ARTICLE

Extraction and Formulation of Macroalgal Phenolic Compounds for Cosmetic Application

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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 µM 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 a 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
Solid-liquid extraction
Cosmetic formulations

1. Introduction

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]. 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.

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 those with natural origin, but aiming at keeping the increased performance of synthetic substances [2]. Natural extracts help improve skin tone, texture, and appearance by delivering nutrients necessary for healthy skin. In this context, marine algae have gained significant attention as sources for skin care. They represent one of the richest marine resources considered safe, with negligible cytotoxicity and many beneficial effects on humans [3]. Regarding sustainability, seaweeds are considered a viable alternative feedstock of natural bioactive compounds offering a wide range of primary and secondary metabolites with beneficial properties as antioxidant capacity, pigmentation inhibition, and antimicrobial activity, beneficial in cosmeceutical preparations [4].

In this work, the extraction of phenolic compounds, an important group of chemical compounds present in seaweeds varying quantitatively and qualitatively for each specimen of red, brown or green seaweeds[5], will be carried out envisaging their application in green cosmetics. 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 as: anti-diabetic, anti-inflammatory, anti-microbial, anti-viral, anti-allergic, anti-diabetic, antioxidant, anti-photoaging, and anticancer properties [6].

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 in industry is one of the main solutions to improve sustainability, as well as selection of green alternatives [7]. One interesting alternative is the use of natural deep eutectic solvents (NADES), seen as a promising, green alternative to

synthetic organic solvents to produce plant extracts. It has been amply shown that these solvents bring new challenges and opportunities to produce plant extracts with novel phytochemical compositions and biological activities[8,9]. 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 [10].

This study is focused on the extraction of phenolic compounds, well known antioxidant agents, using novel green solvents (NADES) aqueous solutions and their test in a cosmetical formulation.

2. Materials and Methods

2.1. Materials

The green seaweed *C. tomentosum*, known as Velvet Fingers, was produced in an Integrated Multi-Trophic Aquaculture system and grounded to a powder (ALGAplus, Ílhavo, Portugal) Portions of *C. Vermilara* were identified in the sample, therefore the name *Codium* Sp. was given to the whole mixture. *G. corneum*, red seaweed 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 (Krups, Solingen, Germany) and stored at room temperature. Afterwards, *G. Corneum* was ground with liquid nitrogen to get a powder.

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, Lynge, Denmark). The dried algal material was ground into a powder in a grinder which was stored protected from light, at room temperature.

D-Fructose and L-Proline were purchased from Alfa Aesar (Kandel, Germany). The Folin-Ciocalteau Reagent was obtained from AppliChem (Darmstadt, Germany). Sodium carbonate was obtained from VWR Chemicals (Pennsylvania, USA). Trypticase Soy Broth, Tryptic Soy Broth and Leeming-Notman were purchased from Merck KGaA (Darmstadt, Germany). All the other materials were obtained from Sigma-Aldrich (Steinheim, Germany).

For the W/O emulsion, purified water was obtained by reverse osmosis (Millipore, Elix 3, Millipore Corporation, Billerica, MA). Liquid paraffin, almond Oil and glycerin were obtained from António M. S. Cruz, Material de Laboratório, Lda. (Lisbon, Portugal). Polyglyceryl-3 Dicitrate/Stearate (Tego Care PSC3®), cetearyl alcohol (Tego Alkanol 1618®), decyl oleate (Tegosoft DO®) were obtained from Evonik Industries AG (Essen, Germany). Methylparaben (Nipagin®) and propylis parahydroxybenzoas (Nipasol®) were obtained from Fagron Iberica S.A.U. (Barcelona, Spain).

2.2. 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, using a heating plate MR HeiTec (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.

2.3. SLE extraction

Seaweed material was weighted using 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. After the initial NADES screening, all extractions were performed in triplicate.

2.4. Total Phenolic Content (TPC)

The quantification of the TPC of each extract was determined according to the Folin-Ciocalteu procedure proposed by Singleton et al. [11], using gallic acid as standard. The results were extrapolated from a standard curve obtained with gallic acid and thus expressed as milligram of gallic acid equivalents per milliliter of extract (mg GAE/mL).

