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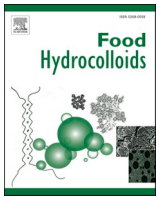

Hydrothermal extraction and thorough characterization of carrageenans and proteins from *Gigartina pistillata*

Milena Álvarez-Viñas [a,b], Fernanda Zamboni [b,c], Guido Domingo [d], Candida Vannini [d], María Dolores Torres [a,*], Maurice N. Collins [b,c], Herminia Domínguez [a]

[a] CINBIO, Department of Chemical Engineering, University of Vigo (Campus Ourense), Edificio Politécnico, As Lagoas, 32004, Ourense, Spain
[b] Stokes Laboratories, School of Engineering, Bernal Institute, University of Limerick, Limerick, V94 T9PX, Ireland
[c] Health Research Institute and AMBER University of Limerick, Limerick, V94 T9PX, Ireland
[d] Department of Biotechnology and Life Sciences, University of Insubria, Via J. Hdunant 3, 21100, Varese, Italy

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ABSTRACT

Carrageenan, an interesting biopolymer from red seaweed, possesses a myriad of applications in food, nutraceutical, or pharmaceutical industries. Although its conventional extraction used to be performed with alkaline solvents, water extraction under subcritical conditions is an alternative eco-friendly technique that has gained popularity in recent years. This work evaluates the recovery and characterization of carrageenan and proteins from *Gigartina pistillata*. The highest number of proteins was identified at the lowest processing tempeartures. Extracted carrageenan exhibits molecular weights greater than 500 kDa and good rheological behaviour, with interest for food applications and for the formulation of hydrogels when mixed with KCl. The carrageenans and carrageenan hydrogels presented interesting properties such as good thermal stability until 170 °C (TGA-DTG) and show characteristic bands of kappa/iota carrageenans in FTIR studies. Greater cell viability than 70% were achieved on NIH/3T3 fibroblast at carrageenan concentrations of 0.05 and 0.025%, whereas carrageenan extracted at 160 °C (concentration of 0.025%) displays a lower inflammatory action than other samples. In this sense, in this work the eco-friendlier extracted carrageenan was thoroughly characterized and its potentiality to be used in the biomedical field was evaluated.

1. Introduction

The awareness of the need for natural molecules with biological potential of sustainable origin has increased rapidly due to the growing concerns around climate change, population growth and unsustainable practices in general. Among different sources, seaweeds are considered an interesting option due to: being ubiquitous, presenting a high photosynthetic efficacy, not competing for arable land, little water consumption, great potential for obtaining high-added value products, among others … (Rodríguez-Jasso et al., 2013; Ruiz et al., 2013). Additionally, marine algae present diverse applications, including environmental indicators of water quality, vegetable biostimulants, food or even as source of proteins, fiber, minerals, polysaccharides, and other compounds of interest for the food, cosmetic or pharmaceutical industries. All this converts seaweed in a multifaceted resource (Mateos-Aparicio et al., 2018).

Red seaweeds are also known as Rhodophyta, which is considered the oldest phylum and has the most diversity of species. They comprise great amounts of pigments (chlorophyll *a* and *d*, carotenoids, phycoerythrin, phyllocyanin and allophycocyanin), proteins and polysaccharides, highlighting the carrageenan and agar (Carpena et al., 2021). Carrageenan has been widely studied due to its potential as anticoagulant, antithrombotic, antiviral, antitumor, antimicrobial, antioxidant, biological properties both in vitro and *in vivo* (Carpena et al., 2021).

Gigartina pistillata is an edible underexplored carrageenophyte which produces a heterogeneous type of sulfated carrageenans, which as in other *Gigartina* species, are determined by the life cycle phase (Mateos-Aparicio et al., 2018), presenting either kappa/iota carrageenan (gametophyte phase) or lambda carrageenan (tetrasporophytic phase) (Cotas et al., 2020). The carrageenans obtained vary depending on the species used, normally producing complex hybrids rather than pure carrageenans. The structure of carrageenan type is defined by the presence of 3,6-anhydro-D-galactose, the number and position of sulfate

* Corresponding author.
E-mail address: matorres@uvigo.es (M.D. Torres).

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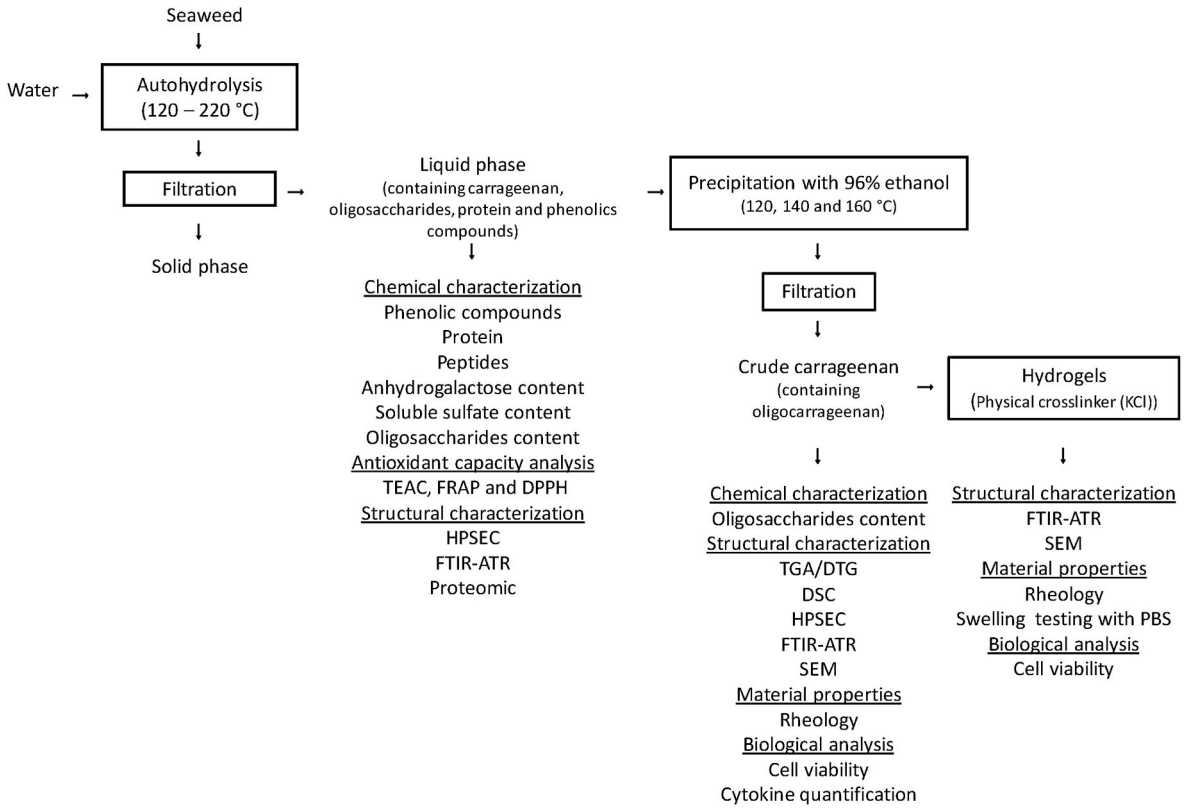

Fig. 1. Flow diagram of the experimental and analytical stages in the present study.

Table 1
Characterization of the raw material *Gigartina pistillata,* expressed in dry basis, except moisture.

