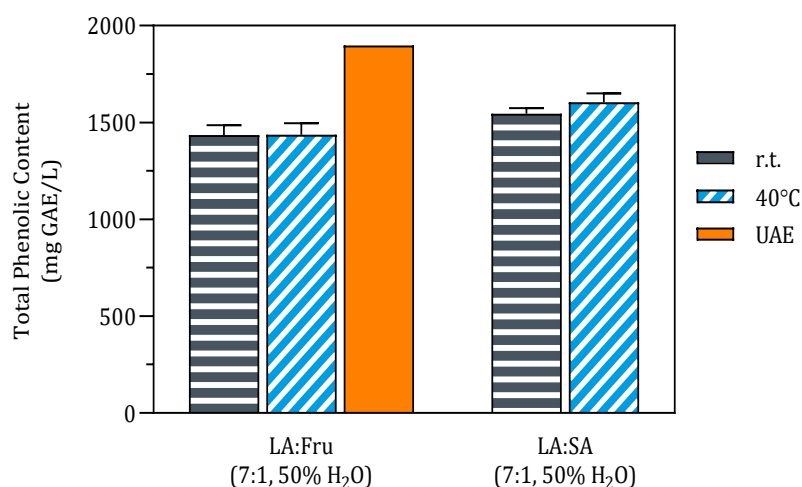


Figure 24: Effect of temperature on *Sargassum muticum* extractions. Results are the mean \pm SD of three independent experiments, except for UAE.

In agreement with the results obtained for *Gelidium corneum*, the operating temperature of 40 °C did not improve TPC of the extracts. Interestingly, UAE resulted in high TPC, 1898 mg GAE/L, suggesting that combining the use of NADES with other novel technologies for phenolic extraction could be useful.

When analyzing the results of NADES screening, Table 6 - 8, together with the results from NADES optimization it is possible to understand that the seaweed *S. muticum* has a higher phenolic content, probably due to a strong presence of phlorotannins in its composition, which are mainly found in brown seaweeds [39]. The most concentrated extracts from this seaweed were lactic acid:sodium acetate (7:1, 50% H₂O v/v) extracting 1546 mg GAE/L and 1436 mg GAE/L for lactic acid:fructose (7:1, 50% H₂O v/v).

An additional *S. muticum* extract was produced using a 1:3 g/mL SLR. The objective here was to maximize phenolic concentration without risking the extraction procedure, especially the separation of seaweed and extract. This extract resulted in a phenolic concentration of 2099 mg GAE/L, being the most concentrated extract, which will be, therefore, considered for the next steps of this work.

From all the extracts produced for *G. corneum*, the most concentrated were lactic acid:fructose (5:1) 408.4 mg GAE/L and lactic acid:glucose (5:1) 334.8 mg GAE/L, both with 25% water content (v/v), for *G.*

corneum phenolics extraction. In this seaweed the increase in water content did not improve extraction, suggesting that phenolic compounds here extracted are less polar.

To simplify results presentation and understanding, the most promising extracts and NADES selected for further work are listed in Tables 9 and 10, with the respective codes. The NADES used in these extracts will also be evaluated and, therefore, are listed in Table 10. These will be evaluated regarding their biological activities: antioxidant, antimicrobial and cytotoxicity in the following sections.

Table 9: Sample codes for extracts and NADES carried over to biological assays.

Sample Code	Seaweed	NADES		Molar Ratio (n ₁ :n ₂)	Water content (%, v/v)	SLR (g/mL)
		Compound 1	Compound 2			
CLF	<i>C. tomentosum</i>	Lactic Acid	Fructose	5:1	25	1:5
CLS			S. Acetate	7:1	25	
GLF	<i>G. corneum</i>	Lactic Acid	Fructose	7:1	25	
GLG			Glucose	1:1	25	
SLF3*					50	1:3
SLF5					50	1:5
SLF20			Fructose	7:1	50	1:20
SLFHT (40°C)	<i>S. muticum</i>	Lactic Acid			50	1:5
SLFUS (UAE)						50
SLS5					50	1:5
SLS20			S. Acetate	7:1	50	1:20

* Sample with the highest phenolic content

Table 10: Codes of the NADES used in the selected extracts for biological evaluation.

NADES code	Compound 1	Compound 2	Molar ratio (n ₁ :n ₂)	Water content (%, v/v)
LA:Fru (7:1)	Lactic Acid	Fructose	7:1	50
LA:Fru (5:1)			5:1	25
LA:SA (7:1)		Sodium Acetate	7:1	50
LA:Glu (5:1)		Glucose	5:1	25

5.4. Evaluation of the biological activities

A total of eleven extracts and four NADES were selected according to the previous section, NADES optimization. Several bioactivities related to cosmetic/cosmeceutical applications were analyzed to understand the potential of these extracts in this field, namely their antioxidant and antimicrobial activities, as well as their cytotoxic profile. In the following result figures, samples are grouped by seaweed and NADES used where the first column of each group (in bold) represents the results of that assay for the isolated NADES of that group, this is, without seaweed extract.

5.4.1. Antioxidant activity

In order to describe the antioxidant potential of each extract, two assays were accomplished including the evaluation of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity and the ferric reducing antioxidant power (FRAP). Additionally, the total phenolic content (TPC) was assessed allowing to establish a relationship between the antioxidant potential and phenolic content of each sample.

I. Quantification of Total Phenolic Content (TPC)

The TPC of each extract was evaluated in all samples and results are presented in Figure 25.

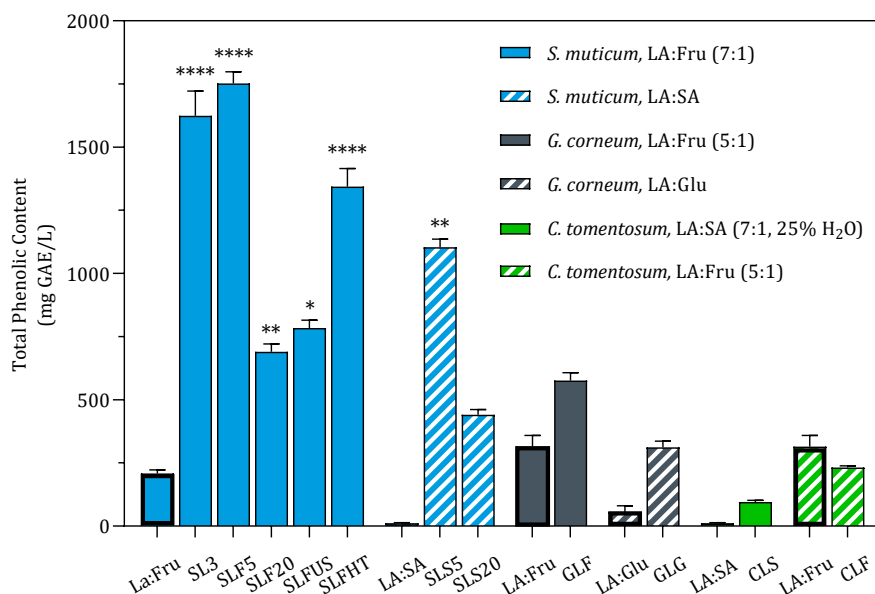


Figure 25: Total Phenolic Content of seaweed extracts and corresponding NADES. The values correspond to mean \pm SEM of three independent experiments. Symbols represent significant differences (Kruskal–Wallis test, Dunn’s test; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$) when compared to the solvent used in each group (*).

Results show that each extract has a higher antioxidant potential than the respective NADES used. The most promising extracts were SLF3 and SLF5, with 1620 ± 10 and 1750 ± 5 mg GAE/L, respectively (significantly different from the other extracts, $p < 0.0001$ or less). The extract SLFHT also resulted in promising antioxidant activity with 1340 ± 7 mg GAE/L, also with significant differences, $p < 0.0001$. The three extracts with higher activities were all obtained from the combination of *S. muticum* and the NADES

lactic acid:fructose (7:1, 50% H₂O v/v). The resulting extract from the NADES lactic acid:sodium acetate (7:1, 50% H₂O v/v) and *S. muticum* also revealed significant differences ($p < 0.01$). However, for *G. corneum* and *C. tomentosum* extracts no significant differences were found between extracts and NADES.

II. DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Radical Scavenging Activity

Regarding the DPPH radical scavenging ability, a concentration dependency was verified, with higher reduction of DPPH with more concentrated extracts (extracts with higher SLR). In general, almost all extracts showed over 20% radical scavenging activity, Figure 26. In contrast, all NADES showed no reduction of the DPPH radical, over 10%. Extracts of *S. muticum* and lactic acid:fructose (7:1, 50% H₂O v/v) resulted in higher reduction activities, three of which with strong scavenging activities over 60%, SFL3, SLF5 and SLFUS, significantly different from the control, $p < 0.0001$ or less. The extracts containing high levels of total phenolic content, SLF3 and SLF5, were also potent DPPH radical scavengers, suggesting that the extracted polyphenols may be the principal constituents responsible for antiradical properties of these extracts. Extracts of *G. corneum* and *C. tomentosum* did not show significant differences from the control regarding the DPPH radical scavenging activity.

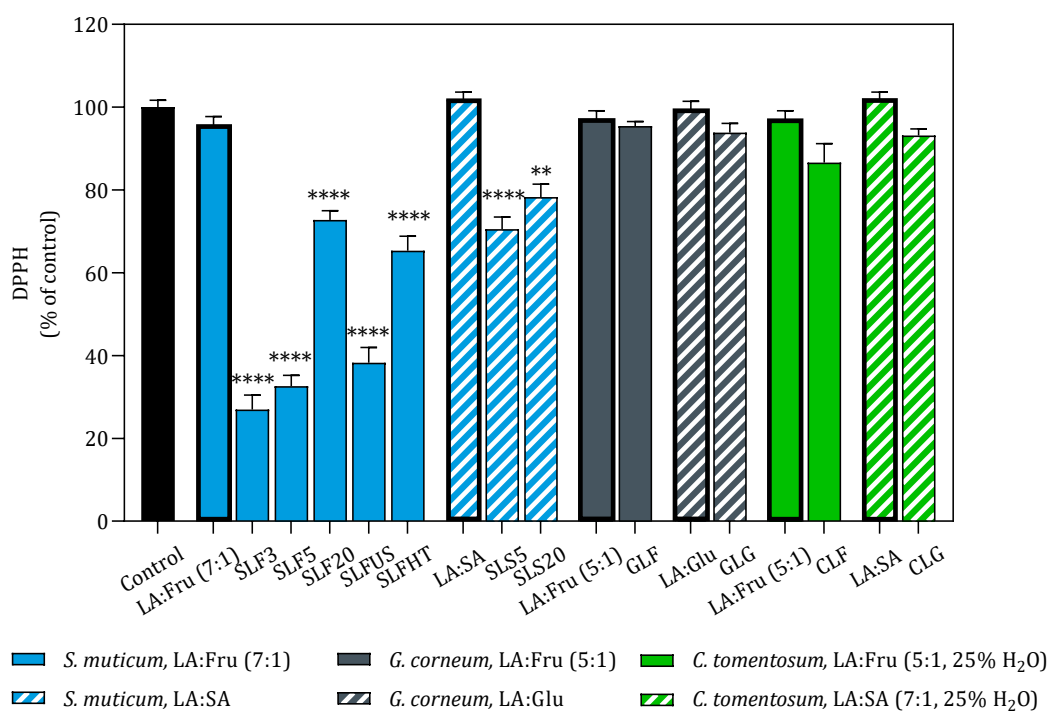


Figure 26: DPPH reduction results. The results are the mean \pm SEM of three independent experiments. The values correspond to mean \pm SEM of three independent experiments. Symbols represent significant differences (Kruskal-Wallis test, Dunn's test; *** $p < 0.001$, **** $p < 0.0001$) when compared to the control (*).