In macroscale, 0.1 mL of sample, blank or standard, 7.9 mL of distilled water and 0.5 mL of Folin-Ciocalteau 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).

For microscale, briefly, 2 µL of extract was added to 158 µL of distilled water and 10 µL of Folin Ciocalteu reagent, vortexed and 30 µL of 20% Na_2CO_3 (*w/v*) was 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), Equation *(1.1*:

$$Abs(nm) = 1.154 \cdot 10^{-3}\, TPC(mg\ GAE/L) - 26.43 \cdot 10^{-3} \qquad (1.1)$$

TPC – total phenolic content

Every sample was evaluated with triplicates and read against a blank sample containing the NADES used for that extraction, to avoid method interferences [12,13].

2.5. 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.

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

The DPPH radical scavenging activity was performed according to Brand-Williams and co-workers adapted for microplate [14]. 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%, IC_{50} values (mg DW/mL) were determined, defining the concentration of sample extract that produces a 50% reduction of the DPPH radical absorbance.

2.7. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed accordingly with Benzie and Strain's with slight modifications [15]. This method measures the ability of the antioxidants to reduce ferric-tripyridyl-triazine (Fe^{3+}–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, $FeCl_3$, 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, $FeSO_4{\cdot}7H_2O$, was used as the control (0 – 10 µM) and the results are expressed in µM $FeSO_4$.

2.8. Evaluation of the biological activities on *in Vitro* cellular models

2.8.1. 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% CO_2 (Unitherm, Planegg, Germany), at 37°C. Subculture was performed according to biobank instructions whenever cultures reached 80–85% confluence.

2.8.2. 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 µL) 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 in [16], 100 µL 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.

2.8.3. 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 µL) was determined during the exponential growth, at 600 nm. Results were expressed as percentage of control.

2.8.4. Statistical Analysis

Non-parametric analyses were performed using the Kruskal–Wallis test, followed by multiple comparisons using Dunn's tests. Results are present 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).

2.9. Preparation of topical formulations

The O/W emulsions were prepared by adding the oily and non-oily compounds to different bowls and placing both in a water bath at 70–80 °C until total homogenization. All ingredients are listed in Table 1. Afterwards, the oily ingredients were verted to the bowl containing the non-oily ingredients, and the mix was subjected to ultra-turrax for 2–3 min. The parabens were added while the emulsion was still warm. The emulsion was manually stirred until cold, then the extract (or solvent) was added, and the mix was stirred until homogeneous.

Table 1: Percentage of ingredients in Sargassum muticum extract formulation.

Ingredients	Quantitative Composition (%, *w/w*)
Polyglyceryl-3 Dicitrate/Stearate	3.0
Cetearyl Alcohol	7.0
Liquid Paraffin	2.5
Decyl Oleate	4.5
Sweet Almond Oil	5.0
Glycerin	5.0
Purified Water	71.8
Methylparaben	0.18
Propylparaben	0.02
Sargassum Liquid Extract	1.0

Blank emulsions and emulsions containing lactic acid:fructose (7:1), 50% (*v/v*) water NADES and *S. muticum* extract were named BC, SC and EC, respectively. 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.

2.10. Physicochemical characterization of topical formulations

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. Three replicates of measurement were performed. 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).

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 ± SD; n = 5). Measurements were performed 8 days after preparation of the emulsions (t = 192 hours).

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

3. Results and Discussion

3.1. NADES preparation

Despite the many possible combinations of natural compounds to form NADES, the use of some 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 Cosmetics Regulation (EC) No 1223/2009 of the European Commission, CosIng - database for information on cosmetic substances and ingredients. If any compound is listed in Annex II of this regulation, it should to be discarded from cosmetic formulations [17].

