Journal of Applied Phycology (2025) 37:2793–2807
https://doi.org/10.1007/s10811-025-03529-6

RESEARCH

Application of wet route extraction and its potential for attaining biologically active compounds from seaweed biomass: The case-study of *Gracilaria gracilis*

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Received: 7 November 2024 / Revised: 11 April 2025 / Accepted: 22 April 2025 / Published online: 13 May 2025
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Abstract

Seaweeds have been targeted as sustainable feedstock for a biorefinery approach. However, extraction is a challenge and the wet route approach (without drying) may be more sustainable and economically viable. The edible red seaweed *Gracilaria gracilis* is an excellent case-study, since it is produced for agar preparation. Hence, this study tests different extraction techniques and operational parameters in a wet route approach and compares it with the conventional procedure. The best yield results (> 40% dry matter yield) were registered when wet route was coupled either with overnight agitation (biomass:70% ethanol ratio of 1:10, w:v), pH-shift method (biomass:1 M HCl, 1:10), or ultrasound (biomass:70% ethanol, 1:10), reaching 47.1 ± 0.5%, 46.9 ± 0.1%, and 41.8 ± 0.0%, respectively. Applying overnight agitation to wet instead of dry biomass generally produced higher polyphenol contents: up to an increase from 46.3 ± 6.2 to 287.7 ± 36.9 mg GAE (100 g)$^{-1}$ dw. Ultrasound-assisted extraction of wet biomass yielded up to 309.1 ± 68.1 mg GAE (100 g)$^{-1}$ dw. The overnight agitation (wet biomass:70% ethanol, 1:10) and ultrasound-assisted extraction (wet biomass:70% ethanol, 1:10) were chosen for characterization as the best extracts and compared to a conventional dry route extract (biomass:70% ethanol, 1:20). ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) identified a benefit of wet route extraction, with 4469.5–5296.3 µmol Trolox Eq. (100 g)$^{-1}$ dw. Whereas selected ultrasound-assisted extract had a strong anti-inflammatory activity, reaching 38.3 ± 12.5% of COX-2 inhibition, the dry route extract displayed only 6.8 ± 3.0%. Results show the potential of wet route extraction processes, at least to *G. gracilis*, paving the way for scale-up and industrial development.

Keywords Rhodophyta · Bioactive compounds · Antioxidant activity · Anti-inflammatory activity · Process optimization

Introduction

The red seaweed *Gracilaria gracilis* (Gracilariales, Gracilariaceae) has a wide global distribution, being known as a source of agar and used as food in Vietnam and other Asian countries (Pereira 2016; Algaebase 2018). Its commercial importance has led to a growing interest in its production and to testing different cultivation methods (Capillo et al. 2018). In general, Gracilariales are considered to be efficient bio-filters for aquaculture waste water treatment, removing nitrogen and phosphorous salts (Huo et al. 2012; Capillo et al. 2018). They are viewed as adequate candidates for Integrated Multi-Trophic Aquaculture (IMTA), a type of aquaculture system that is more environmentally sustainable and brings together different organisms and trophic levels into the tanks/ponds of farmed fish production.

In particular, *G. gracilis* has an additional advantage of being a well-known source of biologically active compounds, such as fatty acids, sterols, peptides, polyphenols, flavonoids, essential vitamins, and many others (Francavilla et al. 2013; Capillo et al. 2018; Ebrahimzadeh et al. 2018). These compounds display a diverse array of biological activities, including antioxidant, anti-inflammatory, antifungal,

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antibacterial, and anti-tumour activities (Agatonovic-Kustrin and Morton 2017; Ebrahimzadeh et al. 2018). Moreover, it has been reported that the overall activities of *G. gracilis* extracts, obtained with either organic solvents or water, may present a clear seasonality (Francavilla et al. 2013). This may be considered an additional reason to consider *G. gracilis* cultivation, since it may enable a control of environmental conditions that ensures a more constant and reliable level of biologically active compounds, thereby improving the chances of finding further industrial application for this seaweed beyond agar.

Extracting the biologically active compounds and attaining extracts with high activity remains a key challenge, not only in algal biomass poor in such components and properties, but also in seaweeds rich in them, such as *G. gracilis* (Reboleira et al. 2020). For instance, Reboleira et al. (2020) studied extraction conditions in an optimization effort that encompassed different independent variables, such as solid-to-liquid ratio, extraction time, and ethanol percentages, with Response Surface Methodology (RSM) used to estimate the effect of each variable on the tested biological activities. This study only focused on solid–liquid hydroethanolic extraction, but other extractive technologies have been explored by others (Matos et al. 2021; Carpena et al. 2023; Perez-Vazquez et al. 2023). Some technologies may be advantageous in that are more effective in disrupting the cell wall and releasing cell contents but may also be deleterious if too drastic and destructive of more sensitive —namely more thermolabile or oxidizable— compounds (Jacobsen et al. 2019; Carpena et al. 2023; Perez-Vazquez et al. 2023). Drastic processes may be necessary as exemplified by sugar extraction in *G. verrucosa*, whose sugar content was better extracted after citric acid-catalysed pretreatment (0.1 M citric acid, 150 °C, 1 h), yielding 50.9% of total reducing sugar (Kwon et al. 2016). In this case, physical disruption was not sufficiently effective in sugar extraction (Kwon et al. 2016; Khoo et al. 2019). Recently, alternative methods and approaches have been proposed, aiming not only at improving process performance, but also at being less hazardous to the environment and consumers. In this context, the so-called green solvents have been suggested, that is, solvents that are neither toxic nor generate toxic substances that are harmful to the environment (Chemat et al. 2019). Furthermore, a biorefinery approach has also been proposed as advantageous regarding economic and environmental issues, leaving less residues after production and coming much closer to the recommended zero waste system (Matos et al. 2021).

Similarly, with a purpose to be environmentally sustainable, the so-called wet route extraction of targeted compounds has been recently proposed as a less energy-intensive and effective way to implement a biorefinery approach in the case of algal biomass (Khoo et al. 2019; Rahman et al. 2022). In a wet route process, cell disruption procedures are performed prior to extraction in order to facilitate the release of valuable biomolecules from the disrupted algal cells (Rahman et al. 2022). Furthermore, dewatering and drying steps —performed prior to extraction in a typical biorefinery— are avoided. These are energy-intensive steps, since the energy required accounts for more than 80% of the total energy consumption in the extraction and they are major bottlenecks in algal processing. Besides being energy-sparing, the wet route may also be more effective, ensuring higher extractive yields than the dry route. Indeed, in order to overcome the presence of a thick and robust cell wall, extraction from wet biomass is being proposed as an alternative and innovative solution, according to which cell wall disruption is more effectively carried out by mechanical and/or non-mechanical techniques (Rahman et al. 2022).

Accordingly, this study aimed to assess the feasibility of applying the wet route extraction to *G. gracilis* biomass and its advantages and drawbacks regarding the dry route. Additionally, different wet route processes were compared, using the composition and biological activity of the attained extracts as guiding criteria.