The concentration of extracts required to scavenge 50% of DPPH radicals, called effective concentration (EC₅₀) was also calculated for the most reducing extracts, SFL3, SFL5 and SFLFUS, Figure 27.

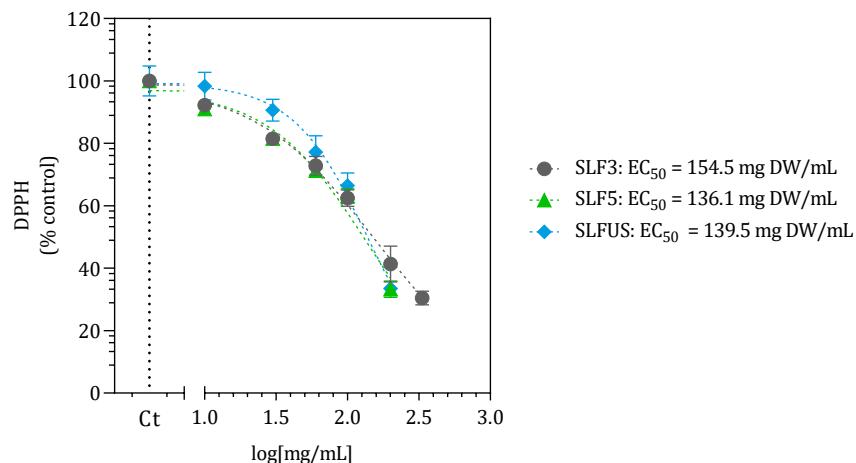


Figure 27: DPPH reduction results. Concentration dependence (330 - 10 mg DW/mL). EC₅₀ of 2,2-diphenyl-1-picrylhydrazyl (DDPH) radical scavenging activity is expressed as mg of *Sargassum muticum* per milliliter of NADES (mg DW/mL). Values are expressed as means with 95% confidence intervals.

III. Ferric Reducing Antioxidant Power (FRAP) Assay

Results of the FRAP assay are presented in Figure 28, where the first column of each group represents the result for the NADES used in that group of extracts. Similarly, highest antioxidant activity estimated by FRAP assay was also shown by *S. muticum* extracts, specially SLF3 ($29.9 \pm 1.4 \mu\text{M FeSO}_4$), SLFUS ($28.8 \pm 1.7 \mu\text{M FeSO}_4$), SLFHT ($24.1 \pm 1.1 \mu\text{M FeSO}_4$) and SLS5 ($19.7 \pm 1.4 \mu\text{M FeSO}_4$), significantly different from the respective NADES, $p < 0.0001$ or less. As observed in the other antioxidant assays, extracts of *G. corneum* and *C. tomentosum* did not show promising results.

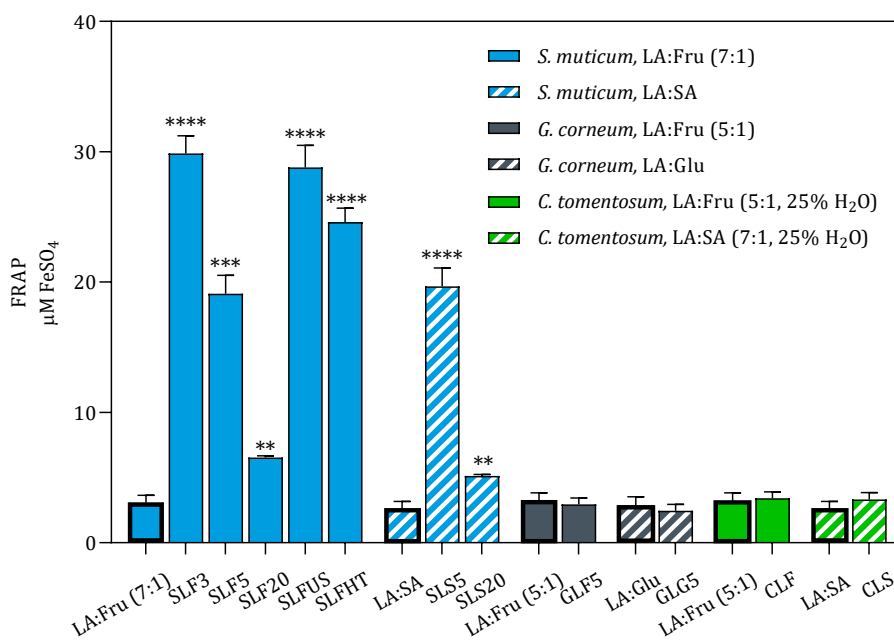


Figure 28: FRAP assay results. The values correspond to mean \pm SEM of three independent experiments. Symbols represent significant differences (Kruskal-Wallis test, Dunn's test; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) when compared to the solvent used in each group (*).

Combining the phenolic content assay and both antioxidant assays it is possible to conclude that *S. muticum* extracts revealed higher TPC as well as antioxidant activities, probably due to the presence of phlorotannins, exclusively found in brown seaweeds [23]. From these, the extracts SLF3 and SLF5 arouse higher interest, since they were synthesized with a bigger SLR, 1:3 and 1:5 grams of *S. muticum* per milliliter of NADES lactic acid:fructose (7:1, 50% H₂O), resulting in more concentrated extracts with higher TPC and better antioxidant activities.

5.5. Evaluation of the biological activities on *in vitro* cellular models

5.5.1. Cytotoxicity evaluation

The cytotoxic activity of seaweed samples and NADES (first column of each group) were evaluated on HaCaT cells, and the results are presented in Figure 29. Cells' viability was significantly ($p < 0.0001$) affected by all NADES, except lactic acid:glucose (5:1) only reducing cells' viability to about 85%. Nevertheless, some extracts were able to maintain cells' viability at around 50-70% (SLF3, SLF5, SLFHT, SLS5 and SLS20) being considered safe for application. Extracts of *G. corneum* and *C. tomentosum*, in general, presented cytotoxicity, significantly reducing cells' viability by about 80%. From these two seaweed extracts, GLF was the less cytotoxic extract but still presented significant differences from the control ($p < 0.01$).

The cytotoxicity evaluation was performed after a pH adjustment with NaOH (40 % w/v) to increase the pH values of the extracts to around 5 - 6. Since all the used NADES have a high content of lactic acid, the original pH levels of the extracts were extremely low, around 2, affecting the cytotoxicity evaluation.

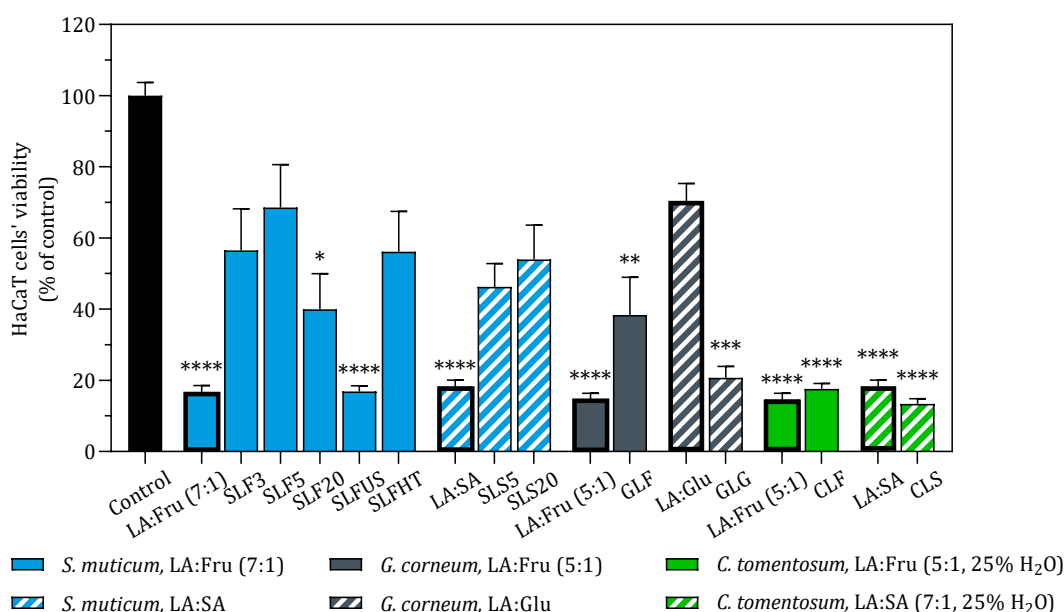


Figure 29: Cytotoxic potential of seaweed extracts on HaCaT cells. Cells' viability was evaluated after 24 h of exposure to 2 μ L of extracts and the results are expressed as % of the control. The values correspond to mean \pm SEM of three independent experiments. Symbols represent significant differences (Kruskal-Wallis test, Dunn's test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) when compared to the control (*). All samples were adjusted for pH levels of 5 to 7 with sodium hydroxide (NaOH 40% w/v).

5.5.2. Antimicrobial activity

The antimicrobial activity of seaweed samples was evaluated against two Gram (+) bacteria, *S. epidermidis* and *C. acnes*, and one fungus, *M. furfur*. Results are presented in Figures 30 to 32.

Due to the high acidity of the NADES used, pH values around 2, microorganisms' growth was very affected when extracts were tested without any pH adjustment. In Appendix C are presented the results of antimicrobial activity of seaweed extracts and NADES with their original pH. Since topical formulations are employed with pH levels between 5 and 7, seaweed extracts were adjusted with NaOH (40 % w/v) until these values were reached [115].