	Component	Content
	Moisture (%, d.b.)	8.75 ± 0.20
	Ash (%, d.b.)	19.87 ± 0.03
	AIR (%, d.b.)	–
	Proteins (%, d.b.)	12.89 ± 0.07
	Carbon (%, d.b.)	29.45 ± 0.27
	Hydrogen (%,d.b.)	4.59 ± 0.08
	Sulfates (%, d.b.)	11.73 ± 0.01
	Extractives (%, d.b.)	4.51 ± 0.03
Carbohydrates (%, d.b.)	Glucose in polymeric units	7.69 ± 0.31
	Galactose in polymeric units	40.30 ± 0.53
Minerals (mg/kg)	Calcium (Ca^{2+})	4578
	Potassium (K^{+})	1458
	Magnesium (Mg2+)	5659
	Sodium (Na^{+})	20,299
	Phosphorous (P3+)	1458
	Zinc (Zn^{2+})	94.50
	Iodine (I^{-})	–
Heavy metals (mg/kg)	Arsenic (As^{2+})	11.20
	Cadmium (Cd^{2+})	0.40
	Copper (Cu^{+})	3.90
	Mercury (Hg^{+})	<20
	Iron (Fe^{2+})	85.40
	Lead (Pb^{2+})	0.50

Data are given as mean ± standard deviation, except for minerals and heavy metals where standard deviations were <2% in all cases and were independently studied.

groups, and the conformation of the pyranosidic ring (Gómez-Ordóñez & Rupérez, 2011). For instance, kappa carrageenan is usually obtained from *Kappaphycus alvarezii* (commercially known as "cottonii"), iota carrageenan from *Euchema denticulatum*, and lambda carrageenan from different species of *Gigartina* and *Chondrus* (Pereira & van de Velde, 2011). Depending on the type of carrageenan the chemical composition and, hence, their rheological properties can differ. Kappa and iota carrageenan contain 3,6-anhydro-galactose units, being kappa carrageenan able to form hard, strong, and brittle gels, while iota carrageenan forms soft and weak gels. Lambda carrageenan does not have the ability to form gels (it only contains galactose residues), and it is used as a thickening agent (Pereira & van de Velde, 2011). Considering all the above, and although the controversial around the use of carrageenan, it has become a promising biopolymer to be used in the food, pharmaceutical and biomedical industries via the manufacture of flexible edible films and coatings (Sedayu et al., 2019), as thickening/gelling agent (Zia et al., 2017), or as drug delivery system (Li et al., 2014) amongst others.

Although conventional methods for extracting carrageenan can be effective, they are based on the use of alkali mixtures such as KOH (Rafiquzzaman et al., 2016) or NaOH (Al-Alawi et al., 2011; Azevedo et la., 2013) which has environmental consequences. In contrast, the employment of subcritical water is an interesting alternative, green technology. High temperatures enable the recovery of water-soluble compounds due to the self-ionization of water (Cheng et al., 2021). The carrageenan obtainment by water extraction methods is still an under explored strategy; however, Gereniu et al. (2018) studied the use of subcritical water in combination with ionic liquids to recover carrageenan from Solomon Islands red seaweed, while Bahari et al. (2021) evaluated the temperature (22–90 °C) and extraction time (2–8 h).

The current work aims to study the potential of the red seaweed *Gigartina pistillata* as a source of proteins and carrageenan. For that reason, it was processed by an environmentally friendly process such as subcritical water, and the carrageenan was subsequently precipitated with ethanol. The biopolymer was thoroughly characterized to determine its physiochemical properties and further analyzed via an ELISA test to determine their influence on the immune response and were subsequently utilized to produce hydrogels which were further characterized in terms of physiochemical properties and cell viability studies.

Table 2
Chemical composition and antioxidant capacity of water *Gigartina pistillata* extracts with carrageenan after hydrothermal extraction.

		Temperature (°C)					
Parameter		120	140	160	180	200	220
Severity (S_0)		1.44	1.99	2.55	3.24	3.82	4.45
Solid yield (g solid residue/100 g dry alga)		48.84	45.19	28.40	19.58	8.96	13.60
Liquid yield (g extract/100 g dry alga)		51.16	54.81	71.60	80.42	91.04	86.40
pH		6.66 ± 0.01	6.57 ± 0.01	6.50 ± 0.01	2.33 ± 0.01	1.45 ± 0.01	1.42 ± 0.01
Conductivity (eq-g NaCl/L)		3.31 ± 0.23	3.96 ± 0.17	1.21 ± 0.06	4.66 ± 0.09	11.96 ± 0.13	14.07 ± 0.23
Phenolic content (eq g GAE/100 g extract)		0.07 ± 0.02	0.12 ± 0.01	0.26 ± 0.00	1.57 ± 0.16	2.03 ± 0.07	3.72 ± 0.07
Antioxidant capacity	**TEAC (g Trolox/100 g extract)**	–	–	0.20 ± 0.08	1.37 ± 0.11	4.54 ± 0.14	8.36 ± 0.82
	FRAP (g ascorbic acid/100 g extract)	0.70 ± 0.15	0.94 ± 0.06	0.56 ± 0.02	0.45 ± 0.01	1.70 ± 0.06	4.42 ± 0.13
	DPPH (IP (%))	–	–	–	2.85 ± 0.00	10.68 ± 0.00	22.63 ± 0.00
Protein content (g BSA/100 g extract)		0.51 ± 0.05	0.57 ± 0.04	0.72 ± 0.02	0.73 ± 0.01	1.03 ± 0.01	0.51 ± 0.02

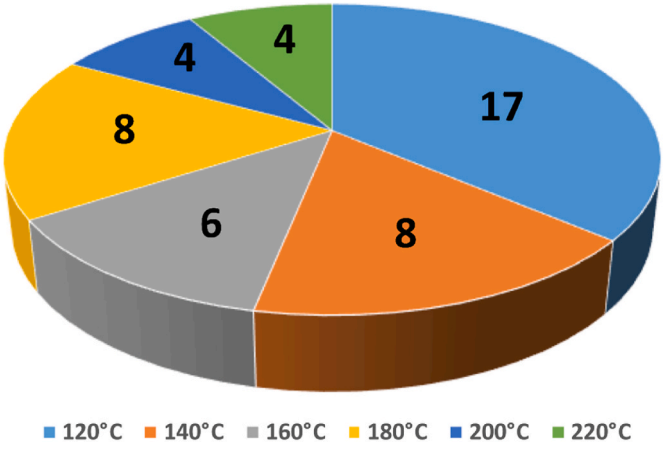

Fig. 2. Influence of the hydrothermal treatment temperature on the number of proteins identified in the crude extracts.

This work explores the potential of *Gigartina pistillata* as alternative red seaweed to other more commonly employed, the extraction of carrageenan by environmentally friendly processes (in comparison to the alkaline process usually used) and its characterization and evaluation as possible source to be used in the biomedical field.

2. Materials and methods

2.1. Materials

Gigartina pistillata was pursached to Mar de Ardora (A Coruña, Spain) and maintained frozen until further processing.

Dulbecco's modified Eagle Medium (DMEM), trypsin-EDTA 0.25%, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine and antibiotics and all other reagents used in this work were of analytical grade purchased from Sigma Aldrich (St. Louis, MO, USA). Alamar Blue (AB) cell viability reagent was purchased from Invitrogen (Paisley, UK). NHI3T3 fibroblast cell line derived from mouse was purchased from commercial source. Mouse granulocyte macrophage colony stimulating factor (GM-CSF) ELISA MAX Deluxe set as purchased from BioLegend (San Diego, CA, USA). Commercial carrageenan was purchased from Santa Cruz Biotechnology (sc-205622 B).

2.2. Autohydrolysis or water extraction with water under subcritical conditions

The frozen algae was dried at 50 °C until constant weightpreviously to the extraction uisng subcritical water treatment in a non-isothermal regime by means of a pressurized reactor (Parr Instruments, Illinois, USA) . A liquid:solid ratio of 30:1 (w/w) was used and the samples were heated up to a maximum temperature in the range of 120–220 °C. Then, the reactor was cooled down and the liquid and solid phases were

Table 3
List of all identified proteins in the extracts.