Several NADES of interest were prepared but not all were stable at room temperature. A selection of five NADES to be used in the extraction of phenolic compounds from seaweed was done: lactic acid:fructose (5:1), lactic acid:glucose (5:1), lactic acid:sodium Acetate (7:1), and glycerol:proline (1:1), lactic acid:proline (1:1), tested in the molar ratios found in the literature to ensure feasibility [8,18,19]. 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.

3.2. NADES screening

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 and ethanol (70:30, *v/v*) was used for comparison purposes. Results of extract concentration in (mg GAE/L) were obtained with the Total Phenolic Content (TPC).

Overnight extractions with conventional solvents, water and 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.

Traditional seaweed extraction usually employ high quantities of organic solvents (1:10, 1:20, 1:30 or as high as 1:100 g/mL) [20–22]. However, these extractions are commonly followed by solvent removal processes, such as evaporation. Since one of the objectives of this work is the incorporation of NADES solvents in the final formulation, avoiding high temperature steps and taking advantage of some of the beneficial properties that these NADES might have, our key result is related to the phenolic concentration (mg GAE/L) rather than total phenolic content (mg GAE/g DW).

From Table 2 it can observed 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 1466 mg GAE/L. Despite the difference in the phenolic compounds present in the 3 seaweeds under study, it can be concluded that the presence of lactic acid favours the extraction of phenolic compounds.

Table 2: Phenolic content results of NADES screening for: *Codium tomentosum*, *Gelidium corneum* and *Sargassum muticum*.

Solvent	Molar Ratio	SLR (g/ml)	*C. tomentosum*	*G. corneum*	*S. muticum*
HBD : HBA (for NADES)			**Phenolic Content** (mg GAE/L)		
H_2O/EtOH	-----	1:20	59.1	65.1	130
H_2O/EtOH (Overnight)	-----	1:20	59.0	67.4	127
H_2O/EtOH	-----	1:5	127.5	168.8	446
Lactic acid:fructose	5:1	1:5	135.9	383.7	1466
Lactic acid:glucose	5:1	1:5	90.2	299.0	1153
Lactic acid:sodium acetate	7:1	1:5	134.7	253.0	1193
Lactic acid:proline	1:1	1:5	145.1	57.3	529
Glycerol:proline	1:1	1:5	32.8	---*	425

* Inconclusive results due to interference

3.3. NADES optimization

The initial screening of the selected NADES enabled to identify the most suited NADES for TPC extractions and 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: NADES molar ratio (1:1, 3:1, 5:1 and 7:1), water content (25, 35 and 50% *v/v*) and extraction temperature (r.t. and 40°C).

Also, to be mentioned that for *S. muticum,* the performance of Ultrasound Assisted Extraction (UAE) was also evaluated.

Experimental conditions were tested sequentially, and, after each test, the most suitable conditions were maintained throughout the following tests.

3.3.1. *Gelidium corneum* extract optimization

For phenolic extraction of *G. corneum*, extractions employing lactic acid:fructose, lactic acid:glucose and lactic acid:sodium acetate were carried with different extraction conditions. Figure 1 represents the parametric optimization on *G. corneum* extraction, evaluating NADES' composition, water content and extraction temperature.

The molar ratio 5:1 resulted in higher phenolic extraction for lactic acid:fructose (408.4 mg GAE/L) and lactic acid:glucose (334.8 mg GAE/L), while lactic acid:sodium acetate showed lower extraction performances and the best suited ratio was that with the highest content of lactic acid, 7:1 (286.6 mg GAE/L.

Water is often added as a third component of NADES, in order to decrease viscosity and promote the solubilization of solid organic compounds [10], allowing the confident use of a liquid DES. Both NADES, lactic acid:fructose (5:1) and lactic acid:glucose (5:1) showed the highest phenolic extraction performance, 408.4 mg GAE/L and 334.8 mg GAE/L, respectively, with only 25% (*v/v*) water.

At last, the temperature effect was evaluated and extractions at room temperature and 40°C were carried out seeking for high phenolic extractions due to reduced viscosity and surface tension of the solvents [23]. Yet, temperatures higher than room temperature did not improve the extraction efficiency of TPC.