The fungus *M. furfur*, as other *Malassezia* species, habit the human skin as commensals, as they are harmless and benefit the skin in healthy conditions, but in adverse conditions can be associated with multiple skin disorders, such as pityriasis versicolor, folliculitis, dandruff, atopic dermatitis, and psoriasis [116, 117]. The results of the antimicrobial assay against this fungus are presented in Figure 30. None of the samples showed a strong inhibition of *M. furfur* growth, in fact, almost all extracts and NADES maintained or stimulated the growth of the microorganisms. The most affecting sample was the NADES lactic acid:sodium acetate (7:1), reducing microorganisms' growth around 50%. No significant differences were found between all samples and the control, $p < 0.05$ or less.

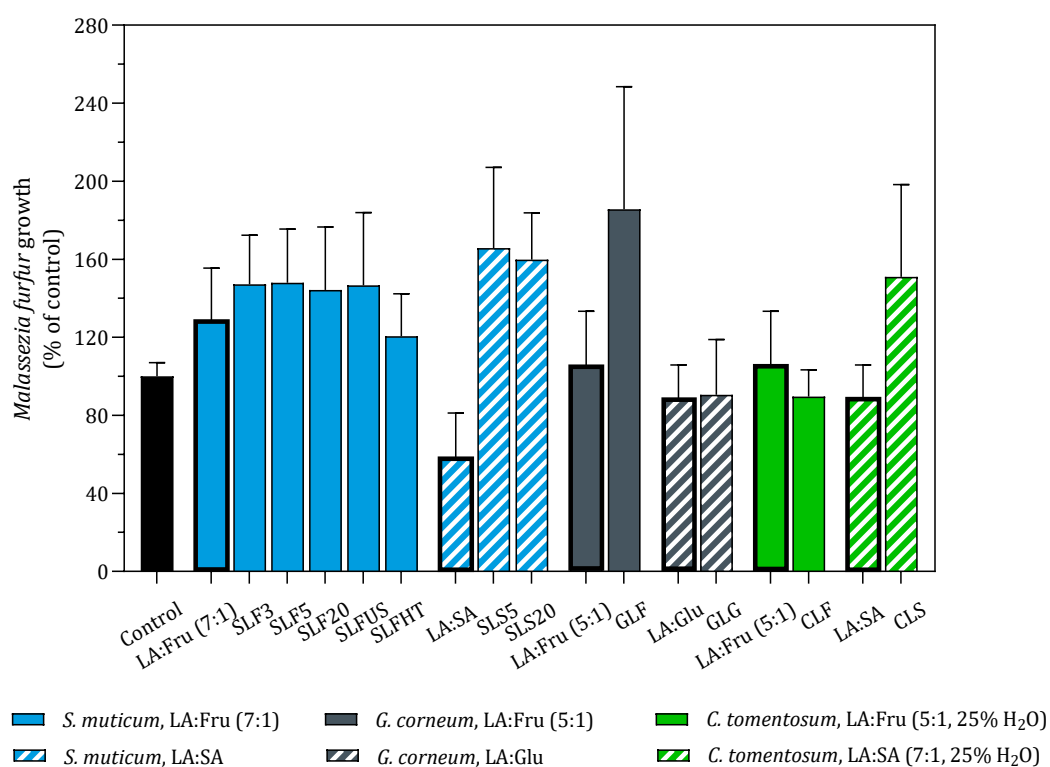


Figure 30: Antimicrobial activity of seaweed extracts (2 μ L) against the fungus *Malassezia furfur*. The values correspond to mean \pm SEM of three independent experiments. No significant differences were found (Kruskal-Wallis test, Dunn's test; * $p < 0.05$) when compared to the control (*). All samples were adjusted for pH levels of 5 to 7 with sodium hydroxide (NaOH 40% w/v).

The Gram positive bacteria, *S. epidermidis* is a facultative anaerobic bacteria and, despite being harmless for healthy people, it may constitute a threat to patients with a compromised immunological system, as it is seen as an opportunistic pathogen [116, 118]. Results regarding antimicrobial activity against this bacterium are presented in Figure 31. The NADES lactic acid:sodium acetate (7:1) and lactic acid:fructose (5:1, 25% H₂O) showed statistically significant results, $p < 0.0001$, reducing microorganisms' growth to around 66% and 43%, respectively. Regarding seaweed extracts, only the *C. tomentosum* extract CLF showed a statistically significant effect, reducing microorganisms' growth to 81%. Excepting for CLF extract, all extracts were able to maintain microorganisms' growth without significant differences.

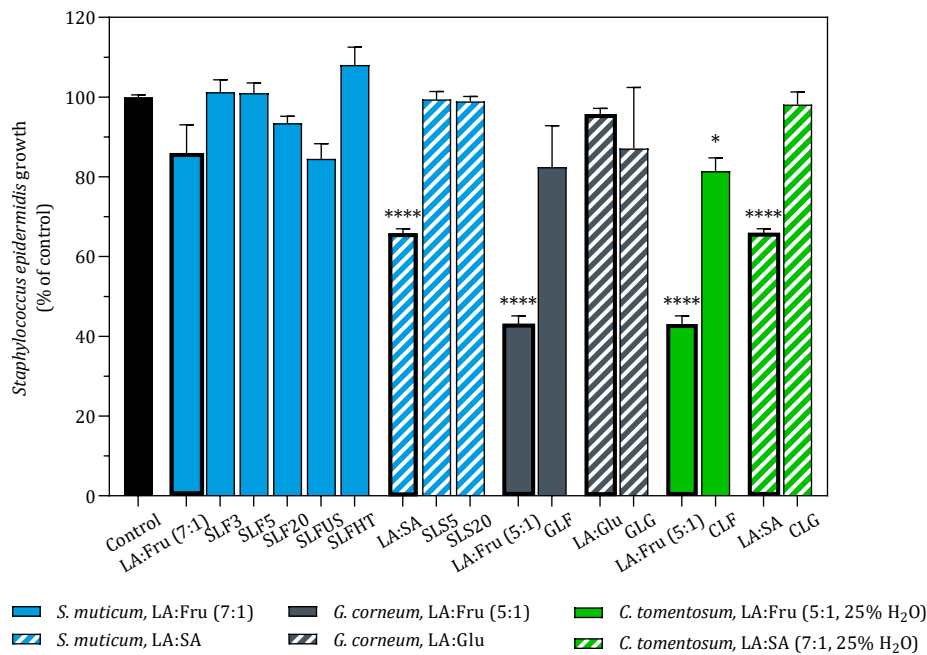


Figure 31: Antimicrobial activity of seaweed extracts (2 µL) against the bacteria *Staphylococcus epidermidis*. The values correspond to mean ± SEM of three independent experiments. No significant differences were found (Kruskal–Wallis test, Dunn's test; * $p < 0.05$, **** $p < 0.0001$) when compared to the control (*). All samples were adjusted for pH levels of 5 to 7 with sodium hydroxide (NaOH 40% w/v).

C. acnes is mainly found within follicles and pores, although it also lives in the surface of healthy skin. It uses skin sebum and cellular metabolic by-products as nutrients. In the past, over-colonization of *C. acnes* have been identified as one of the main triggers of acne. However, not all *C. acnes* strains are responsible for the acne lesion, in fact, acne might be triggered by the selection of a subset of *C. acnes* strains, as the acne-associated phylotype IA1 [116]. In healthy skin *S. epidermidis* controls the proliferation of *C. acnes*. Nevertheless, an unbalanced equilibrium between these bacteria in pilosebaceous units, favoring the phylotype IA1 acnes strains, may not allow *S. epidermidis* to fully play its role as a regulator of the natural skin homeostasis in limiting the growth of *C. acnes*, causing acne inflammatory lesions [118].

The results of the antimicrobial assay regarding *C. acnes* are presented in Figure 32. In this assay, three of the four NADES significantly affected the growth of *C. acnes*, lactic acid:fructose (7:1, 50% H₂O) and lactic acid:sodium acetate (7:1) reduced microorganisms' growth by around 50% ($p < 0.01$) and lactic acid:fructose (5:1, 25% H₂O) resulted in a more severe effect reducing *C. acnes* growth to around 20% ($p < 0.0001$), while lactic acid:glucose (5:1) did not affect microorganisms' growth. The overall use of seaweed

extracts did not affect microorganisms' growth, only the *S. muticum* extracts obtained with UAE and high temperature extraction resulted in bacteria growth lower than 80% (SLFUS – 74% and SLFHT – 47%).

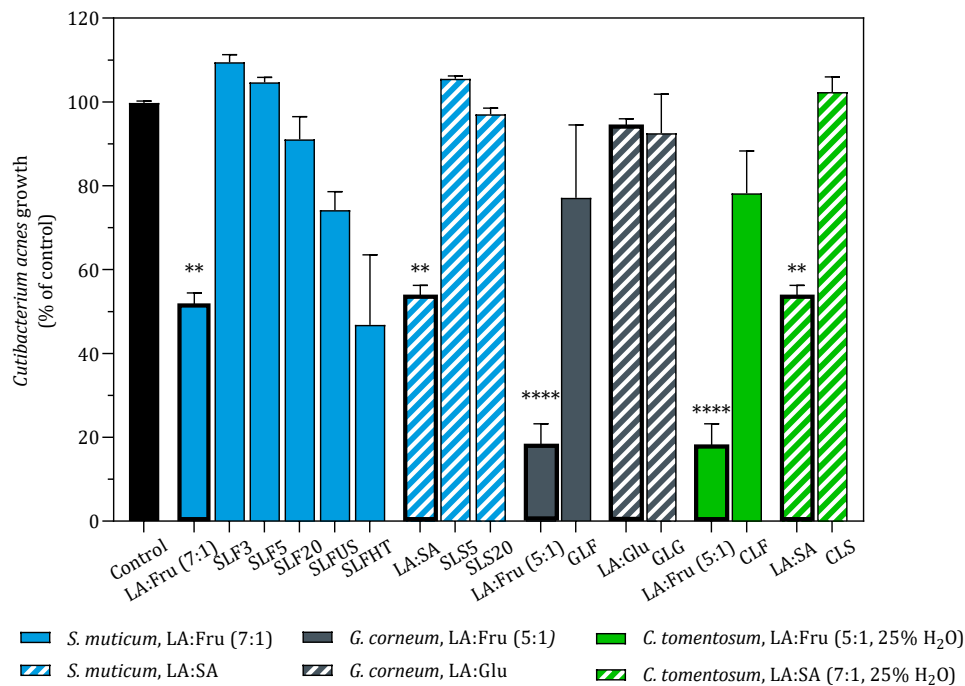


Figure 32: Antimicrobial activity of seaweed extracts (2 µL) against the bacteria *Cutibacterium acnes*. The values correspond to mean ± SEM of three independent experiments. No significant differences were found (Kruskal–Wallis test, Dunn’s test; ** $p < 0.01$, **** $p < 0.0001$) when compared to the control (*). All samples were adjusted for pH levels of 5 to 7 with sodium hydroxide (NaOH 40% w/v).