			Intensity					
Sequence coverage [%]	Mol. Weight [kDa]	Total Intensity	120 °C	140 °C	160 °C	180 °C	200 °C	220 °C
1.9	64.643	11,264,000	5,208,500	2,415,700	0	2,103,400	0	1,536,600
4.6	14.438	1,951,100	1,521,500	224,020	205,630	0	0	0
11.2	17.466	1,816,000	1,367,500	111,070	222,150	115,190	0	0
14.3	17.459	2,238,200	1,178,600	327,650	647,700	84,335	0	0
1.2	138.28	2,058,100	1,161,900	157,100	0	307,680	383,860	47,562
19.6	5.8395	686,960	686,960	0	0	0	0	0
9.7	11.469	649,850	649,850	0	0	0	0	0
5	23.872	424,980	424,980	0	0	0	0	0
3.2	24.745	702,730	366,650	0	336,080	0	0	0
0.4	358.92	351,580	351,580	0	0	0	0	0
0.8	187.45	195,030	195,030	0	0	0	0	0
17.5	21.72	535,970	191,350	62,230	0	0	282,390	0
11.9	18.586	190,030	70,804	0	119,230	0	0	0
2.2	105.87	53,734	53,734	0	0	0	0	0
0.6	157.1	231,830	33,495	129,270	20,531	26,227	0	22,304
1.1	200.78	55,374	28,340	0	0	0	27,034	0
12.9	18.656	1,400,400	18,053	0	0	1,382,300	0	0
1.3	105.62	28,614	0	28,614	0	0	0	0
2.4	65.496	43,202	0	0	0	43,202	0	0
1.2	67.207	366,660	0	0	0	0	0	366,660
0.8	93.83	0	0	0	0	0	0	0
1.1	100.27	10,838	0	0	0	0	10,838	0
5.1	30.827	113,540	0	0	0	113,540	0	0

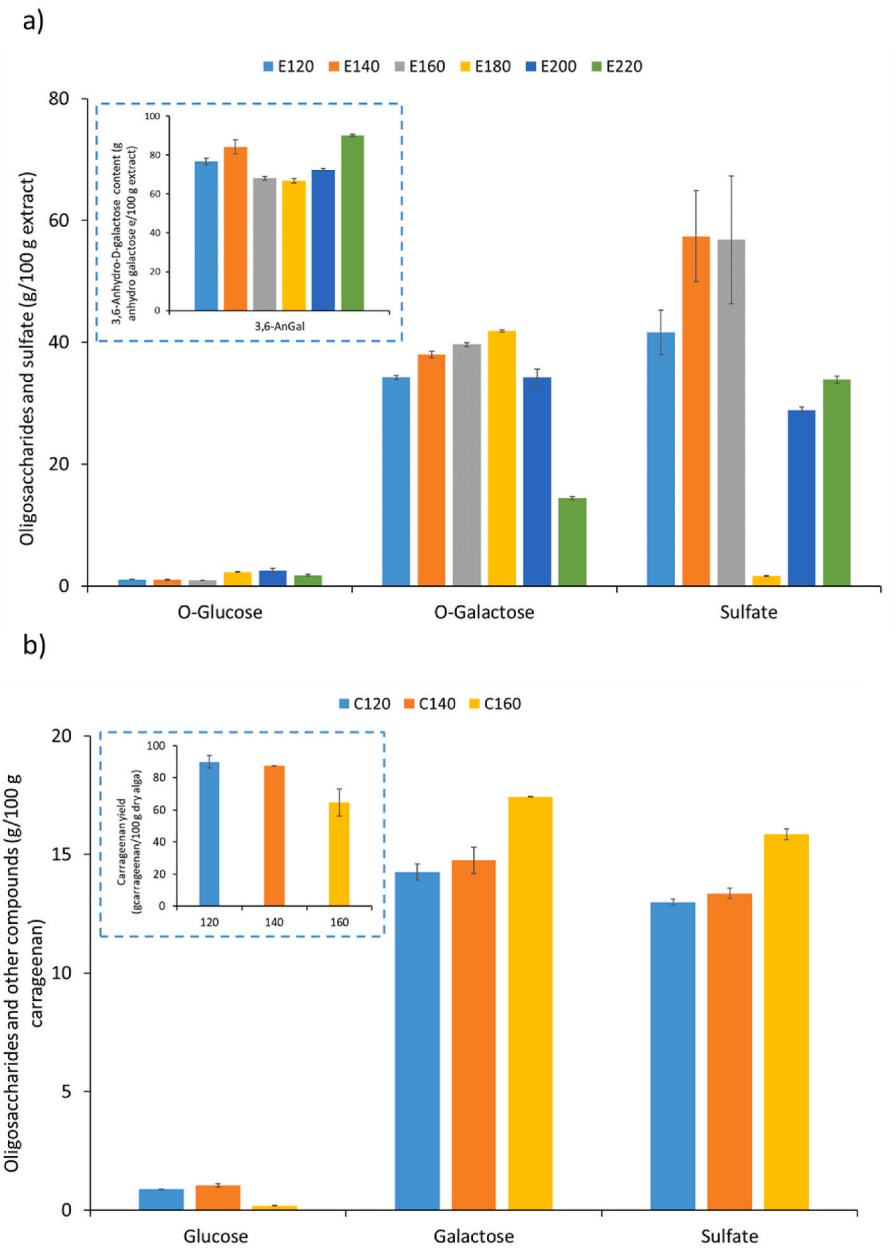

Fig. 3. Influence of the hydrothermal treatment temperature on the characterization of (a) crude extract obtained at 120–220 °C (E120-E220) and (b) crude carrageenan obtained at 120–160 °C (C120–C160).

separated by filtration. The carrageenan solubilized in the liquid phase was precipitated by adding 96% ethanol in a liquid:ethanol ratio 1:1.5 (v/v) (Álvarez-Viñas et al., 2022), vacuum filtered, washed with ethanol, dried and ground, and then stored at room temperature until analysis. The process flow diagram and corresponding product analysis are summarized in Fig. 1. BY MEANS

2.3. Hydrogel formulation

The previously extracted carrageenan (named C120, C140, and C160 when extracted at 120, 140 and 160 °C) was dissolved in distilled water at 90 °C with vigorous stirring. Once the carrageenan has been dissolved, it was poured into circular molds and the solution (3 M KCl) was added. They were left in the refrigerator (4 °C) overnight. For the formulation of the hydrogels, the minimum necessary amount of carrageenan was used to obtain a manageable hydrogel (C120 = 1.5%; C140 = 2.5%; C160 = 10%).

2.4. Analytical methodology

2.4.1. Chemical analysis

2.4.1.1. Characterization of the raw material. Chemical characterization of the red seaweed was performed employing the methods described by NREL for moisture (NREL/TP-510-42,621) (Sluiter et al., 2008a), Sluiter et al., 2008b ashes (NREL/TP-510-42,622) (Sluiter et al., 2008b), Sluiter et al., 2008b extractives (NREL/TP-510-42,619) (Sluiter et al., 2008d) and determination of structural carbohydrates (NREL/TP-510-42,618) (Sluiter et al., 2008c). This last process involves the generation of a hydrolyzed liquid phase and an insoluble solid phase (also known as acid insoluble residue-AIR). The liquid phase would be filtrated by 0.45 μm membranes and injected in HPLC for the determination of monomers using a 1100 series Agilent HPLC (Santa Clara, CA, USA) equipped with an Aminex HPX-column 87H (300 × 7.8 mm, BioRad, Hercules, CA, USA) operating at 60 °C with a mobile phase composed of 3 mM H_2SO_4

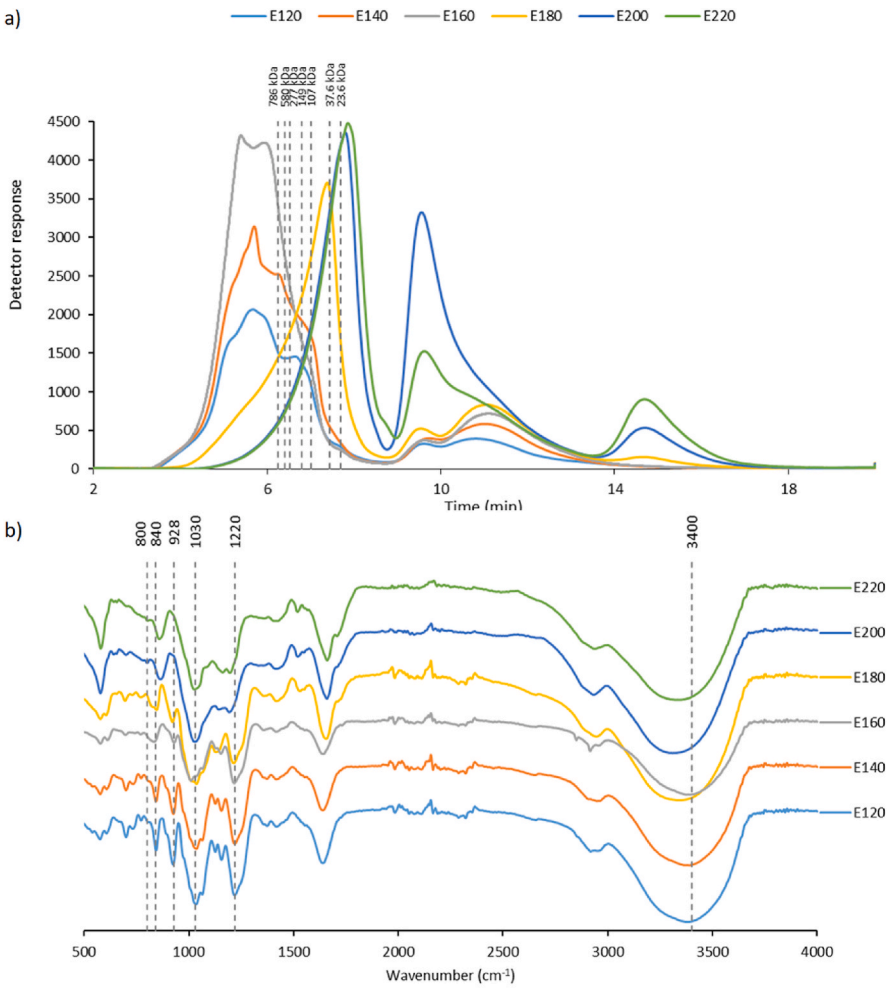

Fig. 4. Influence of hydrothermal treatment on *G. pistillata* crude extracts (obtained at 120–220 °C (E120-E220)) on (a) HPSEC and (b) FT-IR profiles.

at a flow rate of 0.6 mL/min, and a refractive index detector at 35 °C.