Materials and methods

Experimental design

This study was designed to enable a direct comparison between the dry route (with drying of the seaweed and with the corresponding extracts whose acronym begins with D) and wet route (utilization of the thawed and moist seaweed and corresponding extracts whose acronym begins with W) for extraction from *G. gracilis* biomass as well as across specific technological extractive processes. These were: (i) conventional solid–liquid extraction with overnight agitation (coded as OA and using water, *w*, or ethanol:water 7:3, *ew*); (ii) solid–liquid extraction with intense homogenization (drastic physical process and coded as H) and encompassing so-called green solvents (water, *w*, ethanol:water 7:3, *ew*, ethyl acetate, *ea*, and isoamyl acetate, *ia*); (iii) pH-shift extraction (coded pHS); (iv) ionic liquid multi-step extraction (IL); and (v) ultrasound-assisted extraction (UA, using water, *w*, or ethanol:water 7:3, *ew*). Different proportions of seaweed biomass to extracting liquid (1:5, 1:10 or 1:20, w/v, depending on the rheological properties of the extractive mixture) were tested. All these technologies and operational parameters (Fig. 1) were used with the purpose to optimize the wet route process, taking the most conventional dry route process as control (solid–liquid extraction with overnight agitation). As all these extraction technologies were already much studied

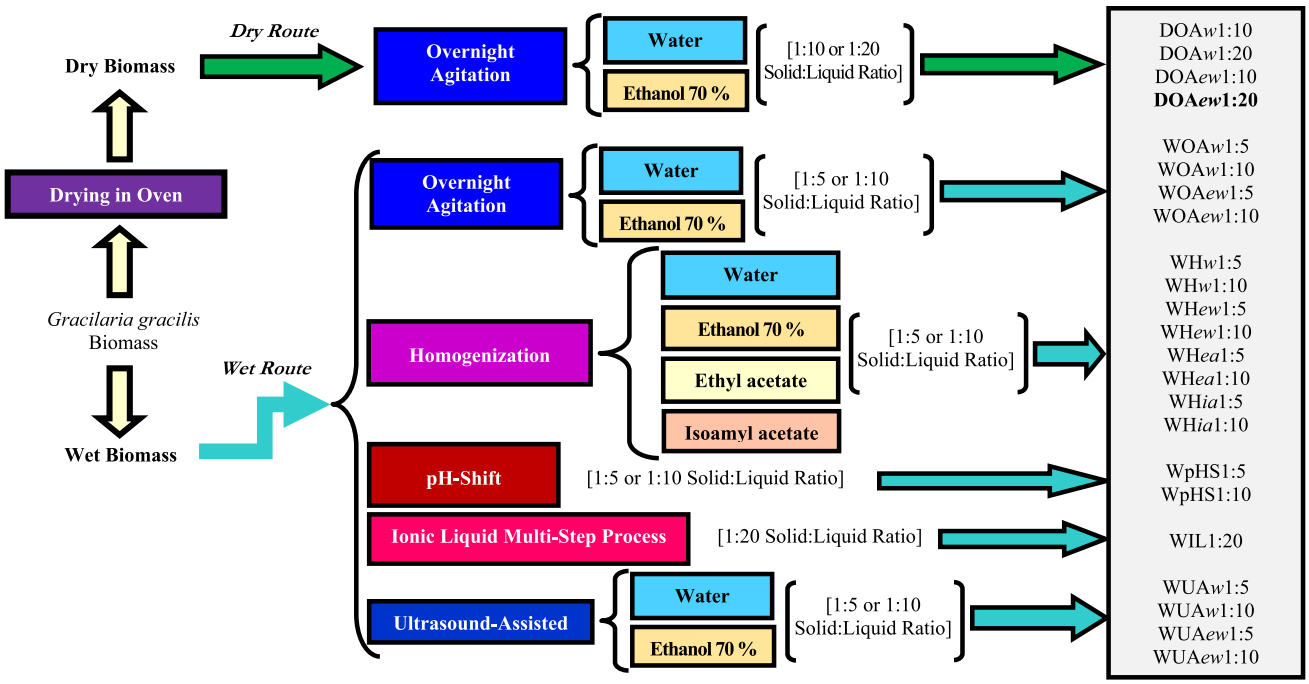


Fig. 1 Experimental design of the study on the comparison between dry route and wet route extraction methodologies (reference dry route extraction is indicated by bold code)

in the dry route, they were not tested in the D experimental batches (Ummat et al. 2021).

After preparation of all these extracts, moisture and ash contents were determined and used to calculate the yields in terms of dry and mineral matter extraction. Total polyphenol content was also determined for all extracts. Afterwards, the best extracts, that is, those with higher yields and total polyphenol content, were selected for a more thorough characterization of their biological activity. The overall experimental scheme and detailed codes are shown in Fig. 1.

Seaweed source, collection and preparation

As starting material *Gracilaria gracilis*, that grew in ponds belonging to a fish farm and whose environmental conditions were defined by an IMTA system, was chosen. Biomass growth is favoured by a high content of nutrients in the water derived from the cultivation of gilthead seabream. Besides its IMTA form of cultivation, other reasons for the selection of this species were its edibility, presence as marketed product after drying in specialty shops, and richness in biologically active compounds (Francavilla et al. 2013; Capillo et al. 2018; Ebrahimzadeh et al. 2018). The taxonomic identification was by the Portuguese producer company, Aqualvor – Actividades em Aquacultura, Lda. (Lagos, Algarve, Portugal). Specific traits that led to its phenotypic identification were the characteristics of the fronds (cartilaginous, cylindrical, and dull purple), their length (up to 500 mm), and the small, fleshy, and perennial discoid holdfast. Moreover, seaweed branching was irregular, with branches to 2 mm diameter and pointed apices. This seaweed originated from Vale da Lama (Lagos, Algarve, Portugal), collected from the Aqualvor fish farm in April 2024. The seaweed biomass was sent fresh in thermal insulated boxes to the IPMA laboratory in Lisbon. Immediately after reception, the biomass was frozen in vacuum-packaged bags at −80 °C and stored at this temperature. Finally, adequate amounts of *G. gracilis* were ascribed to the dry route and wet route processes, with the former thawed, dried in an oven (Memmert, Germany) at 60 °C for 3 days, minced into a fine powder with a Retsch GM200 knife mill (Retsch GmbH, Germany), packaged under vacuum, and stored at −80 °C until analysis and the latter thawed, left dripping for 2 h at room temperature (15 °C) until constant weight, thereby ensuring that all melted ice from freezing and packaging and not belonging to the seaweed was removed from the biomass to be processed—, minced in the same knife mill, and directly used in analysis.

Seaweed extraction methodologies

Five different methodologies were used in the wet route extraction study of *G. gracilis*, either based on essentially physical processes or rather chemical treatments. In the case of the former, overnight agitation, homogenization with a high velocity rotating disperser rod, and ultrasound-assisted extraction. As to chemical processes, pH-shift and ionic

liquid multi-step extraction were tested. For the dry route extraction, as explained above, only the conventional overnight agitation was applied.

Overnight agitation (OA)

This is the most conventional solid–liquid extraction process and was applied both in the case of the wet (W) and dry (D) route. Indeed, a typical extraction procedure involves addition of a hydrophilic or hydrophobic solvent to dried seaweed at a given proportion and a following lengthy agitation step (Yang et al. 2012). In this study, water (*w*) and ethanol:water (70%, v/v) (*ew*) were added either at 1:5 and 1:10 ratios, w/v, to wet *G. gracilis* (WOA*w*1:5, WOA*w*1:10, WOA*ew*1:5, and WOA*ew*1:10) or at 1:10 and 1:20 ratios, w/v, to dry *G. gracilis*, requiring more solvent due to its dryness (DOA*w*1:10, DOA*w*1:20, DOA*ew*1:10, and DOA*ew*1:20, being this latter taken as reference of a typical extraction). For this extraction, 5 g of *G. gracilis* was weighed and then the required volume of solvent was added. After preparing all extracts, the beakers containing the solid:liquid mixtures were placed in an IKA KS260 orbital shaker (IKA-Werke, Germany) and left shaking (200 rpm) overnight (12 h) at 20 ºC. The following day, extracts were transferred to tubes and centrifuged at 3,000 × *g* and 20 ºC for 10 min. Subsequently, supernatant (extract) was transferred to new tubes and stored in a refrigerator at 4 ºC until analysis.