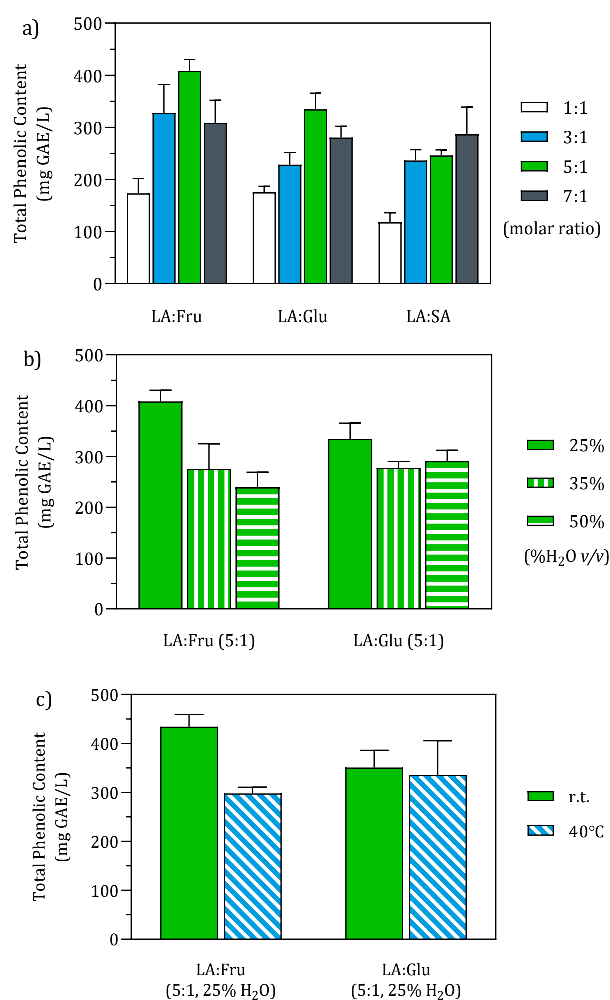

Figure 1: Effect of solvent and extraction conditions on *Gelidium corneum* extractions: a) NADES' molar ratio, b) water content and c) extraction temperature. LA:Fru – lactic acid and fructose; LA:Glu – lactic acid and glucose; LA:SA – lactic acid and sodium acetate. Results are the mean ± SD of three independent experiments

Results show that the best NADES for phenolic extraction on *G. corneum* were obtained with lactic acid:fructose (5:1, 25%

H_2O *v/v*, r.t.) with 408.4 mg GAE/L, and lactic acid:glucose (5:1, 25% H_2O *v/v*, r.t.) 334.8 mg GAE/L.

3.3.2. *Sargassum muticum* extract optimization

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 of extraction optimization on *S. muticum* are presented in Figure 2.

It can be observed that high concentration 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.

Contrary to the results obtained for phenolic extraction from *G. corneum,* in this case higher water content revealed higher phenolic extraction for the two NADES used, which suggest the extraction of more polar compounds. Lactic acid:sodium acetate (7:1) extracted 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. In this case the NADES with 50% (*v/v*) water content was selected since it brings economic and environmental benefits to the process.

In agreement with the results obtained for *Gelidium corneum,* the operating temperature of 40°C did not improve the extractions. 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.

Analysing the results of NADES screening in Table 2, together with the results from NADES optimization, it is possible to understand that the seaweed *S. muticum* has a higher TPC, probably of phlorotannins, in its composition[24]. The most concentrated extracts from this seaweed were those obtained using lactic acid:sodium acetate (7:1, 50% H_2O *v/v*) extracting 1546 mg GAE/L and 1436 mg GAE/L for lactic acid:fructose (7:1, 50% H_2O *v/v*).

An additional *S. muticum* extract was produced using a 1:3 g/mL SLR. The objective was to maximize TPC 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, and therefore, will be considered for the next steps.

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 non-polar.

3.4. Evaluation of the biological activities

Several bioactivities related to cosmetic/cosmeceutical applications are here analysed to understand the potential of the most promising extracts, namely their antioxidant and antimicrobial activities, as well as their cytotoxic profile. The results of the TPC, and their antioxidant capacity assessed through the different assays (DPPH and FRAP) are presented in Table 3.