The protein content was calculated based on the nitrogen content measured by the Kjeldahl method and the conversion parameter regarding red seaweed (4.92) was applied (Lourenço et al., 2002). The sulfate content was measured by ion chromatography (Metrohm Advanced IC-861, Herisau, Switzerland) (Gómez-Ordóñez et al., 2010). The content of minerals and heavy metals was evaluated by acid digestion (HNO_3 and H_2O_2 at 1600 W for 15 min and 200 °C for 10 min) using a Marsxpress microwave (CEM, Charlotte, NC, USA). Na and K were measured by Atomic Emission Spectrophotometry (AES), while Zn, Ca, Mg, Fe and Cu were measured using Atomic Absorption Spectrophotometry (AAS) (220 Fast Sequential Spectrophotometer, Varian, CA). Cd and Pb were measured by inductively coupled plasma mass spectrometry (ICP-MS) (X Series, Thermo Scientific, USA) (Álvarez-Viñas et al., 2022).

2.4.1.2. Characterization of the liquid phase and carrageenan. Incubation (15 min) of the liquid phase together with the Bradford reagent (Sigma, Spain) enabled to measure the soluble protein at 595 nm, using bovine serum albumin (BSA, Sigma, Spain) as standard, following the manufacturer specifications.

The incubation of the sample with Folin-Ciocalteau reagent (1 N) and 10% sodium carbonate in the dark at room temperature for 1 h enabled to measure the total phenolic content at 730 nm, using gallic acid (Sigma, Spain) as standard (Singleton & Rossi, 1965).

The liquid phase was subjected to an acid posthydrolysis (121 °C for 20 min) in order to measure the oligomeric fraction. The obtained hydrolyzed liquid phase was filtrated and analyzed by HPLC as explained in a previous section for monomers quantification.

2.4.1.3. Antioxidant properties. The antioxidant capacity assays of the liquid phase were performed in an Evolution 201 UV-VIS spectrometer (Thermo Scientific, Germany) following the methods reported by Benzie and Strain (1996) to quantify the ferric reducing antioxidant power (FRAP), by Re et al. (1999) to determine the Trolox equivalent antioxidant capacity (TEAC) and by Von Gadow et al. (1997) to determine the α,α-diphenyl-β-picrylhydrazyl (DPPH·) free radical scavenging capacity.

All experiments were carried out in triplicate.

2.4.1.4. LC-MS/MS analysis and downstream bioinformatic analysis. The proteins present in the carrageenan extracts were characterized by LC-MS/MS analysis. Protein samples were loaded onto a standard Laemmli-type polyacrylamide gel, allowed to stack and enter the resolving gel but not to separate. At the end of the run, gel pieces were excised and subjected to in-gel digestion as reported elsewhere (Domingo et al., 2023). Tryptic peptides were reconstituted in LC/MS-grade water containing 0.1% (*v/v*) formic acid and analyzed by a hybrid high-resolution LTQ- Orbitrap Elite mass spectrometer coupled to a Proxeon Easy-nLC 1000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA). Peptides separation was performed onto a reverse phase column (PepMap® RSLC C18, 2 μm, 100 Å, 75 μm × 50 cm, Thermo Fisher Scientific, Waltham, MA, USA) with an ACN gradient of 5–30% containing 0.1% formic acid for 240 min. The tandem mass spectrometer scan was performed in positive ion mode, with top 15 at

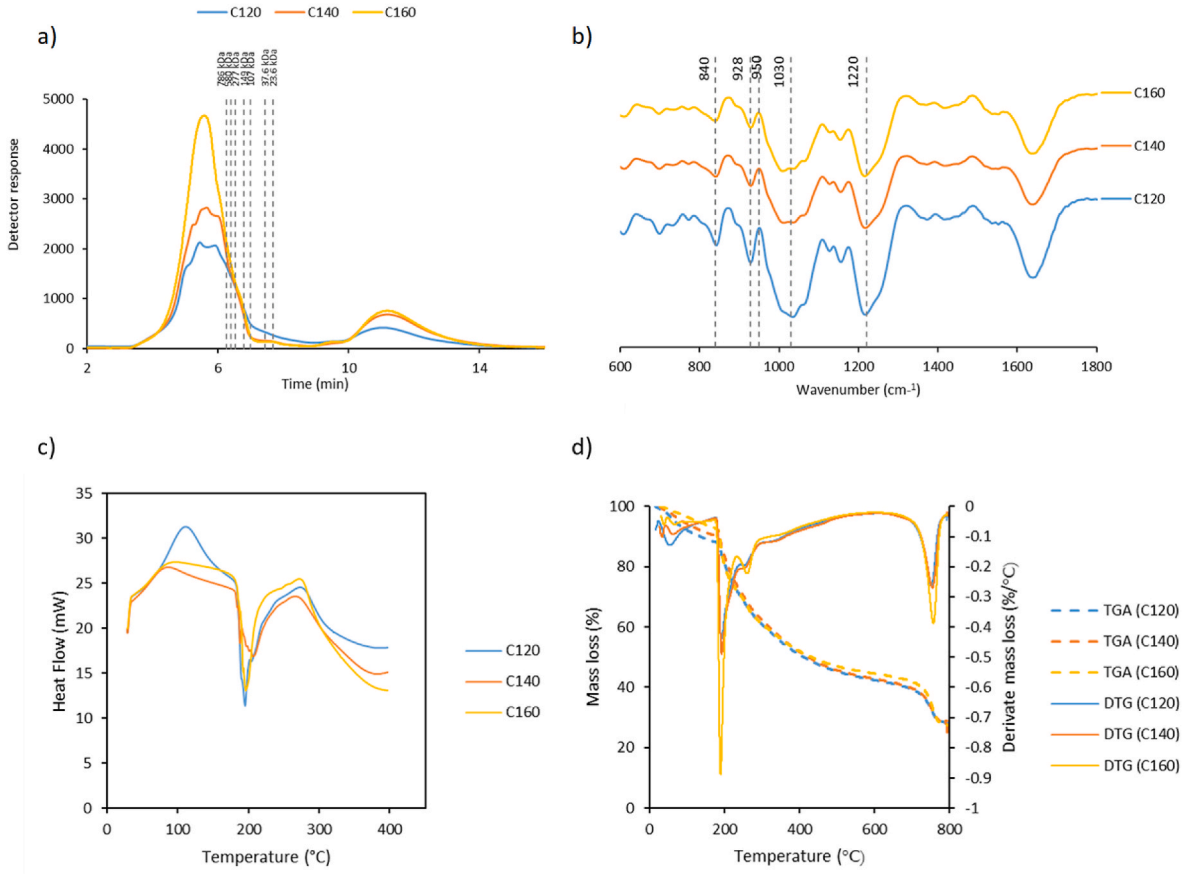

Fig. 5. Influence of hydrothermal treatment on *G. pistillata* carrageenan obtained at 120–160 °C (C120–C160) on (a) HPSEC; (b) FT-IR profiles; (c) DSC thermogram and (d) TGA/DTG.

35% normalized collision energy and a dynamic exclusion time at 30 s. The minimum signal threshold was set at 1000, the resolution at 30,000, and the isolation width at 2.0 Da. A full MS scan was performed from 390 to 1700 m/z with resolution at 120,000.