Homogenization (H)

Wet *G. gracilis* biomass was used for this extraction procedure and this solid–liquid technique (Ummat et al. 2021) used four alternative green solvents (water, ethanol:water, 70%, v/v, ethyl acetate, and isoamyl acetate) added either at a 1:5 or 1:10 ratio, w/v, to the biomass (WH*w*1:5, WH*w*1:10, WH*ew*1:5, WH*ew*1:10, WH*ea*1:5, WH*ea*1:10, WH*ia*1:5, and WH*ia*1:10). Briefly, 5 g of wet *G. gracilis* was poured in an analytical balance and the required volume of solvent was added. Then, the prepared mixtures were homogenized using a CAT Unidrive X1000D homogenizer equipped with a large diameter rod (Ingenieurbüro CAT, M. Zipperer GmbH, Germany). After three 5-min cycles at 30,000 rpm, the solutions were centrifuged at 3,000 × *g* and 20 ºC for 10 min. Afterwards, the supernatant was transferred to new tubes and stored in a refrigerator at 4 ºC until analysis.

pH-Shift (pHS)

Wet *G. gracilis* biomass was also used in the pH-shift extractive method. This method entails a solid–liquid extraction with an acidic aqueous solution followed by a pH neutralization step (Harrysson et al. 2018). The acidic solution is added either at a 1:5 or 1:10 ratio, w/v, to the biomass (WpHS1:5 and WpHS1:10). Briefly, 5 g of wet *G. gracilis* was weighed and the amount of 1 M HCl necessary to obtain the desired proportions was added. Then, the attained solid–liquid mixtures were placed in an orbital shaker and left shaking (200 rpm) overnight. The following day, pH of the mixture was measured using a Mettler-Toledo SevenCompact S220 pH meter (Mettler-Toledo, Switzerland) and the extracting mixture was carefully neutralized by adding 5 M NaOH solution dropwise until a pH between 6.8 and 7.2 was obtained. After neutralization, the tubes were centrifuged at 3,000 × *g* and 20 ºC for 10 min. Finally, the supernatant was transferred to new tubes and stored in a refrigerator at 4 ºC until analysis.

Ionic liquid multi-step extraction (IL)

The utilization of an ionic liquid (1-butyl-3-methylimidazolium acetate) represents a specific modality of a solid–liquid extraction (Martins and Ventura 2020), at a 1:20 ratio, w/v, to the biomass, which is followed by sequential separative steps (WIL1:20). Briefly, 0.5 g of wet *G. gracilis* was placed in tubes to which 10 mL of ionic fluid was added —given the logistically limited amounts of available ionic liquid, IL trials were down-scaled in relation to other techniques. Then, the tubes were placed in a shaking bath at 75 ºC for 2 h and then centrifuged at 3,000 × *g* and 20 ºC for 10 min. The supernatant was collected into a new tube and 25 mL of pure acetone was added. The supernatant + acetone mixture was filtered through Whatman nº1 filter paper. The filtrate was evaporated under vacuum using a Heidolph 401 Digital rotary evaporator (Heidolph Instruments, Germany), operated at 40 °C and 400 mbar. After this step, the concentrated filtrate was precipitated using 25 mL of Milli-Q water. The tubes were then placed in the refrigerator at 4 ºC overnight under a nitrogen atmosphere. The following day, the mixture was filtered through Whatman nº1 filter paper. This last filtrate was transferred to new tubes and stored in a refrigerator at 4 ºC until analysis.

Ultrasound-Assisted extraction (UA)

Wet *G. gracilis* biomass was also used in this solid–liquid extraction technique coupling two solvents (water and ethanol:water, 70%, v/v) added either at a 1:5 or 1:10 ratio, w/v, to the biomass (WUA*w*1:5, WUA*w*1:10, WUA*ew*1:5, and WUA*ew*1:10) and ultrasound treatment (Maia et al. 2023). Briefly, 5 g of wet *G. gracilis* was poured into a beaker and the required amount of solvent was added to obtain the intended solid–liquid ratio. After extract mixture preparation, all beakers were placed in an Emerson/Bransonic CPX 5800-E ultrasound bath (Emerson Electric Co., USA), applying 2 cycles of 20 min each of 160 W at an ultrasound frequency of 40 kHz. Afterwards, mixtures were centrifuged at 3,000 × *g* and 20 ºC for 10 min. Finally, the supernatant was transferred to new tubes and stored in a refrigerator at 4 ºC until analysis.

Determination of moisture and ash and calculation of yields

The moisture and ash contents in percentage were determined in accordance to the methods of the Association of Official Analytical Chemists (AOAC 2000). Moisture was determined in the initial biomass (including after drying) and in extracts. The respective dry matter and mineral matter yields were calculated through the formulas:

$$Dry\ Matter\ Yield(\%) = ((([Dry\ Matter]_{ext}/100) \times Ext.\ Wt.) / (([Dry\ Matter]_{in}/100) \times In.Wt.)) \times 100$$

where, $[Dry\ Matter]_{ext}$ = dry matter content in % in the extract; $[Dry\ Matter]_{in}$ = dry matter content in % in the initial *G. gracilis* biomass; *Ext. Wt.* = weight of attained extract in g; and *In. Wt.* = weight of the initial *G. gracilis* biomass used in the extraction in g.

$$Mineral\ Matter\ Yield(\%) = ((([Ash]_{ext}/100) \times Ext.\ Wt.) / (([Ash]_{in}/100) \times In.Wt.)) \times 100$$

where, $[Ash]_{ext}$ = mineral matter (ash) content in % in the extract; $[Ash]_{in}$ = mineral matter (ash) content in % in the initial *G. gracilis* biomass; *Ext. Wt.* = weight of extract in g; *In. Wt.* = weight of the initial *G. gracilis* biomass used in the extraction in g.

All final results were expressed on the basis of dry matter terms for the purpose of comparability regardless of being used dry or wet route processes.

Total polyphenol content

The total polyphenol content was determined by the Singleton and Rossi (1965) method using Folin-Ciocalteu reagent. Briefly, 100 µL of each extract was used in triplicate. Gallic acid (GA) was used as a standard and the phenolic content was expressed as gallic acid equivalents (mg GAE $(100\ g)^{-1}$ dw of initial *G. gracilis*) according to a gallic acid calibration curve. It should be noted that all results were expressed on the basis of dry matter terms — moisture was determined in order to calculate parameters in effective dry matter terms even in the case of dried material— for the purpose of comparability regardless of being used dry or wet route processes (the same applies to the other analytical methods).

Antioxidant activity as measured by DPPH method

The antioxidant activity was measured by determining the radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Miliauskas et al. 2004). A 1 mL volume of the extract (selected for a more thorough characterization of the biological activity) was prepared in triplicate for each sample and 2 mL of DPPH (Sigma, Germany) in 0.15 mM methanolic solution was added and thoroughly mixed. After 30 min incubation at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated using the following formula:

$$\%\ Inhibition = \left(\left(A_0 - A_{sample}\right)/A_0\right) \times 100$$

where, A_0 = absorbance of the blank; A_{sample} = absorbance of the sample.

On the basis of the ascorbic acid calibration curve, results were then expressed in mg of ascorbic acid equivalents (AA Eq.) per 100 g dw.

Antioxidant activity as measured by FRAP method

The antioxidant activity was also determined by the method of the Ferric Reducing Antioxidant Power (FRAP) using a modified version of Benzie and Strain (1996). In brief, 100 µL of extract was used in triplicate. Based on a $FeSO_4$ calibration curve, results were calculated as µmol Fe^{2+} equivalent (Eq.) g^{-1} dw.

Antioxidant activity as measured by ABTS method

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging activity was determined using the method described by Re et al. (1999). For this, 20 µL of extract was used in triplicate. The ABTS radical scavenging activity of the samples was expressed as a percentage of inhibition as follows:

$$\%\ Inhibition = \left(\left(A_0 - A_{sample}\right)/A_0\right) \times 100$$

where, A_0 = absorbance of the blank; A_{sample} = absorbance of the sample.

On the basis of a trolox calibration curve, results were then expressed in µmol of trolox equivalents (Trolox Eq.) per 100 g dw.