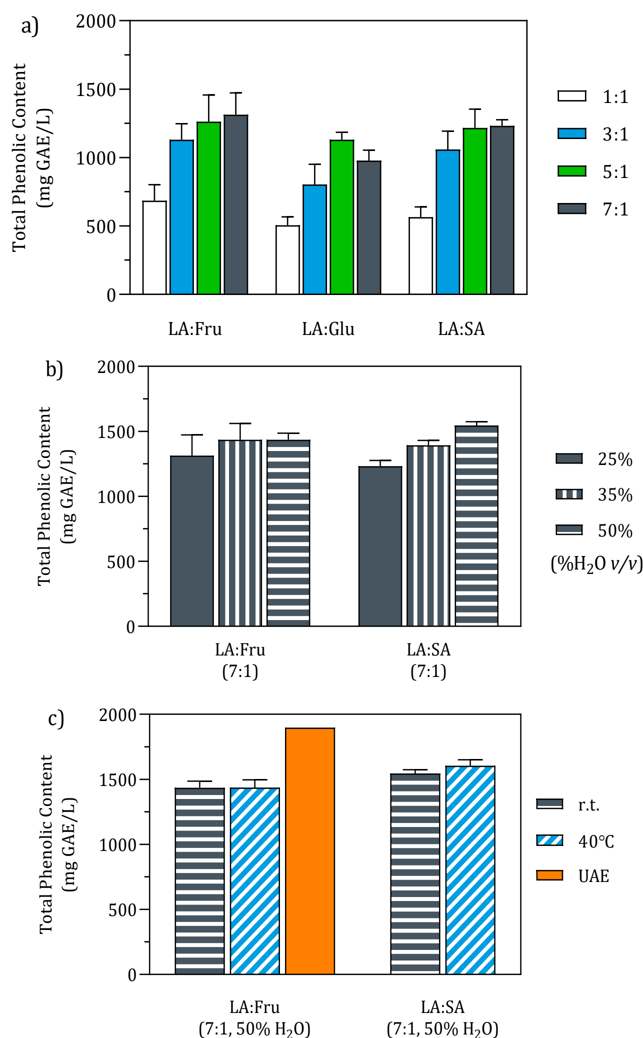

Figure 2: Effect of solvent and extraction conditions on *Sargassum muticum* extractions: a) NADES' molar ratio, b) water content and c) extraction temperature. LA:Fru – Lactic acid and Fructose; LA:Glu – Lactic acid and Glucose; LA:SA – Lactic acid and Sodium Acetate. Results are the mean ± SD of three independent experiments

Results show that every extract has a higher antioxidant potential than each of the NADES used. The most promising extracts are SLF3 and SLF5, with 1624 ± 98 and 1752 ± 46 mg GAE/L, respectively. The extract SLFHT also resulted in promising antioxidant activity with 1344 ± 71 mg GAE/L, but it was not higher than room temperature extracts. The three extracts with the highest activities were all produced using *S. muticum* and the NADES lactic acid:fructose (7:1, 50% H_2O *v/v*). For *G. corneum* and *C. tomentosum* extracts results show very low phenolic content.

Regarding the DPPH radical scavenging ability, a concentration dependency was verified, with higher reduction of DPPH for more concentrated extracts (extracts with higher SLR). In general, almost all extracts showed over 20% radical scavenging activity. In contrast, all NADES showed no significant reduction of the DPPH radical, around 10%. Extracts of *S.*

muticum and lactic acid:fructose (7:1, 50% H_2O *v/v*) resulted in higher reduction activities.

Table 3: Antioxidant capacity of seaweed extracts and NADES used.