Raw data were searched by using the MaxQuant program (v.1.5.3.3, http://www.Coxdocs.org/doku.php?id=maxquant:start, accessed on January 18, 2023) against the Rhodophyta database (downloaded on January 8, 2024 from www.uniprot.org). The search criteria were set as follows: two missed cleavages, fixed modification of cysteine (carbamidomethylation), variable modifications of methionine (oxidation) and phosphorylation on serine, threonine and tyrosine, minimum peptide length of six amino acids, precursor mass tolerance 4.5 ppm for the main search. The match between runs (time window of 0.7 min) and target-decoy search strategy (revert mode) options were enabled. A false discovery rate (FDR) of 1% was accepted for peptide and protein identification, respectively. The raw data obtained were processed using an in-house tool (Mancini et al., 2022). Incorrect identifications, such as contaminants and not consistent identifications, were filtered out.

2.4.2. Structural analysis characterization

2.4.2.1. Fourier-Transform Infrared Spectroscopy. The freeze-dried liquid phase, carrageenans and hydrogels were subjected to Fourier-Transform Infrared Spectroscopy (FTIR) using a Nicolet 6700 spectra. The obtained spectra were logged in a wavelength from 400 to 4000 cm^{-1} using a resolution of spectrum of 4 cm^{-1} and 32 scans min^{-1} using the OMNIC software (Álvarez-Viñas et al., 2022).

2.4.2.2. High performance size exclusion chromatography. The high-resolution size exclusion chromatographic technique (HPSEC) was used to determine the molar mass distribution of the crude extracts and the extracted carrageenans. Two TSKGel SuperMultipore PW-H columns in series (6 × 150 mm) (Tosoh Bioscience, Germany) equipped with a TSKGel SuperMP (PW)–H guard column (4.6 × 35 mm) and a refractive index detector were used. Milli-Q water (0.6 mL/min) was used as the mobile phase. Poly (ethylene oxide) between $2.36{\cdot}10^4$ and $7.86{\cdot}10^5$ g/mol (Tosoh Bioscience, Japan) was used as standards (Álvarez-Viñas et al., 2022).

2.4.2.3. Differential scanning calorimetry (DSC). Crude carrageenans were subjected to differential scanning calorimetry using a PerkinElmer DSC 6 using a nitrogen gas flow of 20 mL/min, and a temperature profile 30–400 °C at a heating rate of 10 °C/min (Chiani et al., 2023).

2.4.2.4. Thermogravimetric analysis (TGA-DTG). Crude carrageenans were subjected to thermogravimetric analysis using a PerkinElmer TGA 4000, determining the weight loss against temperature modulation. Samples were purged under nitrogen at 20 mL/min and a heating rate of 10 °C/min from room temperature to 800 °C (Chiani et al., 2023).

2.4.2.5. Swelling studies. Carrageenan-based hydrogels were freeze-dried and submerged in phosphate buffer saline (PBS, pH 7.4) at a temperature of 37 °C. The swelling ratio against time was evaluated by weighing the hydrogels at set times after being dried with blotting paper (Chiani et al., 2023).

The swelling ratio (SR) was determined as:

$$SR = \frac{W_s - W_D}{W_D} \times 100\%$$

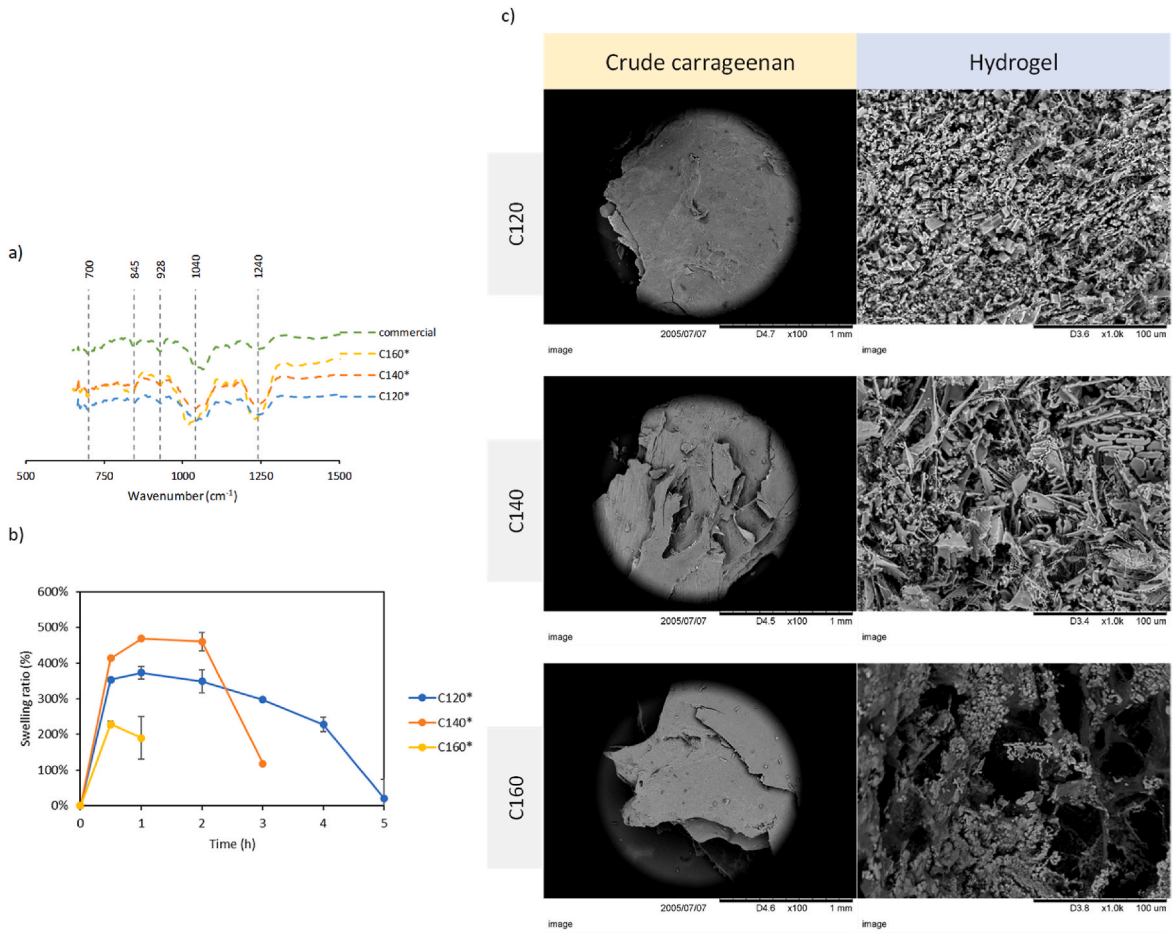

Fig. 6. (a) FT-IR profiles; (b) swelling ratio and (c) SEM images comparing crude carrageenan obtained at 120–160 °C (C120–C160) and carrageenan hydrogels (C120*-C160*).

where W_D reflects the dry weight and W_s represents the weight of the sample after the incubation time. All swelling measurements were performed in triplicate.

2.4.2.6. Rheological studies. Biopolymer-based aqueous systems and hydrogels were subjected to rheology measurements using a Discovery Hybrid Rheometer (DHR-2, TA Instruments, Delaware, USA) at room temperature. Disposable aluminium plates of 25 mm were used as measurement geometry with 1 mm gap between plates. Steady state shear determinations were carried out at different shear rates from 0.1 to 10 s^{-1}. Small amplitude oscillatory shear determinations were made within the linear viscoelastic region (1 % strain) defined though the corresponding strain sweeps conducted at 1.6 Hz (Chiani et al., 2023).

2.4.3. Biological studies

2.4.3.1. In vitro cell proliferation study. Crude carrageenan solutions (0.5%, 0.05% and 0.025%) and hydrogels were evaluated by in vitro cell viability using the Alamar Blue test (Zamboni et al., 2022).

The crude carrageenan was sterilized by autoclave at 123 °C for 15 min while the hydrogels were exposed to UV light and submerged in DMEM (Dulbecco's Modified Eagle Medium) to attain equilibrium at 37 °C for 24 h.