Anti-inflammatory activity

The anti-inflammatory activity of the best —with the highest activities and yields— among the *G. gracilis* extracts selected for a more thorough characterization was determined. A novel aqueous extract for the anti-inflammatory activity was attained from approximately 200 mg of the extract (previously prepared within the scope of Seaweed extraction methodologies) with 2 mL of Milli-Q water. Afterwards, this extract was subjected to heat treatment (80 ºC for 1 h) and then centrifuged (3,000 × *g* and 4 ºC for 10 min). The supernatant was collected and the solvent was evaporated using a vacuum rotary evaporator and a water bath at a temperature

of 65 °C. The residue was directly dissolved in 100% dimethyl sulphoxide (DMSO) to prepare a stock solution with a concentration of 10 mg mL^{-1}. The extract was tested at 1 mg mL^{-1} using a commercial cyclooxygenase (COX) inhibitory screening assay kit (Cayman test kit-560131, Cayman Chemical Company, USA). A 10-µL volume of test extract or DMSO (blank) was used. The results were expressed as the percentage of COX-2 inhibition.

Statistical analysis

To test the normality and the homogeneity of the data variance, the Kolmogorov–Smirnov test and Cochran's C-test were used, respectively. The data were analysed by one-way ANOVA using Tukey´s HSD to determine the difference in yields, contents, and biological activities between extraction methods and operational parameters. For all the statistical tests, the significance level (α) was 0.05. All the data analyses were performed using STATISTICA 7 (Stat-Soft, USA, 2003).

Results

Dry matter and mineral matter yields

Based on the determination of moisture (and, necessarily, dry matter content) and ash content in the initial biomass of *G. gracilis* and in the various extracts (obtained by different techniques and variable operational parameters), it was possible to calculate the total dry and mineral matter yields, which are presented in Table 1. As additional information, it is worth mentioning that moisture content was 85.4%, w/w, in wet and 4.1%, w/w, in dry *G. gracilis*.

The dry matter yield results varied widely, especially as a function of the extractive procedure. Very low yields, not exceeding 10%, were observed in all cases of the dry route (DOA) and in cases with ethyl and isoamyl acetate in the wet route homogenization (WH) as well as with the ionic liquid (WIL). Varying solvent and biomass:solvent proportion in the dry route approach or proportions in the other cases were

Table 1 Total dry matter and mineral matter yields (in %, n = 5) based on the respective initial contents in the biomass of *Gracilaria gracilis* subjected to different extraction methods, including variable operational parameters for each method

Extraction method	Operational parameter	Code	Dry matter yield (%)	Mineral matter yield (%)
Dry Route—Overnight Agitation (DOA)	Biomass:Water 1:10 (w:v)	DOA*w*1:10	7.5 ± 0.2^{f}	18.9 ± 0.7^{de}
	Biomass:Water 1:20 (w:v)	DOA*w*1:20	8.1 ± 0.2^{f}	20.4 ± 0.4^{d}
	Biomass:Ethanol 70% 1:10 (w:v)	DOA*ew*1:10	7.7 ± 0.2^{f}	11.1 ± 0.9^{f}
	Biomass:Ethanol 70% 1:20 (w:v)	DOA*ew*1:20	9.3 ± 0.6^{e}	12.1 ± 0.4^{ef}
Wet Route—Overnight Agitation (WOA)	Biomass:Water 1:5 (w:v)	WOA*w*1:5	30.7 ± 0.2^{c}	59.4 ± 0.0^{a}
	Biomass:Water 1:10 (w:v)	WOA*w*1:10	31.0 ± 0.0^{c}	56.3 ± 0.4^{ab}
	Biomass:Ethanol 70% 1:5 (w:v)	WOA*ew*1:5	34.7 ± 0.1^{b}	49.4 ± 4.9^{b}
	Biomass:Ethanol 70% 1:10 (w:v)	WOA*ew*1:10	47.1 ± 0.5^{a}	43.2 ± 6.7^{bc}
Wet Route – Mechanical Homogenization (WH)	Biomass:Water 1:5 (w:v)	WH*w*1:5	23.0 ± 0.0^{d}	50.3 ± 0.5^{b}
	Biomass:Water 1:10 (w:v)	WH*w*1:10	23.3 ± 0.1^{d}	49.9 ± 0.1^{b}
	Biomass:Ethanol 70% 1:5 (w:v)	WH*ew*1:5	21.3 ± 0.1^{d}	45.2 ± 0.0^{bc}
	Biomass:Ethanol 70% 1:10 (w:v)	WH*ew*1:10	24.1 ± 0.1^{d}	48.0 ± 0.4^{b}
	Biomass:Ethyl Acetate 1:5 (w:v)	WH*ea*1:5	ND^{g}	ND^{g}
	Biomass:Ethyl Acetate 1:10 (w:v)	WH*ea*1:10	2.4 ± 1.3^{fg}	ND^{g}
	Biomass:Isoamyl Acetate 1:5 (w:v)	WH*ia*1:5	ND^{g}	ND^{g}
	Biomass:Isoamyl Acetate 1:10 (w:v)	WH*ia*1:10	ND^{g}	ND^{g}
Wet Route – pH-Shift (WpHS)	Biomass:1 M HCl 1:5 (w/v)	WpHS1:5	23.6 ± 0.0^{d}	ND^{g}
	Biomass:1 M HCl 1:10 (w/v)	WpHS1:10	46.9 ± 0.1^{a}	ND^{g}
Wet Route – Ionic Liquid (WIL)	Biomass:Ionic Liquid 1:20 (w/v)	WIL1:20	ND^{g}	ND^{g}
Wet Route – Ultrasound Agitation (WUA)	Biomass:Water 1:5 (w:v)	WUA*w*1:5	24.1 ± 0.2^{d}	48.8 ± 3.3^{b}
	Biomass:Water 1:10 (w:v)	WUA*w*1:10	30.5 ± 0.3^{c}	51.1 ± 0.4^{b}
	Biomass:Ethanol 70% 1:5 (w:v)	WUA*ew*1:5	32.7 ± 0.2^{b}	49.2 ± 1.0^{b}
	Biomass:Ethanol 70% 1:10 (w:v)	WUA*ew*1:10	41.8 ± 0.0^{a}	38.3 ± 4.3^{c}

Values are presented as the average ± standard deviation. ND – Not detected (no detection of dry or mineral matter in the extracts led to an apparent 0% yield). Different lowercase letters within a column correspond to significant differences ($p < 0.05$) between the different extraction methodologies and conditions.

ineffective. The utilization of water or a 70%/30% ethanol/water mixture in WH did not improve much, since yields remained relatively low. Values above the 30% yield mark, but below 40%, were attained with the combination of wet route with overnight agitation (WOA) and ultrasound agitation (WUA). The best results (> 40% yield) were registered in WOA with 70% ethanol and a biomass:solvent ratio of 1:10 (WOA*ew*1:10), pH-shift method (wet route) with a biomass:1 M HCl solvent ratio of 1:10 (WpHS1:10), and WUA and a biomass:70% ethanol ratio of 1:10 (WUA*ew*1:10), reaching 47.1 ± 0.5%, 46.9 ± 0.1%, and 41.8 ± 0.0%, respectively. These three values were not statistically different ($p > 0.25$).

The mineral matter yield displayed a different set of values and patterns. Nonetheless, there were similarities with the dry matter yield. Namely, the dry route and the WH combined with ethyl and isoamyl acetate (and the WIL) delivered poor results, with yields not exceeding 20.4 ± 0.4% (DOA*w*1:20). Moreover, all yields attained through the pH-shift (WpHS) were extremely low, thus differing from the dry matter results. There was also a whole series of cases with intermediate values between 38.3 ± 4.3% (WUA*ew*1:10) and 49.9 ± 0.1% (WH*w*1:10), covering mostly ultrasound and mechanical homogenization procedures with water and 70% ethanol as extractants. For WOA and ethanol 70% as extractant, values in the same range were observed, 43.2–49.4%. Higher values than these and surpassing 50% mineral matter yield were measured in four instances: WOA*w*1:5, WOA*w*1:10, WH*w*1:5, and WUA *w*1:10. All had in common water as extractant, being the overnight agitation with 1:5 biomass:water ratio, with 59.4 ± 0.0%, higher than WH*w*1:5 and WUA*w*1:10, 50.3–51.1%.