Microbial diversity and relationships in the cutaneous microbiota are essential for the maintenance of a healthy skin. Yet, the cutaneous microbiota is in constant evolution and change over time. The three studied microorganisms are part of this natural microbiome but, in case of disturbance can be related with various skin disorders. For instance, a disturbed balance (dysbiosis) of healthy skin caused by exogenous factors (injury, stress, or pollution) or endogenous factors (hormonal changes, pH alterations) may induce inflammatory skin lesions such as acne, atopic dermatitis, rosacea, and psoriasis.

Both *C. acnes* and *S. epidermidis* are recognized commensals and interact with the host, helping to protect the healthy skin from colonization by pathogens. *S. epidermidis* helps to regulate skin homeostasis and to suppress the pathogenic inflammation that is induced by *C. acnes* (mainly by the phylotype IA1). However, disequilibrium in favor of *S. epidermidis* may represent a threat to patients with a compromised immunological system. *C. acnes* also plays a beneficial role limiting the growth of the pathogens *S. aureus* and *S. pyogenes*. Despite that, over-colonization by specific *C. acnes* phylotypes in the sebaceous unit can lead to different levels of inflammatory acne. Therefore, a balanced microbiota should be the final aim in any acne treatment [118].

The antimicrobial assays in both *S. epidermidis* and *C. acnes* showed that some extracts were able to maintain microorganisms' growth, which can be seen as a positive contribution since both bacteria interact together and are critical in the regulation of skin homeostasis. These extracts were SLF3, SLF5,

SLF20, SLS5, SLS20, GLF, GLG and CLS. Regarding the assay on the fungus *M. furfur*, the seaweed extracts did not show antimicrobial activity, they rather induced microorganisms' growth.

Among the samples here studied, the enriched phenolic *S. muticum* extracts proved to be more suitable for further dermatological applications, combining antimicrobial and antioxidant assays. From these, the extract SLF3 should be highlighted due to its high phenolic content, strong antioxidant activity, as well as to its role in the maintenance of skin microbiota homeostasis, suggesting its inclusion in skin formulations with antioxidant properties.

5.6. HPLC-DAD and LC-MS/MS Characterization of phenolic composition

The polyphenolic composition of *Sargassum muticum* extracted with the NADES lactic acid:fructose (7:1, 50% H₂O v/v, 1:3 g/mL) was investigated using high performance liquid chromatography with diode-array detection coupled on line to a QIT mass spectrometer (HPLC-DAD-MS). Mass spectra were acquired in the ESI negative and positive ion modes. UV-Vis and ionic (negative and positive) chromatograms are depicted in Figure 33. The DAD profile shown in Figure 33 a) clearly indicates that the compound eluting at Rt 6.08 min (λ_{max} 290 nm) is the main compound present in the extract. All the other compounds displayed very low signals in the DAD chromatogram enabling its UV-Vis identification.

Although the major compound gave very low signals in both ESI modes (Figure 33 - b, c), suggesting a compound with low polarity, an extracted ion chromatogram was obtained at m/z 127, assigned to the protonated molecule of a phlorotannin (Figure 34 - c). At Rt 3.75 min it was also identified a small peak with m/z 235 suggesting the presence of another phlorotannin. (Figure 34 - d). Both precursor ions were isolated in the quadrupole ion trap, and collision induced dissociation experiments (CID) were performed, leading to the MS² spectra (Figure 34 - c' and d', respectively).

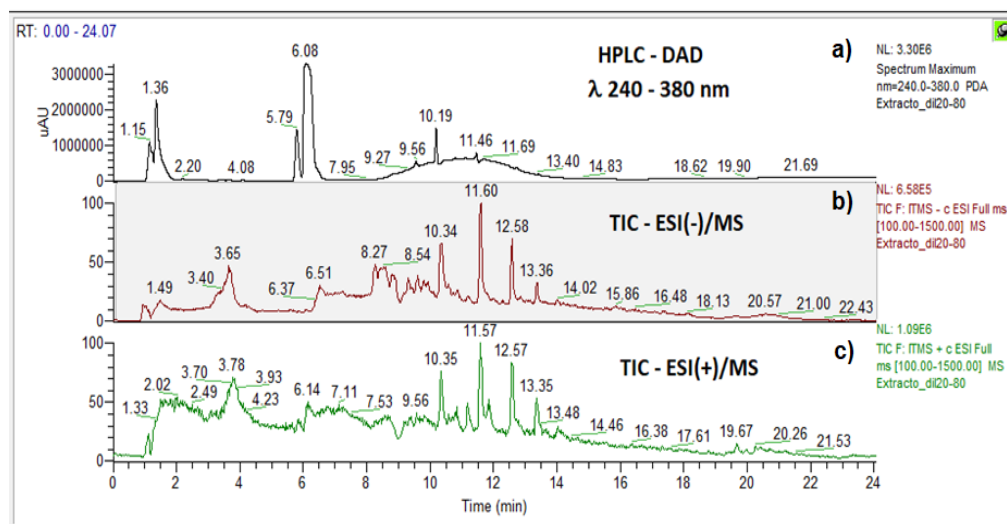


Figure 33: LC-DAD-MS analysis obtained in both ESI modes for an extract of *Sargassum muticum*, in NADES(20:80): a) DAD chromatograms obtained between 240 and 380 nm; b) total ion chromatogram obtained in the ESI negative mode; c) total ion chromatogram obtained between 240 and 380 nm; b) total ion chromatogram obtained in the ESI negative mode; c) total ion chromatogram obtained in the ESI positive mode.

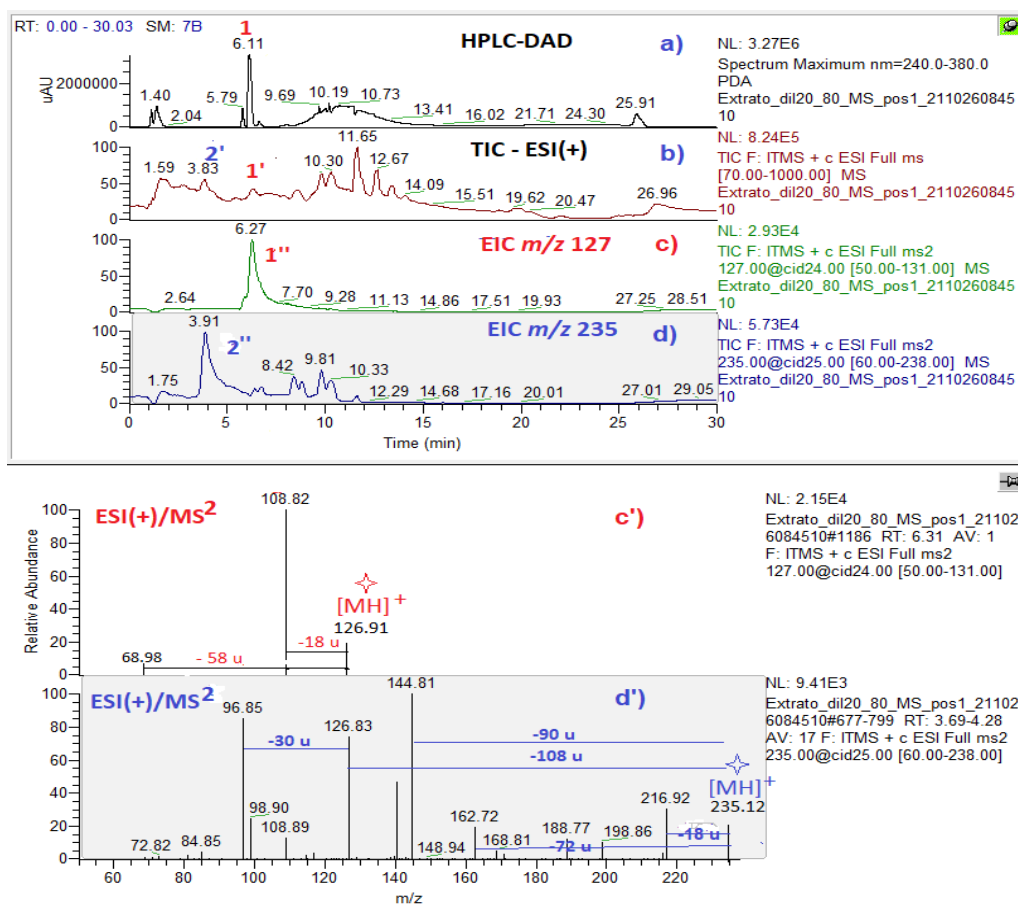


Figure 34: LC-DAD-MS analysis obtained in the ESI positive mode for the extract SLF3 a) DAD chromatograms obtained between 240 and 380 nm; b) total ion chromatogram obtained in the positive mode; c) extracted ion chromatogram and c') MS² spectrum for the precursor ion with m/z 127; d) extracted ion chromatogram, and d') MS² spectrum for precursor ion with m/z 235.

The analysis of the second order mass spectra (Figures 35 and 36) allowed to propose that the precursor ions with m/z 127 and 235 may correspond to the protonated molecules of phloroglucinol and phloroethol, respectively. This conclusion was supported by literature data that reported the presence of phenolic compounds, including phlorotannins in some species of brown algae [48, 119–123].

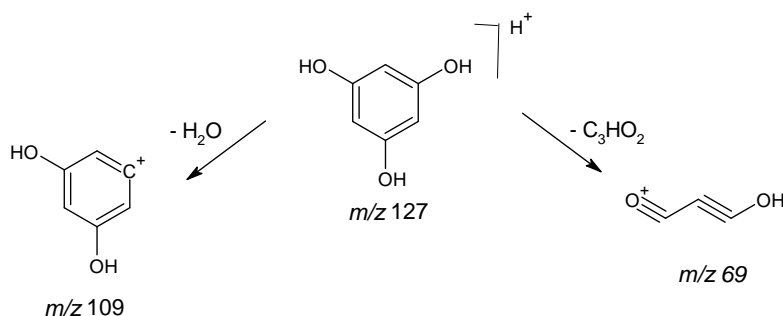


Figure 35: Proposed fragmentation path for the precursor ion with m/z 127 assigned to the protonated molecule of phloroglucinol.