Fibroblast cells (NIH/3T3) were cultured in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1 % antibiotics (penicillin and streptomycin) in a 5% CO_2 environment. Cells were seeded on carrageenan solution or preconditioned hydrogels employing a cell density of 50,000 per well in a 24-well plate and incubated overnight with a supplementation of 1 mL of cell culture medium. The determination of cellular cytotoxicity was assayed at 4 h after adding Alamar Blue™ to a 10% of the well volume. The measurement was carried out by fluorescent cell emission (Agilent BioTek monochromator-based multimode reader (Synergy Mx) with time-resolved fluorescence (UK)) at wavelength of 540–590 nm. Controls were incubated without the presence of carrageenans or their hydrogels.

2.4.3.2. Cytokine quantification by ELISA. Carrageenan solutions (0.5%, 0.05% and 0.025%) were subjected to immune activation assay by ELISA test. NIH/3T3 cells were employed at a cell density of 50,000 per well during 24 h before the cytokine quantitation. After that time, the production of Gm-CSF (cytokines which induces macrophage differentiation as part of the immune response chain) was determined employing mouse granulocyte macrophage colony stimulating factor (GM-CSF) ELISA MAX Deluxe kit from BioLegend (San Diego, CA, USA) as defined by the manufacturer (Zamboni et al., 2022).

3. Results and discussion

3.1. Chemical analysis

3.1.1. Raw material

The proximal composition of *Gigartina pistillata* specimens used in the present study is summarized in Table 1. The extractives, ash, and protein content account for 4.51, 19.87, and 12.89% of the dry weight, respectively. Carbohydrates, expressed as polymers, represented 47.99% of the total content, being galactose the main constituent

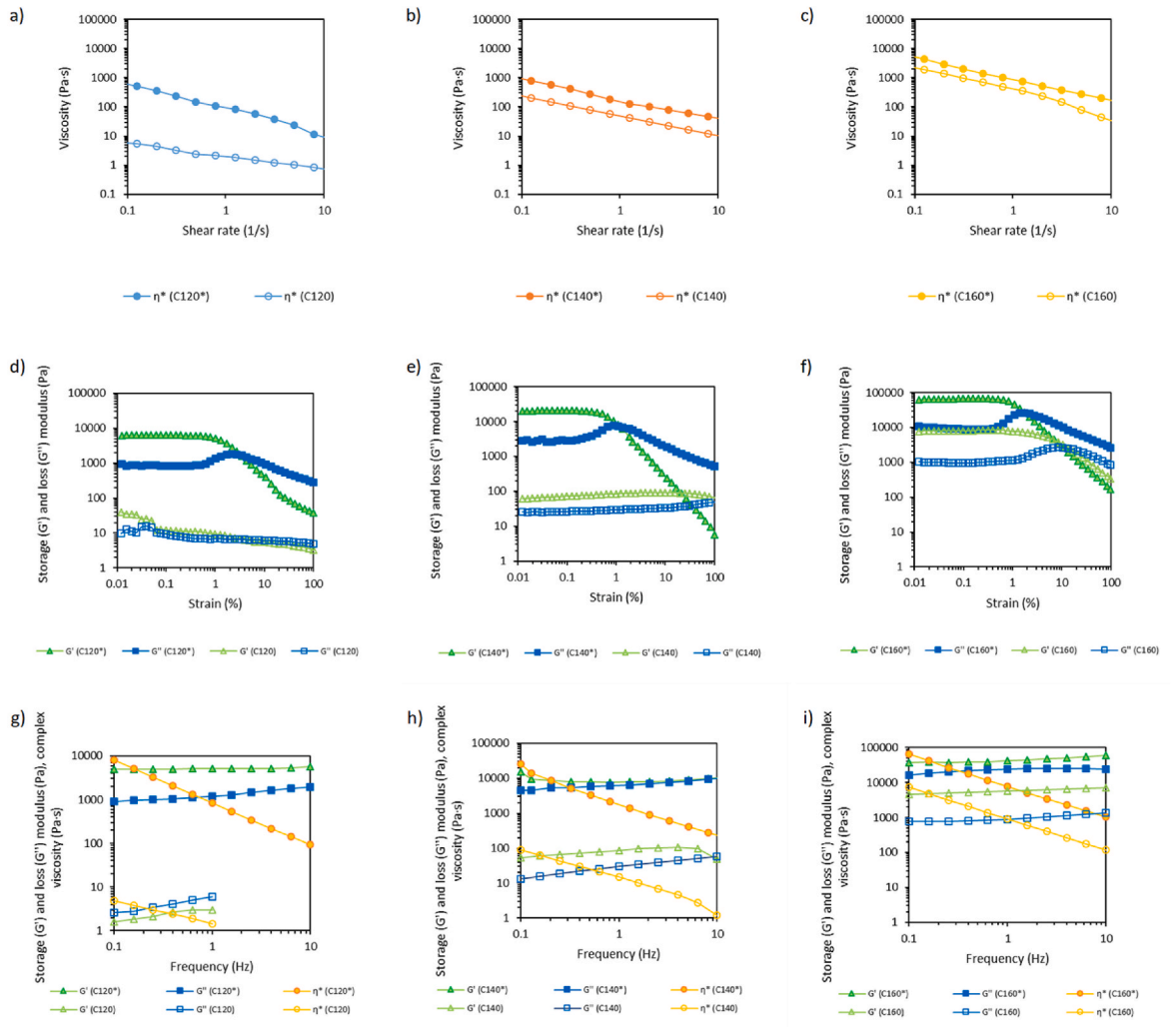

Fig. 7. Comparison of the rheology properties of the aqueous solutions of the carrageenan (C120, C140, C160) and the corresponding hydrogels (C120*, C140*, C160*) in terms of (a, b,c) steady-state shear measurements; (d, e, f) oscillatory shear strain and (g, h, i) oscillatory shear.

saccharide, which accounted for more than 40%. A slightly higher carbohydrate content (67.08%) was attained by Gereniu et al. (2018) when studying the red seaweed *Kappaphycus alvarezii*, although presenting lower ash (15.48%) and protein (5.64%) contents. Similar protein and ash content was attained when characterizing *Gracilaria corticata* (Premarathna et al., 2022). Regarding mineral content, sodium (20.3 g/kg) is by far the most abundant, followed by potassium (14.6 g/kg), magnesium, (5.7 g/kg), and phosphorus (1.5 g/kg). Iodine, zinc, iron, and calcium are found in minor amounts. Similar values of potassium and zinc were found in *Gracilaria corticata* by Premarathna et al. (2022).

3.1.2. Liquid phase and carrageenan

The seaweed was subjected to subcritical water extraction with distilled water. The obtained solid and liquid phases were separated by vacuum filtration.

Table 2 exhibits the extraction yields, phenolic and protein contents besides its antioxidant capacity. Minor differences were found in the extraction yield at the mildest temperatures (120 and 140 °C). Nevertheless, there was a marked increase in the overall extraction yield at higher temperatures, reaching values up to 91% at 200 °C. Overall, similar extraction yields were attained when processing *K. alvarezii* with pressurized hot water, reaching values up to 95% at 300 °C (Gereniu et al., 2018).

At lower temperatures of extraction (120–160 °C), the pH remained neutral, whereas harsher extraction conditions provoked acidification of media, reaching values as low as 1.42, which may correspond to the liberation of acidic groups (such as sulfate or acetyl). On the other hand, the conductivity showed a similar behaviour, maintaining values lower than 4 g eq NaCl/L at temperatures of 120–160 °C up to values of 14.07 g NaCl eq/L at 220 °C. A similar trend on extraction yield and pH was observed by Pangestuti et al. (2019) when subjecting *Hypnea misciformis* to subcritical water extraction.

The influence of extraction temperature on the phenolic content (expressed as gallic acid equivalents per 100 g extract) of the extract showed a continuous increase from 0.07 g/100 up to 3.72 g/100 g at 220 °C. An increase in the phenolic content was also observed by Topuz et al. (2016) when processing the red seaweed *Laurencia obtuse* by ultrasound-assisted extraction.

The reducing power of the ferric ion reached a maximum of 4.42% at the highest temperature, as did the antiradical properties (TEAC and DPPH), which increased notably at the highest temperatures (see Table 2), following an expected trend with the phenolic content. This behaviour was also observed by Gereniu et al. (2018) after processing *K. alvarezii* by pressurized hot water. A similar value of phenolic content, close to 4 g GAE/100 g, was obtained when processing *Hypnea musciformis* by subcritical water at 210 °C, although presenting lower TEAC value of 0.01 g Trolox eq/100 g (Pangestuti et al., 2019) than that obtained in this study.