Total polyphenol content

For all tested extracts (obtained by different techniques and variable operational parameters), it was also possible to determine values of total polyphenol content in them as shown in Table 2. This is indicative of the extractive efficiency of each methodology (and set of parameters) regarding polyphenolic substances.

As in the case of the total dry matter and mineral matter yields, it is possible to divide extractive methodologies in accordance to their effectiveness. The WpHS (pH-shift) and

Table 2 Total polyphenol contents (in mg GAE (100 g)$^{-1}$ dry weight, n = 3) in the various extracts attained from the biomass of *Gracilaria gracilis* subjected to different extraction methods, including variable operational parameters for each method

Extraction method	Operational parameter	Code	Total polyphenol content (mg GAE (100 g)$^{-1}$ g dw)
Dry Route—Overnight Agitation (DOA)	Biomass:Water 1:10 (w:v)	DOA*w*1:10	46.3 ± 6.2^{c}
	Biomass:Water 1:20 (w:v)	DOA*w*1:20	81.9 ± 16.3^{bc}
	Biomass:Ethanol 70% 1:10 (w:v)	DOA*ew*1:10	149.6 ± 18.5^{b}
	Biomass:Ethanol 70% 1:20 (w:v)	DOA*ew*1:20	174.5 ± 22.2^{b}
Wet Route—Overnight Agitation (WOA)	Biomass:Water 1:5 (w:v)	WOA*w*1:5	127.7 ± 4.8^{b}
	Biomass:Water 1:10 (w:v)	WOA*w*1:10	287.7 ± 36.9^{a}
	Biomass:Ethanol 70% 1:5 (w:v)	WOA*ew*1:5	197.4 ± 0.3^{b}
	Biomass:Ethanol 70% 1:10 (w:v)	WOA*ew*1:10	283.6 ± 69.4^{a}
Wet Route – Mechanical Homogenization (WH)	Biomass:Water 1:5 (w:v)	WH*w*1:5	23.9 ± 0.8^{d}
	Biomass:Water 1:10 (w:v)	WH*w*1:10	25.8 ± 0.3^{d}
	Biomass:Ethanol 70% 1:5 (w:v)	WH*ew*1:5	44.6 ± 3.2^{c}
	Biomass:Ethanol 70% 1:10 (w:v)	WH*ew*1:10	76.3 ± 0.3^{bc}
	Biomass:Ethyl Acetate 1:5 (w:v)	WH*ea*1:5	43.1 ± 5.5^{c}
	Biomass:Ethyl Acetate 1:10 (w:v)	WH*ea*1:10	68.3 ± 2.2^{bc}
	Biomass:Isoamyl Acetate 1:5 (w:v)	WH*ia*1:5	51.9 ± 10.9^{c}
	Biomass:Isoamyl Acetate 1:10 (w:v)	WH*ia*1:10	92.5 ± 35.5^{bc}
Wet Route – pH-Shift (WpHS)	Biomass:1 M HCl 1:5 (w/v)	WpHS1:5	5.2 ± 0.3^{e}
	Biomass:1 M HCl 1:10 (w/v)	WpHS1:10	5.8 ± 0.4^{e}
Wet Route – Ionic Liquid (WIL)	Biomass:Ionic Liquid 1:20 (w/v)	WIL1:20	5.7 ± 0.2^{e}
Wet Route – Ultrasound Agitation (WUA)	Biomass:Water 1:5 (w:v)	WUA*w*1:5	138.3 ± 46.3^{b}
	Biomass:Water 1:10 (w:v)	WUA*w*1:10	52.8 ± 7.2^{c}
	Biomass:Ethanol 70% 1:5 (w:v)	WUA*ew*1:5	309.1 ± 68.1^{a}
	Biomass:Ethanol 70% 1:10 (w:v)	WUA*ew*1:10	245.9 ± 31.1^{ab}

Values are presented as the average ± standard deviation. Different lowercase letters within a column correspond to significant differences ($p < 0.05$) between the different extraction methodologies and conditions.

WIL (ionic liquid) results were extremely low, not surpassing 10 mg GAE (100 g)$^{-1}$ dw, thus faring much worse than the dry route values. These (DOA method) varied between 46.3 ± 6.2 mg GAE (100 g)$^{-1}$ dw in DOA*w*1:10 to 174.5 ± 22.2 mg GAE (100 g)$^{-1}$ dw in DOA*ew*1:20, which were statistically different. If the use of water as extracting solvent in DOA is set aside, mechanical homogenization (and WUA*w*1:5 and WUA*w*1:10) in the wet route exhibited poorer results than DOA, varying in the 23.9–138.3 mg GAE (100 g)$^{-1}$ dw range. The application of the overnight agitation procedure to wet algal biomass instead of dry *G. gracilis* generally produced higher polyphenol contents. This was particularly intense in the case of biomass:water in a 1:10 ratio, with an increase from 46.3 ± 6.2 mg GAE (100 g)$^{-1}$ dw in DOA*w*1:10 to 287.7 ± 36.9 mg GAE (100 g)$^{-1}$ dw in WOA*w*1:10. Furthermore, ultrasound application to wet biomass in combination with 70% ethanol was also quite efficient in extracting polyphenol substances, yielding 245.9 ± 31.1 mg GAE (100 g)$^{-1}$ dw and 309.1 ± 68.1 mg GAE (100 g)$^{-1}$ dw in WUA*ew*1:10 and WUA*ew*1:5, respectively. In general, total polyphenol content values in the various extracts agreed with calculated yields for the same extracts —though correlation between phenolic contents and yields was low (Fig. 2).

Biological activity

For a set of selected extracts (DOA*ew*1:20, WOA*ew*1:10, and WUA*ew*1:10) exhibiting better yields and higher polyphenol contents, the antioxidant activity was evaluated by combining and comparing three distinct methods, the DPPH, FRAP, and ABTS techniques, thereby generating the results presented in Tables 3, 4, and 5, respectively. Likewise, for a restricted set of extracts (DOA*ew*1:20, WOA*ew*1:10, and WUA*ew*1:10), the anti-inflammatory activity was determined by inhibition of COX-2, being the percentages shown in Table 6. While DOA*ew*1:20 was selected as representative of a typical dry route extraction, the other two, WOA*ew*1:10, and WUA*ew*1:10, were chosen for their outstanding yields in combination with high polyphenol contents.

The dry route reference extract (DOA*ew*1:20) showed antioxidant activity levels comparable to the best wet route levels in the cases of DPPH and FRAP, which were observed with WOA*ew*1:10, 39.9 ± 1.8 mg AA Eq. (100 g)$^{-1}$ dw and 7.28 ± 0.24 µmol Fe^{2+} Eq. g^{-1} dw, respectively. The utilization of ultrasound extraction in combination with the wet route, even in the selected case corresponding to a high polyphenol content, 245.9 ± 31.1 mg GAE (100 g)$^{-1}$ dw (WUA*ew*1:10), generated lower levels of antioxidant activity as measured by DPPH and FRAP. On the other hand, ABTS' levels highlighted a clear antioxidant advantage of the selected wet route extracts, 4469.5–5296.3 µmol Trolox Eq. (100 g)$^{-1}$ dw, which compare with 2728.4 ± 30.9 µmol Trolox Eq. (100 g)$^{-1}$ dw in the dry route reference (DOA*ew*1:20). In addition, the anti-inflammatory activity achieved with the wet route extracts (WOA*ew*1:10 and WUA*ew*1:10), 26.2–38.3% COX-2 inhibition, clearly exceeded that attained with the reference methodology (DOA*ew*1:20), 6.8 ± 3.0% COX-2 inhibition.