Samples	TPC	DPPH	FRAP
	mg GAE/L	% control	µM $FeSO_4$
SLF3	1624.0 ± 97.8	27.0 ± 3.4	29.9 ± 1.4
SLF5	1752.0 ± 46.3	32.6 ± 2.6	19.1 ± 1.4
SLF20	690.3 ± 31.1	72.8 ± 2.2	6.5 ± 0.1
SLS5	1104.0 ± 32.4	38.2 ± 3.7	19.7 ± 1.4
SLS20	440.8 ± 20.4	65.3 ± 3.5	5.1 ± 0.1
SLFUS	783.8 ± 31.0	70.5 ± 2.9	28.8 ± 1.7
SLFHT	1344.0 ± 70.8	78.3 ± 3.1	24.6 ± 1.1
GLF	576.3 ± 30.5	95.5 ± 1.1	2.9 ± 0.5
GLG	311.7 ± 24.8	93.9 ± 2.2	2.5 ± 0.5
CLS	95.33 ± 6.7	93.2 ± 1.6	3.3 ± 0.5
CLF	231.6 ± 6.6	86.6 ± 4.6	3.4 ± 0.5
LA:Fru (7:1, 50% H_2O)	208.6 ± 13.9	95.7 ± 2.1	1.4 ± 0.1
LA:Fru (5:1, 25% H_2O)	314.8 ± 43.7	96.9 ± 2.2	1.4 ± 0.1
LA:SA (7:1, 50% H_2O)	12.0 ± 1.5	101.8 ± 1.8	0.8 ± 0.1
LA:Glu (5:1, 25% H_2O)	32.25 ± 4.2	99.3 ± 2.1	0.9 ± 0.1

First letter represents the seaweed: S – *S. muticum*, G – *G. corneum*, C – *C. tomentosum*. Second letter (L) represent Lactic acid. Other letters stand for: F – Fructose, G – Glucose, SA – Sodium Acetate, US – ultrasound, HT – high temperature. Numbers represent SLR: 1:3, 1:5 and 1:20 g/mL (when absent 1:5 g/mL was used).

For the three extracts with strong scavenging activities, over 60%, SFL3, SLF5 and SLFUS, IC_{50} values were calculated: 154.5, 136.1 and 139.5 mg DW/mL, respectively, values are expressed as means with 95% confidence intervals.

The extracts containing the highest levels of TPC, 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* showed small DPPH radical scavenging activity.

Similarly, the highest antioxidant activity estimated by FRAP assay was also shown by *S. muticum* extracts, specially SLF3 (29.9 ± 1.4 µM $FeSO_4$), SLFUS (28.8 ± 1.7 µM $FeSO_4$), SLFHT (24.1± 1.1 µM $FeSO_4$) and SLS5 (19.7 ± 1.4 µM $FeSO_4$). As observed in the other antioxidant assays, extracts of *G. corneum* and *C. tomentosum* did not show promising enough results to carried out to the formulation stage.

3.5. Evaluation of the biological activities on in Vitro cellular models

3.5.1. Cytotoxicity evaluation

The cytotoxic activity of seaweed samples and NADES (first column of each group) were evaluated on HaCaT cells after a pH adjustment to values around 5 – 7 with NaOH (40 % *w/v*). Results are presented in Figure 3. Cells' viability was significantly ($p < 0.0001$) affected by all NADES, except lactic acid:glucose (5:1). 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%.

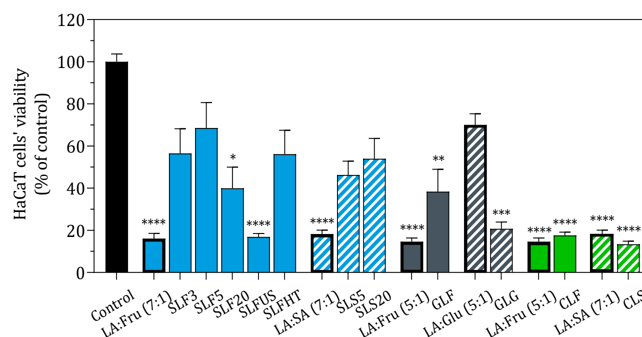

Figure 3: Cytotoxic potential of seaweed extracts on HaCaT cells. Cells' viability was evaluated after 24 h of exposure to 2 µL of extracts and the results are expressed as % of the control. The values correspond to mean ± SEM of three independent experiments. Symbols represent significant differences (Kruskal–Wallis test, Dunn's test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) when compared to the control (*). All samples were adjusted for pH levels of 5 to 7 with sodium hydroxide (NaOH 40% *w/v*).