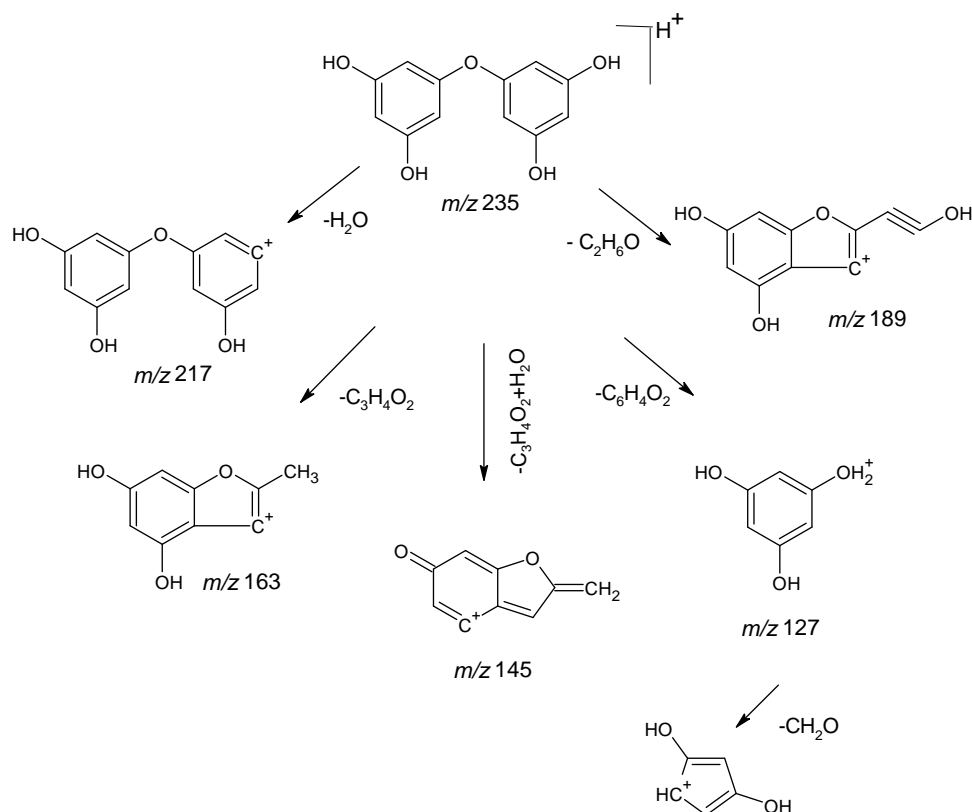


Figure 36: Proposed fragmentation path for the precursor ion with m/z 235 assigned to the protonated molecule of phloroethol.

These results show the presence of phlorotannin compounds in the extracts from *S. muticum*, specially, SLF3. Phlorotannins are the most studied group of seaweed phenolic metabolites due to their interesting bioactivity and they are almost exclusively produced by brown macroalgae, such as *S. muticum*.

Yuan *et al.* [48] have also detected the presence of phlorotannin derivatives through HPLC-DAD-ESI-MS in four brown macroalgae (*Ascophyllum nodosum*, *Laminaria japonica*, *Lessonia trabeculate* and *Lessonia nigrecens*). However, there is still little information on characterization of these compounds [124]. It is known that their antioxidant capacity is up to 10 times higher than other antioxidant compounds, such as ascorbic acid or tocopherol [23], suggesting they are responsible for the high antioxidant activity showed by *S. muticum* extracts.

5.7. Physical characterization of the formulations

To obtain approval for a generic drug, several product's characterizations tests are required to prove equivalence with the reference (control formulation). A detailed product characterization facilitates life-cycle management and, where applicable, supports a claim of equivalence to the comparing product.

After evaluating the biological activities of the extracts, SLF3 was selected to be tested in a topical formulation [125]. W/O emulsions are named after their non-oily (water) and oily fractions and the ingredients used to prepare the formulations are listed in Table 5. Three emulsions were prepared: a blank

emulsion BC, an emulsion containing 1% (w/w) of SLF3 extract, EC, and one containing the 1% (w/w) of lactic acid:fructose 7:1 with 50% (v/v) water, SC.

5.7.1. Appearance, pH, physical stability

Concerning the macroscopic organoleptic characteristics, all formulations presented a homogeneous appearance with bright white color for the BC and SC formulations, while the extract formulation, EC, presented a beige color due to the dark brown color of the *S. muticum* extract, SLF3, Figure 37. All formulations were odorless and in respect to appearance both the solvent and extract creams appear to harden since preparation.



Figure 37: Macroscopic aspect of formulations.

Centrifugation tests were made to check phase separation (Figure 38) verifying the occurrence of phase separation for formulations, SC and EC, respectively. These results indicate that these formulations are not stable, meaning that selected NADES has an impact in formulation stability. Phase separation suggests that this specific formulation is not able to incorporate the amount of NADES used. Due to the high content of lactic acid in the NADES used, solvent and extract formulations, SC and EC, presented low pH values, 2, and were adjusted with NaOH (40% v/v) to pH values of 4.36 and 4.51, respectively. BC formulation was also adjusted from an initial pH value around 9 to 8.12, with HCl (10% v/v). The pH of the skin is usually acidic, ranging between 4 and 6, and topical formulations slightly above the skin pH range do not seem to cause skin irritation, suggesting a safe application. For this reason, pH levels between 5 and 7 are used for these formulations [115].

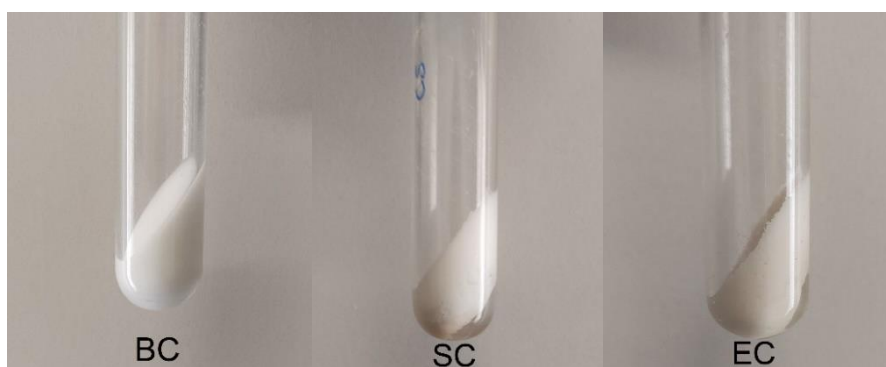


Figure 38: Phase separation of the formulations.

5.7.2. Droplet size analysis

W/O emulsions are two-phase systems containing oil and water, where one of which is dispersed in the other in the form of microscope droplets [126]. Droplet size and the distribution profile of emulsions can be used as an indicator of physical stability. Smaller droplet sizes with tighter and homogenous distributions are associated with higher stability.

Other aspects can also be associated with droplet size such as degradation rate, long-term stability, resistance to creaming, texture, viscosity, or physiological efficiency. The density difference between both fractions also affects stability, and instability issues can be indicated by physicochemical alterations such as creaming or sedimentation, flocculation, Ostwald ripening, coalescence, and phase inversion [127].

Droplet size distributions of the three formulations are represented in

Figure 40 and the corresponding statistical data in Table 11. Results show a monomodal distribution in the BC emulsion, used as the control, while SC and EC emulsions presented bimodal populations. Accordingly with the results of the centrifugation tests, the differences present in the droplet size distribution also point to phase separation issues for SC and EC formulations.

Optical microscope images at a magnification of 20x are presented in Figure 39.

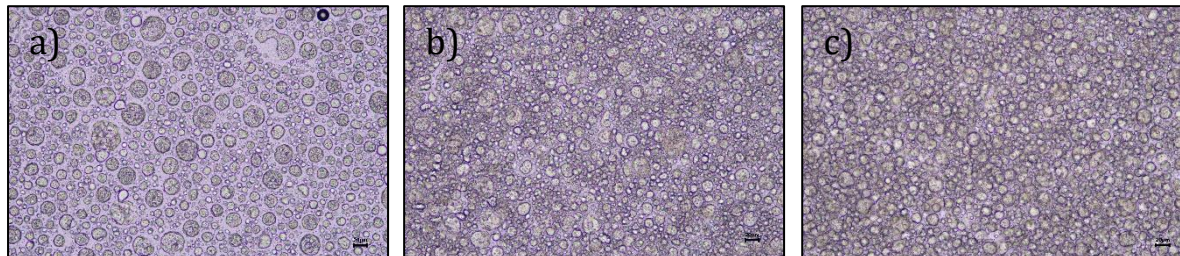


Figure 39: Optical microscope images of formulations at 20x magnification: a) BC, b) SC and c) EC.

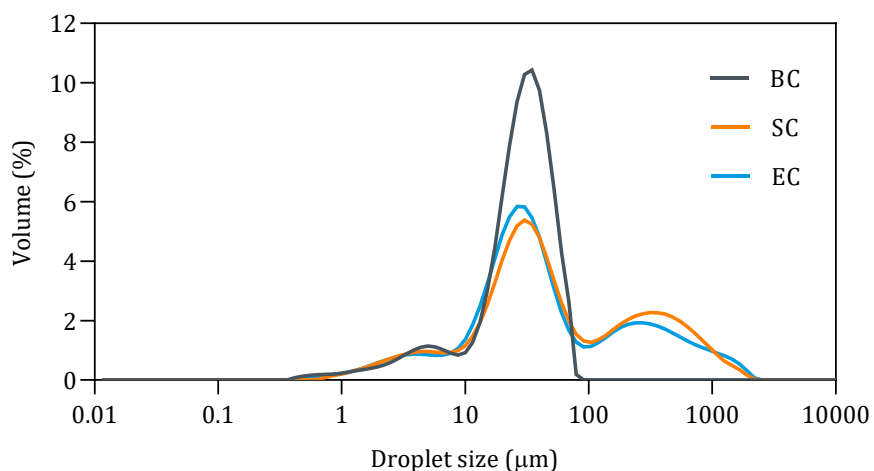


Figure 40: Droplet size distribution of seaweed formulations (n=5).

Table 11: Droplet size distribution. Results are mean \pm SD, n=5.

Formulation	Droplet Size Distribution (μm)			
	Span	d (0.1)	d (0.5)	d (0.9)
BC	1.59 \pm 0.00	5.81 \pm 0.00	27.14 \pm 0.02	48.99 \pm 0.03
SC	13.98 \pm 2.16	6.71 \pm 0.68	38.79 \pm 2.22	546.55 \pm 108.29
EC	13.95 \pm 2.80	6.47 \pm 0.85	31.98 \pm 1.90	452.53 \pm 115.65

d - diameter values corresponding to percentiles of 10%, 50%, 90%.