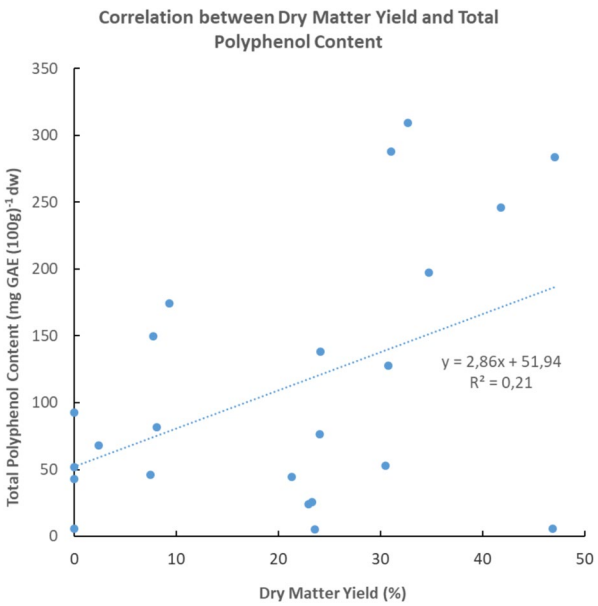


Fig. 2 Depiction of the correlation between dry matter yield (in %) and total polyphenol content (mg GAE (100 g)$^{-1}$ dw)

Discussion

Dry matter and mineral matter yields

Results show that a relatively well-known red seaweed such as *G. gracilis* still has poorly understood and studied aspects that make difficult to design a strategy to set up efficient extractive processes with maximized yields, especially for organic matter. Moreover, though avoiding dewatering and drying steps, which, besides being energy-sparing, may enable higher extractive yields than the dry route through a more efficient cell wall disruption (Rahman et al. 2022), utilization of wet route techniques are still quite uncommon (Le Guillard et al. 2016) and dry route procedures are mostly used (Rodrigues et al. 2015; Gisbert et al. 2021; Panjaitan and Yuliana 2022).

The yields (total extracted matter) reported in the literature vary widely as a function of type of route, extraction technique, operational parameters, and, necessarily, type of seaweed (Rodrigues et al. 2015; Le Guillard et al. 2016; Gisbert et al. 2021; Panjaitan and Yuliana 2022). Regarding dry matter yield, type of seaweed seems to be largely influential, since Rodrigues et al. (2015) reported lower yields with a brown seaweed species, *Sargassum muticum*, never surpassing 32% in a comparison across hot water,

Table 3 DPPH antioxidant activity (in mg AA Eq. (100 g)$^{-1}$ dry weight, n = 3) in selected extracts from *Gracilaria gracilis* subjected to optimized extraction methods

Extraction method	Operational parameter	Code	DPPH (mg AA Eq. (100 g)$^{-1}$ dw)
Dry Route—Overnight Agitation (DOA)	Biomass:Ethanol 70% 1:20 (w:v)	DOA*ew*1:20	33.8 ± 0.8[a]
Wet Route—Overnight Agitation (WOA)	Biomass:Ethanol 70% 1:10 (w:v)	WOA*ew*1:10	39.9 ± 1.8[a]
Wet Route – Ultrasound Agitation (WUA)	Biomass:Ethanol 70% 1:10 (w:v)	WUA*ew*1:10	26.3 ± 4.2[b]

Values are presented as the average ± standard deviation. Different lowercase letters within a column correspond to significant differences ($p < 0.05$) between the different extraction methodologies.

Table 4 FRAP antioxidant activity (in µmol Fe^{2+} Eq. g^{-1} dry weight, n = 3) in selected extracts from *Gracilaria gracilis* subjected to optimized extraction methods

Extraction method	Operational parameter	Code	FRAP (µmol Fe^{2+} Eq. g^{-1} dw)
Dry Route—Overnight Agitation (DOA)	Biomass:Ethanol 70% 1:20 (w:v)	DOA*ew*1:20	7.73 ± 0.25[a]
Wet Route—Overnight Agitation (WOA)	Biomass:Ethanol 70% 1:10 (w:v)	WOA*ew*1:10	7.28 ± 0.24[a]
Wet Route – Ultrasound Agitation (WUA)	Biomass:Ethanol 70% 1:10 (w:v)	WUA*ew*1:10	4.57 ± 0.08[b]

Values are presented as the average ± standard deviation. Different lowercase letters within a column correspond to significant differences ($p < 0.05$) between the different extraction methodologies.

Table 5 ABTS antioxidant activity (in µmol Trolox Eq. (100 g)$^{-1}$ dry weight, n = 3) in selected extracts from *Gracilaria gracilis* subjected to optimized extraction methods

Extraction method	Operational parameter	Code	ABTS (µmol Trolox Eq. (100 g)$^{-1}$ dw)
Dry Route—Overnight Agitation (DOA)	Biomass:Ethanol 70% 1:20 (w:v)	DOA*ew*1:20	2728.4 ± 30.9[b]
Wet Route—Overnight Agitation (WOA)	Biomass:Ethanol 70% 1:10 (w:v)	WOA*ew*1:10	4469.5 ± 207.7[a]
Wet Route – Ultrasound Agitation (WUA)	Biomass:Ethanol 70% 1:10 (w:v)	WUA*ew*1:10	5296.3 ± 1027.3[a]

Values are presented as the average ± standard deviation. Different lowercase letters within a column correspond to significant differences ($p < 0.05$) between the different extraction methodologies.

Table 6 Anti-inflammatory activity (% inhibition of COX-2, n = 4) in selected extracts from *Gracilaria gracilis* subjected to optimized extraction methods and in a reference extract prepared by the dry route

Extraction method	Operational parameter	Code	COX-2 Inhibition (%)
Dry Route—Overnight Agitation (DOA)	Biomass:Ethanol 70% 1:20 (w:v)	DOA*ew*1:20	6.8 ± 3.0[b]
Wet Route—Overnight Agitation (WOA)	Biomass:Ethanol 70% 1:10 (w:v)	WOA*ew*1:10	26.2 ± 5.7[a]
Wet Route – Ultrasound Agitation (WUA)	Biomass:Ethanol 70% 1:10 (w:v)	WUA*ew*1:10	38.3 ± 12.5[a]

Values are presented as the average ± standard deviation. Different lowercase letters within a column correspond to significant differences ($p < 0.05$) between the different extraction methodologies.

ultrasound-assisted, and enzyme-assisted extractions. Red and green seaweed species in this study (Rodrigues et al. 2015) led to yields consistently above 40%, reaching almost 55% in the case of enzyme-assisted extraction using cellulase upon *Osmundea pinnatifida* —a red seaweed just as *G. gracilis* previously shown to have antioxidant properties (Campos et al. 2019)— and between 40 and 50% in the cases of hot water and ultrasound extractions in this same species. This is in accordance with the best results attained for *G. gracilis* in the current study. It should also be noted that Rodrigues et al. (2015) used a (dry) biomass:water ratio of 1:25, which may have enhanced yields. However, Le

Guillard et al. (2016) using another red seaweed and a wet route approach, *Grateloupia turuturu*, achieved yields up to 80% by combining ultrasound- and enzyme-assisted technologies and more than 70% using only ultrasound extraction. These authors (Le Guillard et al. 2016) used an ultrasound frequency, 35 kHz, similar to that applied to *G. gracilis*, 40 kHz, but a much longer time, 6 h *vs* 40 min. Further research may elucidate whether such long times would be helpful in raising yields for *G. gracilis*.