The protein content presented a slight increase up to the temperature of 200 °C, corresponding to a maximum of 1% of the dry weight.

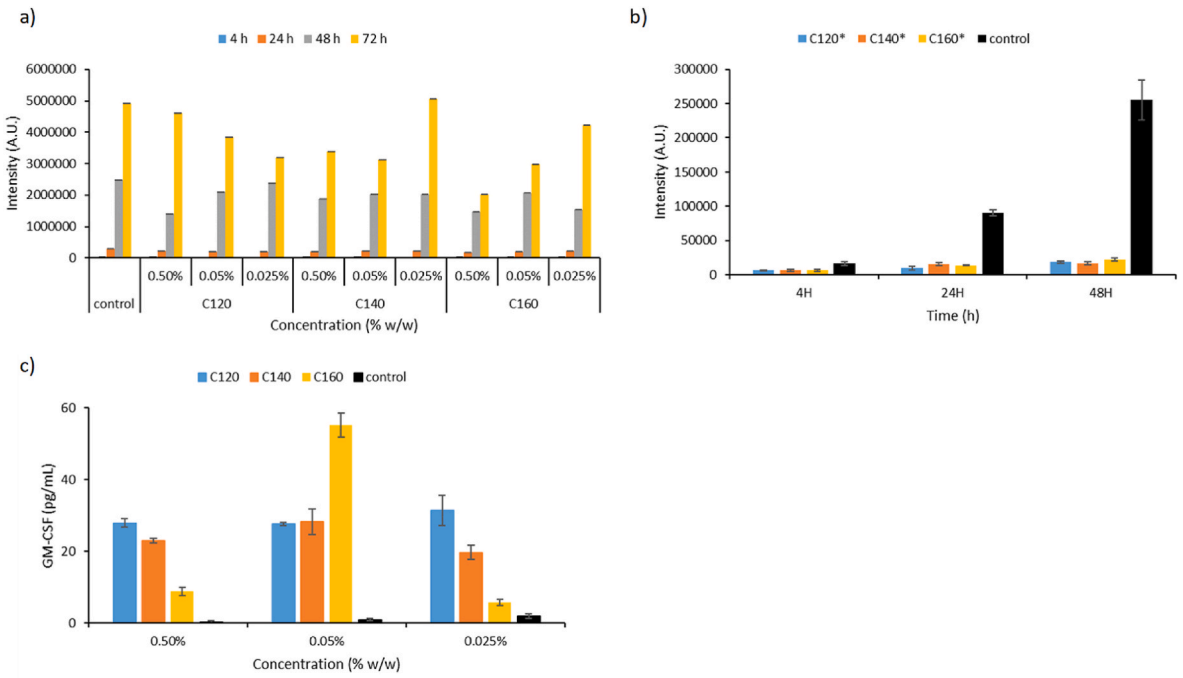

Fig. 8. Influence of hydrothermal treatment with crude carrageenan (C120, C140 and C160) of *G. pistillata* using different solution concentrations on (a) the cell proliferation of NIH/3T3 fibroblasts exposed to different concentrations of carrageenan using the Alamar Blue assay; (b) the pro-inflammatory GM-CSF cytokine production profile using ELISA test and (c) influence of the crosslinker concentration on hydrogel formulation (C120*, C140* and C160*) on cell proliferation of NIH/3T3 using the Alamar Blue assay.

Nevertheless, proteomics analysis revealed that samples produced by water extraction under subcritical conditions at 120 °C yielded the highest number of proteins (Fig. 2). The number of proteins identified decreased as the temperature increased (Table 3). Most of the proteins extracted were phycobiliproteins (PBPs), such as phycocyanins, allophycocyanins, g-cyanins. A gerythrin, protein with high levels of similarity with R-phycoerythrins, according to the BLAST analysis (Fig. 2), was also detected. Phycobiliproteins were present in large quantities, especially in samples treated at temperatures between 120 and 180 °C.

PBPs are water-soluble proteins commonly found in cyanobacteria and algae, such as rhodophytes, which play a crucial role in capturing light energy during photosynthesis and are composed of a complex between proteins and covalently bound phycobilins (Dagnino-Leone et al., 2022).

Due to the harmful effects of many synthetic dyes, there is a growing interest to use natural colors for a wide range of applications and PBPs are of great interest in biotechnological applications in the pharmaceutical, food, and cosmetic industries (Sekar & Chandramohan, 2008). PBPs are also crucial in fluorescent-based detection systems, serving as essential components in a vast industry centered on sensitive fluorescence detection. They are widely utilized in fluorescence-based methods like flow cytometry, enabling high detection sensitivity (Chen et al., 2023; Jung & Dailey, 1989).

Furthermore, increasing reports have described the health-promoting features of phycobiliproteins, demonstrating their pharmaceutical potential. PBPs display a range of bioactivities, including antioxidant, anti-tumoral, anti-inflammatory, neuroprotective, anti-diabetic, anti-viral and hepatoprotective properties. They also hold potential for development as photosensitizers in tumor therapy (Braune et al., 2021; Pendyala et al., 2021; Qiang et al., 2021; Reddy et al., 2000; Soni et al., 2008, 2009).

Although red algae have already been considered excellent sources for the extraction of sustainable and bioactive proteins (Carpena et al., 2023; Gregersen Echers et al., 2022), their use has not yet been fully investigated. In light of the considerable commercial application of PBPs, purity plays a major role and research is being conducted to improve the production, extraction, and purification processes, with a focus on sustainable and scalable methods (Wang et al., 2023). In this regard, the use of water extraction methods for obtaining proteins remains an underexplored strategy.

Fig. 3a exhibits the oligosaccharides content in the extract, besides the sulfate and 3,6-anhydrogalactose content. The predominant polymer in the extract comprises the galactooligosaccharides, which value increases up to 41.86 g/100 g extract at the temperature of 180 °C. However, the processing of the seaweed at higher temperatures triggers the hydrolysis of the oligomers, diminishing the galactooligosaccharides content and probably provoking the formation of galactose monomers or different degradation products, such as hydroxymethylfurfural. A similar trend can be observed in glucooligosaccharides, but reaching lower amounts (up to 2.53 g/100 g extract). Similar tendency was attained when processing *Gelidium sesquipedale* red seaweed by autohydrolysis, increasing the content in galactooligosaccharides up to a maximum at 170 °C for 40 min and increasing the content of degradation products (hydroxymethylfurfural) at harsher conditions (Gomes-Dias et al., 2020).

Carrageenan from the liquid phase was separated by ethanol precipitation, chemically and structurally characterized, and used for the formulation of hydrogels.

Fig. 3b represents the carrageenan yield and the oligosaccharides and sulfate content. Although carrageenan yield after processing at 120 and 140 °C is close to 90%, the carrageenan yield at a higher extraction temperature (160 °C) was lower. However, the galactose content seemed to be higher at 160 °C (17.43 g/100 g) than it was at 120 °C (14.25 g/100 g). Furthermore, the sulfate content follows the same trend as the galactose content, being higher in C160 (15.85 g/100 g) and lower in C120 (12.99 g/100 g). This values are in accordance to the typical sulfate values for red seaweed (Firdaus et al., 2021).

3.2. Structural characterization

3.2.1. Subcritical water extract characterization

The HPSEC technique was employed to assess the size of the

oligosaccharides obtained. As can be seen (see Fig. 4a), the extracts to lower temperatures (120–160 °C) have a size larger than 800 kDa. If it is considered that a greater response implies a greater amount, Fig. 4a would show that at the temperature of 160 °C larger oligosaccharide content would be obtained. On the other hand, from the temperature of 180 °C, the size decreases to range 370 kDa, showing even smaller sizes of 230 kDa gives the higher temperatures (200 and 220 °C).

Fig. 4b displays the FTIR spectra of crude extracts. In this case, the hydrogen bond retching vibrations of *O*–H and *C*–H bonds may be identified in the bands between 3300 and 3500 cm^{-1} and 2900 cm^{-1}, respectively (Castro-Varela et al., 2023). On the other hand, the carbohydrate ring and stretching of glycosidic bond may be represented by bands at 1000-1200 cm^{-1}, although presenting modulations owing to the carrageenan degradation (Castro-Varela et al., 2023). Finally, the asymmetric stretching of sulfated ester groups may be ascribed to the band at 1220 cm^{-1}, which is only visible for extracts of temperatures from 120 to 180 °C (Castro-Varela et al., 2023; Saluri & Tuvikene, 2020). A hybridization degree could be estimated from the FTIR spectra with values of kappa around 50 ± 2.5 mol%.