5.7.3. Rheology

Rheology characterization has become essential for the cosmetic industry since the flow properties of the final product highly influence product application as well as customer acceptance [126]. As a part of quality control, rheology studies assess product stability and several mechanical properties affecting product spreadability and lubricity. Other factors as the use of new ingredients, formulation preparations, material packaging and storage time are also associated with complex material flow [126, 128]. Therefore, to correctly evaluate the possibility of using NADES-based seaweed extracts in cosmetics, rheology assays were carried with the produced creams including dynamic viscosity measurement and oscillation frequency test.

Studying the viscosity of semisolid formulations is very important since it may influence the release of drugs by altering the diffusion rate from the vehicles. The viscosity profile provides important information about the formulations production, processing, and performance and is an indicator of stability of the product being correlated with its internal structure robustness [115].

In Figures 41 and 42 are represented the flow curves (fluid behavior when subjected to increasing shear rates) of the three creams prepared. The BC cream, used as control, showed higher viscosities, although not considerably different from the other formulations, representing higher resistance to the structural breakdown, than SC and EC. At a shear rate of 1 s^{-1} , the apparent viscosity values were 29.95 Pa.s for the BC, 21.54 for the SC and 16.26 for the EC.

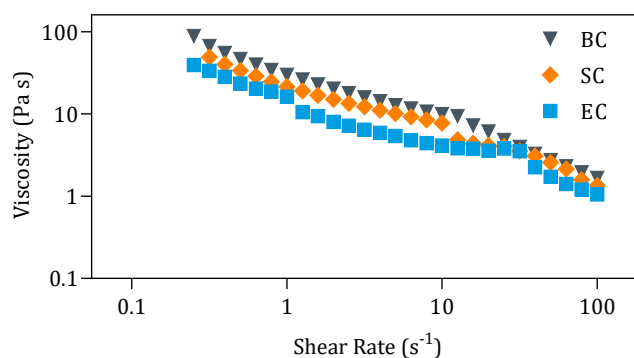


Figure 41: Flow curve of Viscosity vs. Shear Rate of seaweed formulations.

Despite the differences in viscosity, all formulations exhibit a similar behavior to the torque response, represented by similar flow curves. The apparent viscosity decreases simultaneously with the increase of shear rate, a common behavior of non-Newtonian shear-thinning fluids [129]. This is also verified in Figure 42, since the shear stress is defined by Equation 5.2, where τ represents the shear stress, η the apparent viscosity and θ the shear rate.

$$\tau = \eta \theta \quad 5.2$$

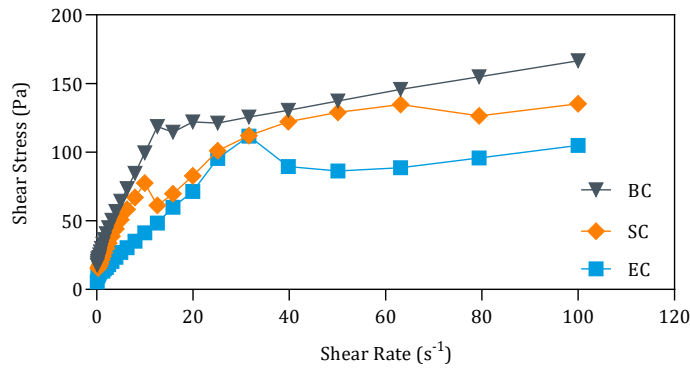


Figure 42: Flow curve of Shear Stress vs. Shear Rate of seaweed formulations.

For lower shear rates, BC shows higher viscosities than SC and EC, this can be associated with the existence of larger droplets in SC and EC emulsions since fine emulsions (smaller droplet sizes) usually have higher viscosities and higher shear-thinning effects than coarse emulsions [130]. Therefore, these results are in accordance with droplet size analysis, BC is monodispersed having smaller droplets and higher viscosities while SC and EC, with bimodal distributions have lower viscosities. For higher shear rates all formulations have closer viscosities due to increased shear-thinning effects of BC, a finer emulsion.

For a more complete flow behavior characterization, various mathematical models (Bingham, Casson, Herschel-Bulkley and Power law) were fitted to the experimental data. Rheological curve fitting helps to decide whether the material tested is within or outside the specifications of the model in question [115].

The fitting parameters of the models are described in Table 22, in Appendix D. Among the four flow models considered in this work, the Herschel-Bulkley was the best for predicting the flow behavior of *S. muticum* extract formulation, Table 12. This model is an extension of a simple power-law flow equation that includes a yield stress term and is useful to quantitatively describe the steady shear flow behavior of several types of soft materials. The results obtained with the Herschel-Bulkley model suggest that all formulations are shear-thinning due to having a flow index value lower than 1. Since they behave as a shear-thinning fluid, all emulsions are suitable for topical administration.

The consistency index (k) in Bingham, Herschel-Bulkley and Power law models, is a measure of the viscous nature of the emulsion, higher k values reflect a stronger emulsion network. The EC and SC had the lowest k values, respectively, on all models indicating a weaker network when compared with the BC, control formulation.

Table 12: Regression parameters from Bingham, Casson, Herschel-Bulkley and Power Law models fitted to the rheological data.

Formulation	Bingham			Casson			
	σ_0 (Pa)	k (Pa · s ⁿ)	R ²	σ_0 (Pa)	η_c (Pa · s)	R ²	
BC	22.3	7.88	0.996	11.4	4.36	0.999	
SC	9.01	3.20	0.995	4.57	1.90	0.994	
EC	10.0	3.13	0.998	5.86	1.54	0.995	
Formulation	Herschel-Bulkley				Power law		
	σ_0 (Pa)	k (Pa · s ⁿ)	η	R ²	k (Pa · s ⁿ)	η	R ²
BC	16.1	13.5	0.79	0.999	31.4	0.47	0.991
SC	7.02	4.90	0.86	0.998	14.7	0.43	0.980
EC	8.97	4.10	0.90	0.999	14.6	0.39	0.966

σ_0 – yield stress (Pa), k – consistency index (Pa · sⁿ), R² – correlation coefficient, η_c – Casson viscosity (Pa · s), η – flow index;

Oscillatory tests offer fundamental rheological information of semisolid formulations. The tests are designed in order not to destroy the structure so that intermolecular and interparticle forces in the material can be measured [115].

The storage modulus (G') represents the elastic behavior of a test material since it is a measure of the deformation energy stored by the sample during the shear process. The loss modulus (G'') is a measure of the energy lost in the deformation of the sample during the shear process, representing the viscous behavior of the material. Usually, for oil-in-water (O/W) creams, $G' > G''$, indicating that the elastic properties exceed the viscous ones [115].

Concerning the oscillatory tests, all formulations resulted in $G' > G''$, meaning the elastic module is superior to the viscous module. It also suggests the existence of a strong network dominated by cohesive forces that allows good spreadability, adhesion and tackiness of emulsions. Results are presented in Figure 43 and further indicate that the formulations SC and EC have higher elastic and viscous modules than the control, BC, meaning these formulations are slightly more structured.

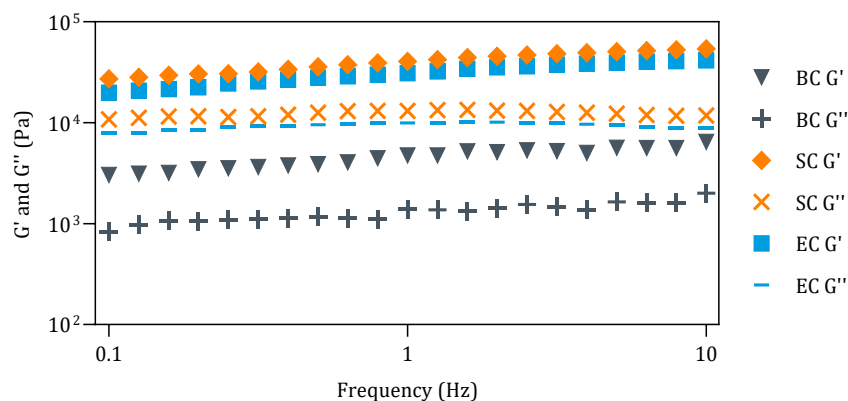


Figure 43: Oscillation frequency sweep test.

Table 13 summarizes G' and G'' values at the frequency of 1 Hz, showing that SC and EC present higher elastic and viscosity modules than BC, the control formulation.

Table 13: G' and G'' values at 1 Hz for all formulations.

Formulation	Frequency sweep test at 1 Hz	
	G' (Pa)	G'' (Pa)
BC	4740	1390
SC	40600	13100
EC	31000	9950

SC and EC creams differ from the BC (control formulation) by containing the NADES lactic acid:fructose (7:1, 50% v/v H₂O) and its *S. muticum* extract, SLF3, respectively. Despite the solvent and extract only represent 1% (w/w) of the SC and EC formulations, this percentage is responsible for the rheological differences between both formulations and the control. Lactic acid is one of the compounds most present in the solvent and in the extract introduced in the formulations and is responsible for lowering the pH values that influences the structural differences observed. NADES, along with the extracted phenolic compounds, mainly phlorotannins, have reduced the apparent viscosity, although maintaining the rheological behavior, increased both elastic and viscous modules and induced phase separation, compared to the control formulation, BC. A bimodal distribution of droplet sizes and bigger droplets were originated with the introduction of the solvent and the extract into the formulations.

6. Conclusions and future prospects

The work developed in this thesis aimed at using natural deep eutectic solvents to face the lack of information concerning their use in the extraction of seaweed phenolic compounds and their application in a topical formulation for dermatological use.

For this, several NADES were tested as extraction solvents on three seaweeds from the Portuguese Atlantic coast, namely: *Codium tomentosum*, *Gelidium corneum* and *Sargassum muticum*. Extraction results with NADES surpassed conventional extraction results, using water/ethanol (70:30, v/v), in all seaweeds. For *C. tomentosum* 145.1 mg GAE/L, 14% higher than the conventional extraction. Due to the higher phenolic content revealed by *G. corneum* and *S. muticum* in NADES screening, the best two NADES for each were optimized with parametric tests resulting in maximum extractions. For *G. corneum* phenolic extraction results revealed 408.4mg GAE/L with lactic acid:fructose and 334.8mg GAE/L lactic acid:glucose, both NADES were synthesized with a molar ratio of 5:1, a 25% water content (v/v) and a 1:5 g/mL SLR, while the conventional extraction resulted in 168.8 mg GAE/L. Phenolic extraction of *S. muticum* resulted in 1546mg GAE/L and 1436±41 mg GAE/L, with lactic acid:sodium acetate and lactic acid:fructose, respectively, both with 7:1 molar ratios, 50% (v/v) of water and a SLR of 1:5 g/mL. UAE was tested in this conditions with the lactic acid:fructose (7:1, 50% H₂O v/v) NADES, resulting in 1898 mg GAE/L, proving the high interest of combining novel technologies for phenolic extraction and suggesting the need of further studies on this matter. A maximum phenolic extraction of 2099 mg GAE/L was yet obtained with of *S. muticum* and the NADES lactic acid:fructose (7:1, 50% H₂O v/v) when applied a SLR of 1:3 g/mL. For this macroalgae, conventional extraction resulted in 445.9 mg GAE/L which was greatly improved with the use of NADES. From the different NADES tested the use of lactic acid was a clear enhancer of phenolic extraction.