Extraction technologies less commonly applied to seaweed, such as pH-shift, seem to perform poorly in extracting total organic matter, being more selective and adequate to specific solutes, such as protein (Kashyap et al. 2024). These authors reported a total solute yield of only ~ 10% with pH-shift applied to wet *Gracilaria* sp. Similarly, the ionic liquid extraction performed poorly in *Gracilaria* sp., thereby diverging from reports asserting the ability of ionic liquids to efficiently dissolve cellulose (Zhang et al. 2017), which may be related to red seaweed from the genus *Gracilaria* being very rich in agar and other sulphated polysaccharides (Niu et al. 2013), but also to the accumulated losses of several successive processing steps that render WIL extraction less straightforward than the other studied techniques. Nonetheless, for instance, Kumar et al. (2013) reported that the residual pulp of *G. verrucosa* biomass leftover after agar extraction contained approximately 40% cellulose. However, this value may represent an overestimation, since a study on 15 agarophytic seaweeds (Baghel et al. 2014) claimed that cellulose and agar contents varied from 3.7 ± 0.1 to 12.2 ± 0.5% dw, and 9.2 ± 0.6 to 25.2 ± 0.5% dw, respectively. The relatively low cellulose contents may help to explain the apparent inefficacy of the WIL technique.

The homogenization of seaweed biomass also presents difficulties that may reduce yield. On the one hand, it may help in disrupting the cell wall and increase surface area for a rapid mass transfer of solutes contained in the algal cells, thus enhancing extract concentration of the targeted compound, as observed with the pigment phycoerythrin in the red seaweed *Chondrus crispus* (Bahari et al. 2021). On the other hand, there are compounds whose extraction is not surface area dependent, thereby suggesting different extraction mechanistic phenomena (Bahari et al. 2021). This was observed in the case of carrageenan in *C. crispus* (Bahari et al. 2021). Adsorption phenomena to a larger surface area of the smaller particles after homogenization —especially if the extracting solvents have low chemical affinity with the targeted compounds, as seems to have been the case with ethyl and isoamyl acetate— may be the determining factor. With more polar solvents, such as water and 70% ethanol, mineral matter yields with mechanical homogenization were relatively similar to those attained with overnight and ultrasound extraction. However, the same did not happen in the case of organic matter, thus resulting in intermediate dry matter yields.

Hence, techniques that are less prone to extremely fragment the seaweed biomass, such as simple solid–liquid overnight and ultrasound extraction methods, may be more effective if they are able to disrupt/permeate the algal cell walls, thereby providing a straightforward route to valuable solute release without extensive adsorption phenomena or deficient centrifugal phase separation. The WUA methodology is also advantageous in that it is a cold extraction technique, has minimal impact on the stability of the target compounds, reduces or eliminates the use of toxic chemicals, and, precisely, is considered to enhance yield and quality of the target products (Kadam et al. 2015). It must be remarked that ultrasound extraction is based on the physicochemical principle of acoustic cavitation and may generate a wide range of phenomena depending on its frequency, duration, and other parameters (Carreira-Casais et al. 2021). Namely, besides fragmentation and erosion —which may pose problems as mentioned above—, ultrasound can promote sonocapillary —an enhanced penetration of solvent into the canals and pores of the matrix (Carreira-Casais et al. 2021)— and sonoporation —an increase in cell membranes permeability to foster the release of intracellular products into the extractive medium, by opening membrane pores (Meullemiestre et al. 2016). The relatively mild WUA conditions (wet biomass coupled with low frequency and short exposure time with an interval between cycles for cooling) used with *G. gracilis* seem to have avoided fragmentation and erosion and promoted sonocapillary and sonoporation, at least by judging from the attained dry matter yields, especially in comparison with WH technique.

An advantage of the wet route techniques in general may also lie in a strong osmotic shock, since the ratio dry matter:solvent is higher, if it is considered that the lower biomass:solvent ratios used in the wet route are combined with very low percentages of dry matter in the algal biomass. It is known that osmoshocking can significantly increase yield as clearly seen in a study on the brown seaweed *Saccharina latissima* (Vilg and Undeland 2017). The osmotic shock helps to break cells and it is considered to facilitate the liberation of algal proteins (Vilg and Undeland 2017).

Total polyphenol content

The polyphenol results are in broad agreement with the yield calculations (Table 1). This is relevant, especially if it is considered that such data convergence is not self-evident. Indeed, even the highest polyphenol contents are less than 0.4%, w/dw, of the *G. gracilis* biomass, which compares with extracted total dry matter contents representing over 40% of the initial biomass. Hence, from a purely quantitative point of view, the amount of extracted polyphenols could

strongly diverge from that of total organic (and inorganic) matter. Therefore, total polyphenol content values show that polyphenol extractability and related mechanisms did not differ so much from overall organic matter. The existence of interactions between polyphenols and polysaccharides — generally, the main constituent of seaweed biomass— may explain the parallel extractability of polyphenols and total organic matter in seaweed (Renard et al. 2017). All this matters in the sense that also informs any production engineer aiming to maximize phenolic extraction yields about the most suitable conditions to be applied.

As in previous studies comparing hydrophilic and hydrophobic extracts (Regal et al. 2020), the utilization of more apolar solvents in the alternative techniques, such as 70% ethanol, is associated to higher phenolic contents in the extracts from *G. gracilis*, being WOA extracts the sole exception. In addition, the largest differences in polyphenol content were produced by the application of alternative extractive technologies. In the cases of WOA and WUA (with exception of WUA*w*1:10) methodologies, measured phenolic contents agree with the relevant literature for a biomass rich in these compounds, ranging from 100 to 500 mg GAE $(100\ g)^{-1}$ dw (Farasat et al. 2013; Julião et al. 2021). Comparable dry route results were mostly lower in the DOA trials. It must be considered that seaweed drying is considered a process that may decrease phenolic content either by binding polyphenols with other compounds (e.g., proteins) or by altering the chemical structure of polyphenols (Mrad et al. 2012). These chemical alterations are sensitive to higher drying temperatures, but only 60 °C (for three days in an oven) were used for drying *G. gracilis*. More specifically, Le Lann et al. (2008) analysed the effects of oven-drying (50–60 °C, 40 h) and freeze-drying (72 h) on the biomass quality of brown seaweed. They observed a higher phenolic content in freeze-dried samples than in oven-dried samples. A decline of phenolic content by 28% in red seaweed *Porphyra* sp. after oven-drying has also been described (Jiménez-Escrig et al. 2001). This may help to explain the contrast between DOA and WOA phenolic results. The thermolability of polyphenols may also be a factor to be equated (Antony and Farid 2022) in explaining low polyphenolic contents in the WIL extraction, which involved applying 75 °C for 2 h. Finally, with respect to WUA's positive results, ultrasound-assisted extraction was also more effective in producing enriched antioxidant extracts from other seaweed species (Dang et al. 2017).

Biological activity

In the assessment of biological activity, especially antioxidant activity, it must be taken into account that different methodologies focus on different antioxidant pathways and, as such, diverge in their results. Namely, ABTS has been considered more sensitive than DPPH and responsive to compounds with alternative chemical affinities, usually yielding different results (Martysiak-Żurowska and Wenta 2012; Julião et al. 2021; Sapatinha et al. 2022). In addition, although phenolic compounds are usually considered to be key antioxidants in seaweed, with even some studies pointing to a high correlation between phenolic content and DPPH and ABTS values, calculating Pearson correlation coefficients of 0.847–0.989 and 0.718–0.991, respectively (Monteiro et al. 2020; García-Oms et al. 2024)—, there are certain pigments (e.g., carotenoids) and compounds in the algal biomass with antioxidant properties (Ragonese et al. 2014). This may explain why DPPH and FRAP values contrasted with phenolic contents in the selected extracts, especially when comparing DOA*ew*1:20 and WUA*ew*1:10. It is possible that the WUA*ew*1:10 extract, while rich in phenolic substances, lacked other antioxidants, thus leading to poorer DPPH and FRAP outcomes. Regarding ABTS, there may be a higher sensitivity to the type of extracted polyphenols and, perhaps, to other antioxidants better extracted with a wet route approach and ultrasound technology.