3.2.2. Carrageenan characterization

In Fig. 5a it is observed that the extracted carrageenans have molecular weights greater than 800 kDa, weights lesser than 236 kDa were also observed. High molecular weight carrageenan (average 200–800 kDa) is approved for food applications (Castro-Varela et al., 2023), these could be proposed for such applications. However, other applications for smaller fractions should be explored.

Fig. 5b shows the FT-IR spectra, where no significant differences are observed in the number of peaks of the extracted carrageenans, although a greater intensity is observed in C120. The typical kappa peak at 840 cm^{-1} was related to D-galactose-4-sulfate and the characteristic iota peak at 805 cm^{-1} was attributed to the presence of a sulfate group on the second carbon of the anhydrous ring. The existence of 3,6-anhydro-D-galactose was also corroborated by a relevant band at approximately 928 cm^{-1}. These results confirm that this alga can provide kappa/iota hybrid carrageenans (Buschmann et al., 2011). Furthermore, all samples show the main functional groups of carrageenan in the region 4000 - 600 cm^{-1} (Relleve & Abad, 2015). The signal at 934 cm^{-1} corresponds to the anhydrous bond in anhydrogalactose, which is specific to iota and kappa-carrageenans (Vandanjon et al., 2023).

Fig. 5c shows the DSC of crude carrageenans. It is observed that the glass transition temperature (Tg) for carrageenans decreases when the extraction temperature of autohydrolysis is higher; nevertheless, it varied in a narrow range between 86 and 110 °C. Conversely, the melting temperature (Tm) is around 197 °C for C120 and C160, while it is approximately 206 °C for C160. These temperatures agree with those reported in the literature for carrageenan transition from semi-crystalline to amorphous structure (Chandika et al., 2021).

The thermogravimetric data of carrageenans (120–160 °C) can be observed in Fig. 5d. The DTG curve shows a first peak at 70 °C approximately that can be ascribed to the evaporation of water. The highest mass loss can be observed at around 190 °C which is attributed to thermal degradation processes (Mahmood et al., 2014). Other polysaccharides degrade at around 250 °C, which may suggest aless amorphous state (Chandika et al., 2021). At temperatures from 700 to 800 °C, another mass loss step occurs, possibly due to the degradation of decomposition products. Only between 22.5 and 28.7 % of the initial material remains at 800 °C as a char.

3.2.3. Hydrogels characterization

Hydrogels were formulated with the extracted carrageenans and were compared with a commercial carrageenan hydrogel as a control.

Fig. 6a shows the FT-IR spectrum. In the region 650 - 1240 cm^{-1} the spectra were similar for the hydrogels with commercial carrageenan and those extracted. The bands at 1040 cm^{-1} (of the sugar ring and the stretching of the glycosidic bond) (Castro-Varela et al., 2023), and 930 cm^{-1}, where *C*–O of 3,6-anhydrogalactose were also observed (Vandanjon et al., 2023). Furthermore, peaks related to sulfates can be identified at 1240 cm^{-1} of asymmetric stretching, at 845 cm^{-1} of *C*–*O*–*S* bending vibration and at 620 cm^{-1} of *O*–*S*–O bending vibrations.

Fig. 6b displays the swelling behaviour of the hydrogels when incubated at 37 °C. The kinetics reflect non-stable hydrogels. Although they swelled to values of around 200–500% in a short period of time, they do not maintain the solvent inside its structure. This behaviour is typical of low crosslink densities where gels tend to swell quickly beyond their equilibrium point but spring back to equilibrium pushing water out once the polymeric chains relax.

The SEM micrographs of crude carrageenans (120, 140, and 160 °C) and hydrogels are shown in Fig. 6c. Regarding the carrageenans, all of them exhibit a compact structure. Alternatively, carrageenan hydrogels present a more honeycomb-like structure (especially C120 and C140).

3.3. Rheological properties of carrageenans and carrageenan hydrogels

The apparent viscosity for the aqueous solutions of the extracted carrageenans (C120, C140, C160) and the corresponding hydrogels (C120*, C140*, C160*) is presented in Fig. 7 (a,b,c). All profiles showed shear thinning behaviour with a drop of the apparent viscosity with the increase of the shear rate. As expected, the apparent viscosity was higher for the prepared hydrogels when compared with the aqueous solutions. The highest values of this parameter were identified for the systems of carrageenan prepared at the highest biopolymer content corresponding with those extracted after hydrothermal treatment at 160 °C followed by 140 and 120 °C.

Fig. 7 (d,e,f) showed that the linear viscoelastic region was determined below 1% for all tested systems. The profiles displayed in the mechanical spectra exhibited a typica gel behaviour for all carrageenan-based hydrogels (Fig. 7 (g, h, i)). Both elastic (G′) and viscous (G″) moduli were almost invariant, with G″ modulus lower than the G' modulus in the whole range of tested frequencies. Hydrogels formulated at the lowest biopolymer content corresponding with the carrageenan extracted after treatment at 120 presented the weakest gel features, followed by those extracted after processing at 140 °C and 160 °C. In all cases, the complex viscosity dropped with rising frequency. Intermediate gel strength was observed for all tested hybrid carrageenans with values comparable to those previously reported for their counterparts extracted under alkali conventional processing (Azevedo et al., 2013).

3.4. Cell viability and ELISA test of carrageenans and carrageenan hydrogels

Fibroblast cells (NIH/3T3) were used to determine the cell viability in the presence of carrageenans at different concentrations (0.50, 0.05 and 0.025%). Fig. 8a shows the influence of different carrageenan concentration on the cell viability. Although a negative effect can be observed when employing carrageenan at a concentration of 0.50% (especially for C160), the cell viability was higher than 70% for the other assays. In fact, the viability remained stable for the other concentrations (0.05 and 0.025%) tested until 72 h of assay. According to the literature, a survival rate greater than 70% does not imply cytotoxicity (Ulagesan et al., 2023). In this sense, no toxic effects were found for carrageenan concentrations of 0.05 and 0.025% during 72 h of exposure, surpassing the 70% of cell viability in all cases.

Fig. 8b exhibits the influence on viability and inflammation markers induced by the set carrageenan samples when ELISA assay was tested. The production of GM-CSF was higher than that observed in the control group. Nevertheless, a lower carrageenan concentration displayed a lower production of GM-CSF, as can be seen at concentrations of 0.025 % (especially with C160). On the other hand, a higher carrageenan concentration revealed a rapid production of GM-CSF within the first 24 h of incubation, corresponding to an acute inflammatory response.

Finally, Fig. 8c collects the cell viability results for hydrogels

synthesized from crude carrageenan. When comparing the control with the samples, it can be observed a severe decrease in the cell viability. This low cell viability can be ascribed to the KCl employed to formulate the hydrogels. However, other studies showed positive results regarding fibroblast proliferation and migration when using kappa carrageenan for the formulation of hydrogels (Dev et al., 2020; Ulagesan et al., 2023).

4. Conclusions

Gigartina pistillata was used as source for the valorization of carrageenan. Its comprehensive analysis reflected molecular weights greater than 500 kDa, ideal for food applications. Carrageenan concentrations of 0.05 and 0.025% enabled a cell viability higher than 70%, whereas a lower inflammatory effect was observed (by ELISA test) for C160 at concentration of 0.025%, remaining constant for C120 and C140 regardless of concentration. Carrageenan hydrogels presented positive rheological properties (appropriate for food applications) although performing cell viability lower than 50 %. The number of identified proteins decreased with increasing hydrothermal treatment. This work opens the path towards the separation and application of carrageenan for food-related applications by a chemical free extraction process.

CRediT authorship contribution statement

Milena Álvarez-Viñas: Writing – original draft, Investigation. **Fernanda Zamboni:** Writing – review & editing, Investigation. **Guido Domingo:** Writing – review & editing, Writing – original draft, Investigation. **Candida Vannini:** Writing – review & editing, Supervision. **María Dolores Torres:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Maurice N. Collins:** Writing – review & editing, Funding acquisition, Conceptualization. **Herminia Domínguez:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The corresponding author on behalf of all the authors of the manuscript declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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