When assessing the biological activities of diverse extracts the *S. muticum* extracts revealed higher antioxidant activities, strongly related to their high phenolic content and due to the large presence of phlorotannins found this seaweed, these extracts were also able to maintain cytotoxicity levels on HaCat cells. Seaweed-based NADES extracts also exhibited good results regarding the antimicrobial assays in both *S. epidermidis* and *C. acnes*, where microorganisms' growth was maintained. On the other hand, extracts did not show antimicrobial activity on the fungus *M. furfur*, they rather induced microorganisms' growth.

Enriched phenolic *S. muticum* extracts proved to be suitable for further dermocosmetic applications, revealing great phenolic content and antioxidant activity. The extract SLF3, (seaweed - *S. muticum*, NADES - lactic acid:fructose, 7:1, 50% H₂O v/v, 1:3 g/mL) was further employed in the preparation of a topical oil-in-water (O/W) formulation for skin care. This formulation was characterized and compared with two controls, one with the NADES used in this extract and other without NADES or extract.

The physical-chemical characterization of the O/W emulsion showed acidic pH value and a shear thinning behavior suggesting it was suitable for skin application. However, the extract formulation

prepared, EC, revealed stability issues and despite proving the possible use of seaweed-based NADES extract in topical formulations for skin care, further work is required to avoid the occurrence of phase separation.

Considering the attained results some limitations were found as well as suggestions for future prospects. Although satisfactory results were obtained with the *S. muticum* seaweed, its full potential should be further explored with other novel extraction techniques and other NADES. It is proven that combining extraction techniques is possible to achieve synergetic effects that favor the extraction. Despite the less promising results regarding phenolic content and antioxidant activity on the seaweeds *C. tomentosum* and *G. corneum* these should not be discarded of future works and should be considered for other beneficial applications. Regarding the use in topical formulations, further work is required. The development and optimization of dermatological formulations is a complex process that should include *in vivo* assays and microbiological evaluation.

As final remarks the outcome of the present work presents important insights toward the valorization of seaweeds as natural ingredients for the dermocosmetic industry as well as the possible use of natural solvents to obtain bioactive compounds from marine biomass.

7. References

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8. Appendix

8.1. Appendix A

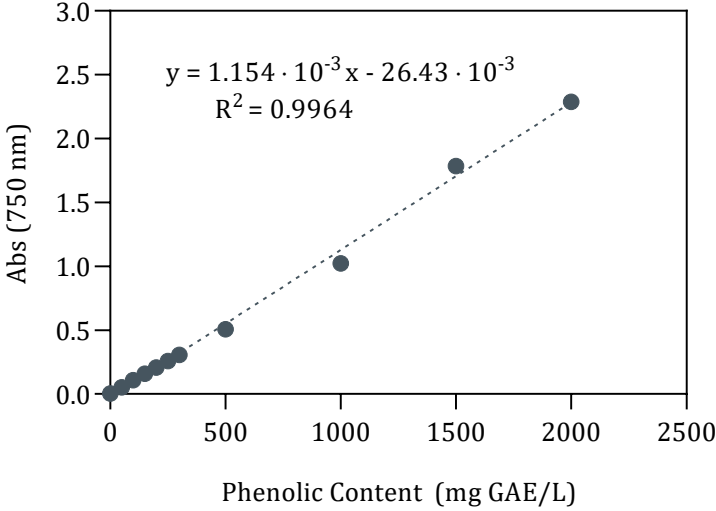


Figure 44: Folin-Ciocalteu method calibration's curve (macroscale).

8.2. Appendix B

Table 14: Optimization data of NADES composition on *Gelidium corneum* extractions. Results are the mean \pm SD of three independent experiments.

NADES	Molar ratio	RSL (g/ml)	Abs. (750nm)	TPC (mg GAE/g DW)	Conc. (mg GAE/L)
Lactic acid:fructose	1:1	1:5	0.174 \pm 0.027	0.87 \pm 0.12	173.4 \pm 23.1
	3:1	1:5	0.352 \pm 0.051	1.64 \pm 0.22	327.9 \pm 44.3
	5:1	1:5	0.445 \pm 0.020	2.04 \pm 0.09	408.4 \pm 17.7
	7:1	1:5	0.330 \pm 0.040	1.55 \pm 0.18	309.2 \pm 35.1
Lactic acid:glucose	1:1	1:5	0.176 \pm 0.011	0.88 \pm 0.05	175.6 \pm 9.3
	3:1	1:5	0.237 \pm 0.022	1.14 \pm 0.10	228.1 \pm 19.1
	5:1	1:5	0.360 \pm 0.029	1.67 \pm 0.13	334.8 \pm 25.1
	7:1	1:5	0.297 \pm 0.020	1.40 \pm 0.09	280.2 \pm 17.7
Lactic acid:sodium acetate	1:1	1:5	0.110 \pm 0.018	0.59 \pm 0.08	117.9 \pm 15.2
	3:1	1:5	0.246 \pm 0.020	1.18 \pm 0.09	236.4 \pm 17.0
	5:1	1:5	0.258 \pm 0.010	1.23 \pm 0.04	246.3 \pm 8.8
	7:1	1:5	0.304 \pm 0.049	1.43 \pm 0.21	286.6 \pm 42.6

Table 15: Optimization data of NADES composition on *Sargassum muticum* extractions. Results are the mean \pm SD of three independent experiments.

NADES	Molar ratio	RSL (g/ml)	Abs. (750nm)	TPC (mg GAE/g DW)	Conc. (mg GAE/L)
Lactic acid:fructose	1:1	1:5	0.76 \pm 0.11	3.42 \pm 0.48	684.9 \pm 95.8
	3:1	1:5	1.28 \pm 0.11	5.65 \pm 0.47	1130.7 \pm 94.9
	5:1	1:5	1.43 \pm 0.18	6.32 \pm 0.79	1263.3 \pm 158.3
	7:1	1:5	1.49 \pm 0.15	6.57 \pm 0.65	1314.3 \pm 129.8
Lactic acid:glucose	1:1	1:5	0.56 \pm 0.06	2.53 \pm 0.24	506.2 \pm 48.5
	3:1	1:5	0.90 \pm 0.14	4.02 \pm 0.60	803.5 \pm 120.2
	5:1	1:5	1.28 \pm 0.05	5.65 \pm 0.22	1130.6 \pm 44.2
	7:1	1:5	1.10 \pm 0.07	4.89 \pm 0.31	978.8 \pm 62.5
Lactic acid:sodium acetate	1:1	1:5	0.63 \pm 0.07	2.83 \pm 0.30	565.1 \pm 60.2
	3:1	1:5	1.20 \pm 0.13	5.29 \pm 0.55	1058.7 \pm 109.6
	5:1	1:5	1.38 \pm 0.13	6.09 \pm 0.56	1217.3 \pm 112.0
	7:1	1:5	1.40 \pm 0.04	6.16 \pm 0.19	1231.9 \pm 37.1

Table 16: Optimization data of water content on *Gelidium corneum* extractions. Lactic acid:fructose: molar ratio 5:1, SLR 1:5 g/mol; lactic acid:glucose: molar ratio 5:1, SLR 1:5 g/mol. Results are the mean \pm SD of three independent experiments.

NADES	Water content (% v/v)	Abs. (750nm)	TPC (mg GAE/g DW)	Conc. (mg GAE/L)
Lactic acid:fructose	25	0.45 \pm 0.02	2.04 \pm 0.09	408.4 \pm 17.7
	35	0.29 \pm 0.05	1.38 \pm 0.20	275.2 \pm 40.2
	50	0.25 \pm 0.03	1.20 \pm 0.12	239.2 \pm 24.5
Lactic acid:glucose	25	0.36 \pm 0.03	1.67 \pm 0.13	334.8 \pm 25.1
	35	0.29 \pm 0.01	1.39 \pm 0.05	277.9 \pm 10.4
	50	0.31 \pm 0.02	1.46 \pm 0.09	291.2 \pm 17.3

Table 17: Optimization data of water content on *Sargassum muticum* extractions. Lactic acid:fructose: molar ratio 7:1, SLR 1:5 g/mol; Lactic acid:glucose: molar ratio 7:1, SLR 1:5 g/mol. Results are the mean \pm SD of three independent experiments.

NADES	Water content	Abs.	TPC	Conc.
	(%, v/v)	(750nm)	(mg GAE/g DW)	(mg GAE/L)
Lactic acid:fructose	25	1.49 \pm 0.15	6.57 \pm 0.65	1314 \pm 130
	35	1.63 \pm 0.12	7.17 \pm 0.51	1435 \pm 103
	50	1.63 \pm 0.05	7.18 \pm 0.20	1436 \pm 41
Lactic acid:glucose	25	1.40 \pm 0.04	6.16 \pm 0.18	1232 \pm 37
	35	1.58 \pm 0.04	6.96 \pm 0.16	1392 \pm 32
	50	1.76 \pm 0.03	7.73 \pm 0.11	1546 \pm 23

Table 18: Data on temperature effect on *Gelidium corneum* extractions. Lactic acid:fructose: molar ratio 5:1, H₂O: 25% v/v; Lactic acid:glucose: molar ratio 5:1, H₂O: 25% v/v. Results are the mean \pm SD of three independent experiments.

NADES	Temp.	Abs.	TPC	Conc.
	(°C)	(750nm)	(mg GAE/g DW)	(mg GAE/L)
Lactic acid:fructose	r.t. (appx. 25°C)	0.45 \pm 0.02	2.04 \pm 0.09	408.4 \pm 17.7
	40	0.31 \pm 0.01	1.44 \pm 0.04	288.3 \pm 8.8
Lactic acid:glucose	r.t. (appx. 25°C)	0.36 \pm 0.03	1.67 \pm 0.13	350.7 \pm 25.1
	40	0.15 \pm 0.03	1.61 \pm 0.25	335.2 \pm 50.0

Table 19: Data on temperature and UAE effect on *Sargassum muticum* extractions. Lactic acid:fructose: molar ratio 7:1, SLR 1:5 g/mol; lactic acid:glucose: molar ratio 7:1, SLR 1:5 g/mol. Results are the mean \pm SD of three independent experiments, except for UAE.