The levels of antioxidant activity achieved in the best *G. gracilis* extracts are comparable to other recent studies also focusing on optimized processes (Maia et al. 2023; García-Oms et al. 2024). For instance, FRAP in the Portuguese *G. gracilis* was within the range of values observed in red seaweed species such as *Palmaria palmata* and *Porphyra* sp. (García-Oms et al. 2024). The ABTS levels compared favourably with those of *C. crispus* ultrasound-assisted extracts (Maia et al. 2023). A comparison to Francavilla et al. (2013), who also studied *G. gracilis*, reveals somewhat higher ABTS levels in hot water (80 °C) extracts attained by mechanical homogenization (but using only 17,000 rpm), varying between 5000 and 15,000 µmol Trolox Eq. $(100\ g)^{-1}$ extract. However, ABTS values in the Portuguese *G. gracilis* extracts are calculated per 100 g of initial dry algal biomass and overlap with the interval reported by Francavilla et al. (2013) in the case of WUAew1:10. It is noteworthy that Francavilla et al. (2013) studied seasonality and determined the highest ABTS values in April and the lowest in July and October. The Portuguese *G. gracilis* biomass was also collected in April. However, comparison with the Italian *G. gracilis* (Francavilla et al. 2013) is complicated by their wild origin, which contrasts with the IMTA conditions of cultivation of the Portuguese seaweed. These conditions may lead to lower levels of algal stress and decreased production of antioxidants (Hasanuzzaman et al. 2020) —according to these authors, high levels of antioxidant are generally associated with high stress tolerance. Manuguerra et al. (2024) studied IMTA-cultivated *G. gracilis* harvested in Summer and phenolic contents and antioxidant properties were modest. Indeed, Vega et al. (2020) showed that hydroethanolic extracts from marine red seaweed cultivated in IMTA

had a lower scavenger activity in comparison to their wild counterparts.

Regarding anti-inflammatory activity, there is also a large variety of determination techniques, which include in vitro assays and in vivo trials (Jin et al. 2006; Yang et al. 2010; Montalvão et al. 2018). This renders comparisons across studies more difficult. Nevertheless, there are studies pointing to the existence of anti-inflammatory biological activity in red seaweed (Montalvão et al. 2018; Yi et al. 2022). In particular, Montalvão et al. (2018) found that an ethanol extract from *G. verrucosa* had anti-inflammatory activity as evaluated in astrocyte cell cultures and Yi et al. (2022) reported that *G. rubra* showed anti-inflammatory functions. These were brought about by the inhibition of nitric oxide (NO) and proinflammatory cytokines production and thwarting of reactive oxygen species (ROS) formation in activated macrophages as well as by the regulation of the expression levels of inducible nitric oxide synthase (iNOS), NADPH-quinone oxidoreductase-1 (NQO-1), heme oxygenase 1 (HO-1), and cyclooxygenase-2 (COX-2) (Yi et al. 2022). The latter were determined through antibodies in an immunoblotting analysis. Hence, the methods used in these studies were different from that used in the Portuguese *G. gracilis*. A direct comparison is possible with the red seaweed *O. pinnatifida*, whose aqueous and ethanolic extracts did not generate any anti-inflammatory activity (Campos et al. 2019), since these authors applied exactly the same technique. Campos et al. (2019) observed a high anti-inflammatory activity, similar to that measured in WUA*ew*1:10 extract —and, to a lesser extent, to that determined for the WOA*ew*1:10 extract —, in an aqueous extract of the brown seaweed *Petalonia binghamiae* (40 ± 7% COX-2 inhibition).

A final overview of the various activity results in *G. gracilis* reinforces the evaluation derived from the yields and indicates not only a high effectivity and technological viability of the wet route approach, but even the achievement of yields and antioxidant and anti-inflammatory properties that outpace those measured in extracts prepared by the dry route. In addition, as advocated and suggested by Rahman et al. (2022), by avoiding energy-intensive steps, namely drying operations, the wet route is also ecologically advantageous. Accordingly, with such an alliance of effectiveness and by-passing expensive operations, an approach based on the wet route is also economically viable.

There is also the question of the extractive technology that better articulates with a wet route approach. Conventional methods may have several drawbacks, such as long extraction times, low yields, co-extraction of non-desirable compounds, and usage of a huge volume of organic solvent that may be harmful to the environment (Getachew et al. 2020). In order to overcome these difficulties, emerging technologies, such as enzyme-assisted extraction, microwave-assisted extraction, pressurized liquid extraction, supercritical fluid extraction, and ultrasound-assisted extraction have been intensively investigated, but often with a dry route approach. The specific outcomes attained with the Portuguese *G. gracilis* in the current study make a case for conjugating wet route and ultrasound-assisted technology. This technique has been successfully employed in the extraction field since a decade ago (Chemat and Zill-e-Huma Khan 2011). With the use of ultrasound, full extractions can be completed in only minutes instead of hours or days as with conventional methods, while ensuring high reproducibility, less manipulation and work-up, and even higher purity of the final product (Getachew et al. 2020). The main hurdles with ultrasound technology may lie in upscaling without entailing overwhelmingly expensive initial investment in equipment and a limited experience of industrial application to seaweeds. The latter may be solved with further optimization studies. In this regard, Maia et al. (2023) provided a valuable contribution that agrees with *G. gracilis*' outcome (WUA) in that, after considering all performed analyses, they concluded that the use of ultrasonic bath (instead of a probe), 40 kHz (frequency identical to the current study), and for 40 min (also identical time) was an efficient way to extract valuable compounds from *C. crispus*.

Conclusions

This study's results enabled comparison of different processing strategies (wet *vs* conventional dry route), technologies, and parameters for a maximization of yields (concerning dry and mineral matter) and antioxidant phenolic contents in seaweed extracts, using *G. gracilis* cultivated under IMTA conditions as example. Wide differences were registered, especially between dry and wet route —favourable to the latter— as well as between poor performing —ionic liquid application, pH-shift, and mechanical homogenization— and effective —overnight and ultrasound-assisted extractions— technologies. A higher biomass:solvent ratio or the choice of a more or less polar solvent had effect upon the studied outcomes, but this was quite variable. After a global evaluation of yields and phenolic contents, the overnight agitation (WOA*ew*1:10) and ultrasound-assisted extraction (WUA*ew*1:10) were selected for further characterization as the best extracts and compared to a conventional dry route extraction (DOA*ew*1:20). Regarding DPPH and FRAP, the wet route extraction performed as well as the dry route extraction and ABTS, in better agreement with total polyphenol content, supported a stronger antioxidant activity in the chosen wet route extracts than in the dry route extract, 4469.5–5296.3 µmol Trolox Eq. $(100\ g)^{-1}$ dw *vs* 2728.4 ± 30.9 µmol Trolox Eq. $(100\ g)^{-1}$ dw. The selected WUA*ew*1:10 extract demonstrated the advantage of combining wet route with ultrasound technology for achieving a high anti-inflammatory activity (38.3 ± 12.5% of COX-2 inhibition),

a multiple of the value attained with DOA*ew*1:20, 6.8 ± 3.0%. Indeed, the Portuguese *G. gracilis*' results show the strengths of applying an ultrasound-assisted extraction within a wet route strategy. Upscaling and a short experience of industrial application to seaweed are weaknesses of this type of extraction, thus justifying further research. Nevertheless, results show the potential of such novel technologies in a wet route approach to process seaweed, urging the need to scale-up and test in industrial conditions.

Author contribution D.P. did the experimental laboratory work, J.M. optimized the used methodologies, C.A. revised and treated the results, and C.C. also revised the results, supervised the experiments, and wrote the main manuscript text.

Funding Open access funding provided by FCT|FCCN (b-on). This study was funded by the project AQUAFISH0.0 (Ref.: EAPA_0062/2022). Researcher Joana Matos was supported by an AQUAFISH0.0 Post-Doctoral Grant, grant number IPMA-2023–041-BIPD; EAPA_0062/2022.

Data availability Data are available from the authors upon reasonable demand.

Declarations

Conflict of interest The authors declare no competing interests.

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