3.5.2. 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 Figure 4.

Topical formulations are employed with pH levels between 5 and 7, therefore seaweed extracts were adjusted with NaOH (40 % *w/v*) until these values were reached [25].

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 [26,27] The results of the antimicrobial assay against this fungus are presented in Figure 4. 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 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 [26,28]. The NADES lactic acid:sodium acetate (7:1) and lactic acid:fructose (5:1, 25% H_2O) 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%. Except for CLF extract, all extracts were able to maintain microorganisms' growth without significant differences.

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 healthy skin *S. epidermidis* controls the proliferation of *C. acnes*. Nevertheless, an unbalanced equilibrium between these bacteria in pilosebaceous units, favouring the phylotype IA1 acnes strains

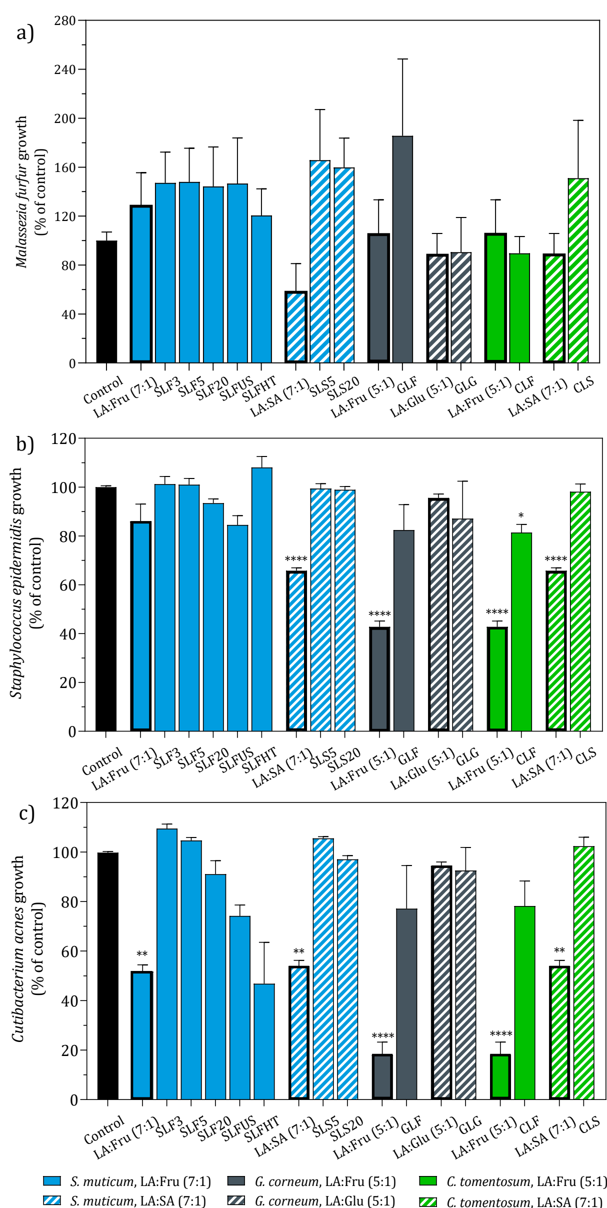

Figure 4: Antimicrobial activity of seaweed extracts (2 µL) against: a) the fungus *Malassezia furfur*, and the bacterias b) *Staphylococcus epidermidis* and, c) *Cutibacterium acnes*. The values correspond to mean ± SEM of three independent experiments. No significant differences were found (Kruskal–Wallis test, Dunn's test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) when compared to the control (*). All samples were adjusted for pH levels of 5 to 7 with sodium hydroxide (NaOH 40% *w/v*).

of *C. acnes*, 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 [28].