NADES	Temp. (°C)	Abs. (750nm)	TPC (mg GAE/g DW)	Conc. (mg GAE/L)
Lactic acid:fructose	r.t. (appx. 25°C)	1.63 \pm 0.05	7.18 \pm 0.20	1436 \pm 41
	40	0.31 \pm 0.01	7.18 \pm 0.24	1437 \pm 49
	UAE	2.16	9.49	1898
Lactic acid:sodium acetate	r.t. (appx. 25°C)	1.63 \pm 0.05	7.73 \pm 0.11	1546 \pm 32
	40	1.83 \pm 0.04	8.03 \pm 0.19	1606 \pm 38

Table 20: Data on solid-liquid ratio assay on *Gelidium corneum* extractions. Lactic acid:fructose: molar ratio 5:1, H₂O: 25% v/v; Lactic acid:glucose: molar ratio 5:1, H₂O: 25% v/v. Results are the mean \pm SD of three independent experiments.

NADES	SLR (g/mL)	Abs. (750nm)	TPC (mg GAE/g DW)	Conc. (mg GAE/L)
Lactic acid:fructose	1:5	0.45 \pm 0.02	2.04 \pm 0.09	408.4 \pm 17.7
	1:10	0.15 \pm 0.01	1.54 \pm 0.04	154.1 \pm 8.9
	1:20	0.06 \pm 0.01	1.49 \pm 0.03	74.5 \pm 6.0
Lactic acid:glucose	1:5	0.36 \pm 0.03	1.67 \pm 0.12	334.8 \pm 25.1
	1:10	0.15 \pm 0.03	1.52 \pm 0.11	151.7 \pm 22.3
	1:20	0.06 \pm 0.00	1.45 \pm 0.01	72.7 \pm 2.4

Table 21: Data on solid-liquid ratio assay on *Sargassum muticum* extractions. Lactic acid:fructose: molar ratio 7:1, H₂O: 25% v/v; lactic acid:glucose: molar ratio 7:1, H₂O: 25% v/v. Results are the mean ± SD of three independent experiments.

NADES	SLR (g/mL)	Abs. (750nm)	TPC (mg GAE/g DW)	Conc. (mg GAE/L)
lactic acid:fructose	1:3	2.40 ± 0.00	6.30 ± 0.00	2099 ± 1
	1:5	1.63 ± 0.05	7.18 ± 0.20	1436 ± 41
	1:10	0.94 ± 0.03	8.37 ± 0.26	837 ± 26
	1:20	0.53 ± 0.02	9.67 ± 0.30	583 ± 15
lactic acid:glucose	1:5	0.36 ± 0.03	7.73 ± 0.11	1546 ± 23
	1:10	0.15 ± 0.03	8.39 ± 0.08	839 ± 8
	1:20	0.06 ± 0.00	10.36 ± 0.29	518 ± 14

8.3. Appendix C

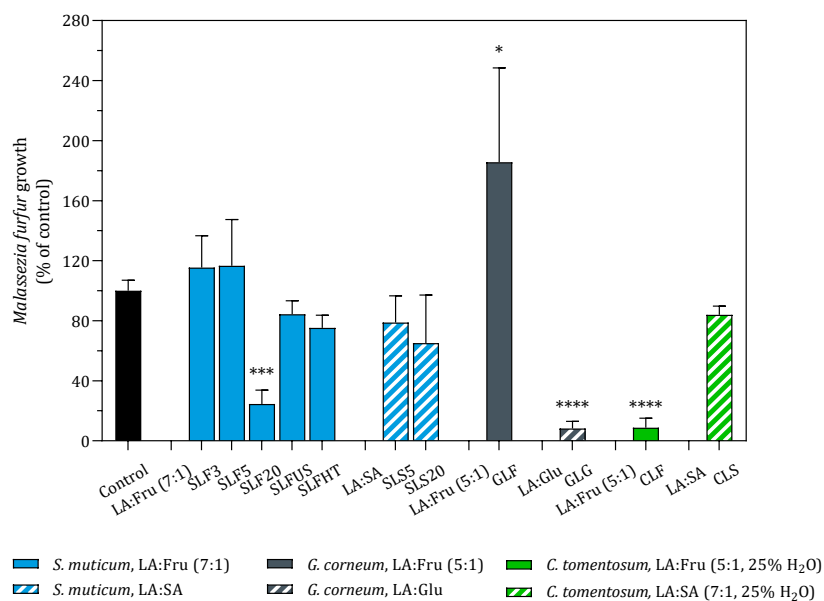


Figure 45: Antimicrobial activity of seaweed extracts (2 µL) against the bacteria *Malassezia furfur*. The values correspond to mean ± SEM of three independent experiments. Symbols represent significant differences (Kruskal–Wallis test, Dunn's test; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$) when compared to the control (*). No pH adjustment was realized in this assay.

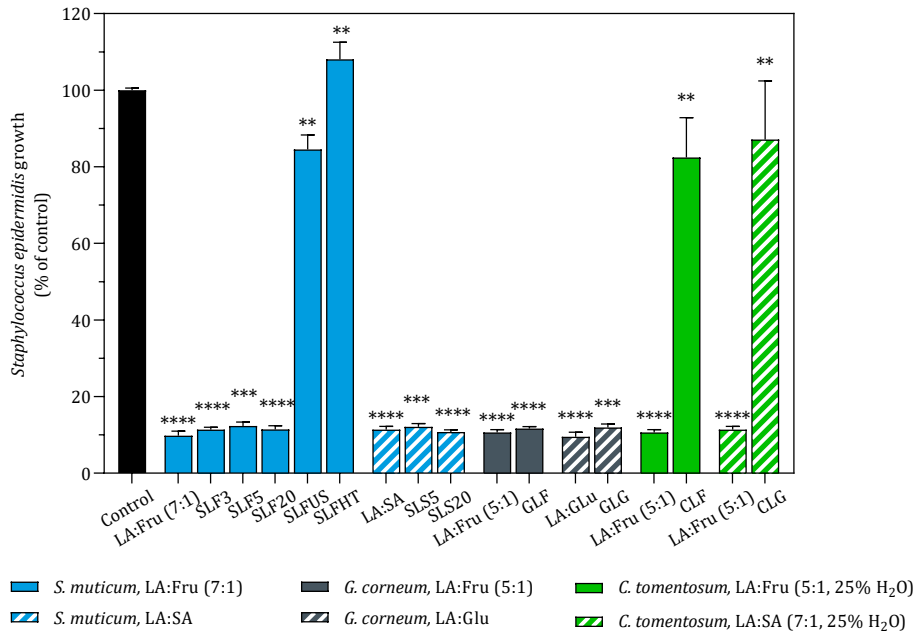


Figure 46: Antimicrobial activity of seaweed extracts (2 μ L) against the bacteria *Staphylococcus epidermidis*. The values correspond to mean \pm SEM of three independent experiments. The values correspond to mean \pm SEM of three independent experiments. Symbols represent significant differences (Kruskal–Wallis test, Dunn’s test; *** $p < 0.001$, **** $p < 0.0001$) when compared to the control (*). No pH adjustment was realized in this assay.

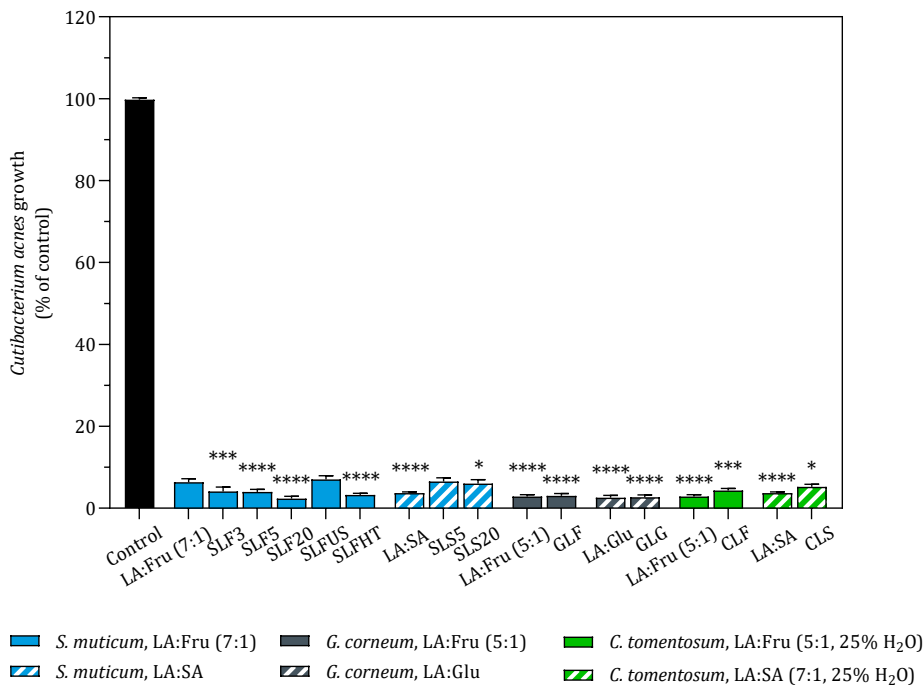


Figure 47: Antimicrobial activity of seaweed extracts (2 μ L) against the bacteria *Cutibacterium acnes*. The values correspond to mean \pm SEM of three independent experiments. The values correspond to mean \pm SEM of three independent experiments. Symbols represent significant differences (Kruskal–Wallis test, Dunn’s test; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$) when compared to the control (*). No pH adjustment was realized in this assay.

8.4. Appendix D

Table 22: Mathematical models used in the fitting of rheological data [115].

Model	Equation	Parameters
Bingham	$\sigma = \sigma_0 + \eta_B \dot{\gamma}$	η_B - Bingham viscosity
Casson	$\sqrt{\sigma} = \sqrt{\sigma_0} + \sqrt{\eta_C \dot{\gamma}}$	σ - shear stress η_C - Casson viscosity
Herschel-Bulkley	$\sigma = \sigma_0 + k \dot{\gamma}^\eta$	σ_0 - yield stress k - consistency index η - shear-thinning index ¹
Power law	$\sigma = k \dot{\gamma}^\eta$	

¹ if $\eta < 1$ material is shear-thinning, if $\eta > 1$ material is shear-thickening.