Lactic acid:glucose (5:1) was the only NADES which did not affect *C. acnes'* growth significantly. 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%).

The three studied microorganisms are part of this natural microbiome but, in case of disturbance can be related with various skin disorders.

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 favour 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 [28].

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 dermo-cosmetic 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.

Results of HPLC-DAD and LC-MS/MS comproved 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*. It is know 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.

3.6. Physico-chemical characterization of topical formulations

To obtain approval for a generic drug, several products characterization tests are required to prove equivalence with the reference. 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 [29]. 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.
Concerning the macroscopic organoleptic characteristics, BC and SC formulations presented a homogeneous appearance with bright white color, while the extract formulation, EC, presented a beige color due to the dark brown color of the *S. muticum* extract, SLF3. All formulations were odorless and in

respect to appearance both the solvent and extract creams appear to harden since preparation.

Phase separation was verified with centrifugation tests confirming that these formulations are not stable, meaning that selected NADES has impact in formulation stability. Phase separation suggests that this specific formulation is not able to incorporate the amount of NADES used. In fact, pH values of the formulations SC and EC were very low, around 2, due to the high content of lactic acid in the NADES used, and therefore 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 [25].

Droplet size distributions of the three formulations are represented in Figure 5. Results show a monomodal distribution in the BC emulsion, used as the control, while SC and EC emulsions presented bimodal populations. Consequently, in agreement with the centrifugation tests, the differences in droplet size distribution also point out phase separation issues for SC and EC formulations.

As a part of quality control, rheology studies assess product stability and several mechanical properties affecting product spreadability and lubricity [30,31]. 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 [25]. In Figure 6 is represented the flow curve 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 BC, 21.54 for SC and 16.26 for EC.

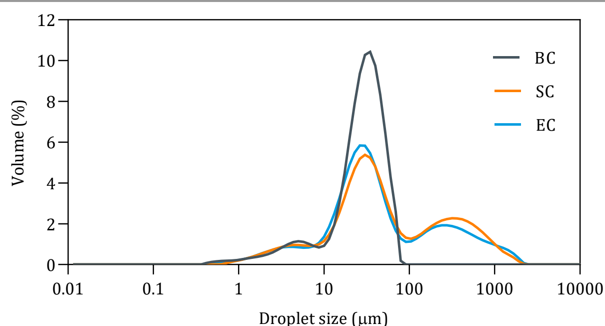

Figure 5: Droplet size distribution of seaweed formulations (n=5).

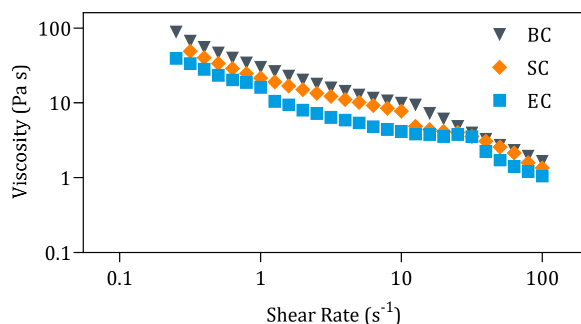

Figure 6: Flow curve of Viscosity vs. Shear Rate of seaweed formulations.

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 [32]

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 [33]. 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 high 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 to decide whether the material tested is within the specifications of the model in question [25].

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. 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 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 [25].

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 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.

SC and EC creams differ from the BC (control formulation) since they contain the NADES lactic acid:fructose (7:1, 50% *v/v* H2O) and its *S. muticum* extract, SLF3, respectively. Despite the fact that 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.

4. Conclusions

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.

Extraction results with NADES surpassed conventional extraction results, using water and ethanol (70:30, *v/v*), in all seaweeds. *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.

Enriched phenolic *S. muticum* extracts proved to be suitable for further dermatological applications, revealing great phenolic content and antioxidant activity. The extract SLF3, (seaweed - *S. muticum,* NADES - lactic acid:fructose (7:1), 50% H_2O *v/v*, 1:3 g/mL) was further employed in the preparation of a topical oil-in-water (O/W) formulation for skin care.

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.

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