applied biosciences

MDPI

Article

Production and Characterization of a Bioactive Rich Extract from the Macroalga *Pyropia orbicularis* for the Development of Novel Functional Ingredients

Carla P. Morales-Guzmán [1], Inés A. Cea-Pavez [2,*], Inmaculada Moscoso-Ruiz [2], David Manteca-Bautista [2], Vito Verardo [2,3,4] and Martin Gotteland [1,*]

[1] Laboratory of Digestive Physiology, Department of Nutrition, Faculty of Medicine, University of Chile, Santiago 8380453, Chile; carlamg@uchile.cl
[2] Research and Development of Functional Food Center (CIDAF), 18016 Granada, Spain; imoscoso@cidaf.es (I.M.-R.); dmanteca@cidaf.es (D.M.-B.); vitoverardo@ugr.es (V.V.)
[3] Department of Nutrition and Food Science, Campus of Cartuja, University of Granada, 18016 Granada, Spain
[4] Institute of Nutrition and Food Technology Jose Mataix, Biomedical Research Center, University of Granada, 18016 Granada, Spain
* Correspondence: ines.cea@cidaf.es (I.A.C.-P.); mgottela@uchile.cl (M.G.); Tel.: +34-958-63-72-06 (I.A.C.-P.); +56-2-29786130 (M.G.)

Abstract

Macroalgae are a valuable source of bioactive substances, particularly phenolic compounds with antioxidant properties. However, conventional extraction methods frequently rely on non-GRAS solvents, limiting their applicability in food and nutraceutical products. This study aimed to develop a *Pyropia orbicularis* extract enriched in bioactive compounds and antioxidant activity using GRAS solvents through a solid–liquid extraction process. Response surface methodology was employed to optimize the extraction process using total phenolic content (TPC) and FRAP assay as response variables. The selected maceration conditions were 80% ethanol, 55 °C, an algae-to-solvent ratio of 1:29.4 (g/mL), and 40 min of stirring. The resulting dried extract exhibited a TPC of 106.9 ± 3.5 mg GAE/g, a flavonoid content of 12.4 ± 0.3 mg QE/g, and antioxidant capacities of 50.0 ± 2.2 µmol TE/g by FRAP and 500.1 ± 8.9 µmol TE/g by ORAC. HPLC-MS analysis revealed a chemically diverse profile, with major compounds tentatively identified as amino acid derivatives, lipids, and organic acids. No cytotoxicity was observed in Caco-2 or HT-29 cells at concentrations up to 1000 µg/mL. Overall, the optimized extraction strategy enabled the recovery of a bioactive-rich extract from *P. orbicularis*, supporting its further investigation as a potential ingredient for food-related applications.

Keywords: response surface methodology; antioxidant capacity; sequential extraction; HPLC-MS; cytotoxicity

Check for updates

Academic Editor: Sanggil Lee

Received: 15 July 2026
Revised: 11 September 2026
Accepted: 20 September 2026
Published: 28 September 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

1. Introduction

Macroalgae have been used in food and nutrition for thousands of years, with evidence of human consumption dating back to 35,000 BC, and documented medicinal applications appearing as early as 300 AD [1]. Among their bioactive constituents, phenolic compounds contribute to the protection of macroalgae against environmental stress through antioxidant and photoprotective mechanisms [1,2].

Beyond their traditional importance in human nutrition and health [3], naturally derived polyphenols have attracted considerable interest for the development of bioactive

materials [4–8]. Owing to their antioxidant and antimicrobial properties, polyphenols can be incorporated into edible coatings and active packaging systems, where they may help limit oxidative and microbial deterioration, thereby helping maintain food quality and extending the shelf life of products such as meat, fruits, and vegetables [4–8]. Moreover, certain polyphenols can impart stimulus-responsive properties to packaging materials, enabling their potential application in intelligent systems for monitoring food freshness [4–8]. These emerging applications highlight the potential of polyphenol-rich extracts as functional components for food protection systems and bioactive materials.

Red algae (phylum *Rhodophyta*) are commercially important sources of hydrocolloids, particularly agar and carrageenan [9]. However, they also contain a diverse range of phenolic compounds [3]. The total phenolic content (TPC) in red algae varies considerably depending on species and extraction methodology, with a mean value of approximately 3.2 mg gallic acid equivalents (GAE)/g dry weight (DW) reported for red algal biomass [10]. Moreover, extracts obtained from different algal species exhibit distinct phenolic profiles and antioxidant capacities [8], emphasizing the importance of studying individual macroalgal species. The main phenolic compounds identified in red algae include phenolic acids such as hydroxybenzoic, *p*-coumaric, caffeic, salicylic, hypogallic, and chlorogenic acids, as well as several flavonoids [10].

Numerous studies have evaluated the TPC of red algae and their potential health-promoting effects [10–12]. For example, extracts from *Eucheuma cottonii* have demonstrated antiproliferative and pro-apoptotic effects in human breast cancer cell lines, while extracts from *Porphyra* species have shown high phenolic content, strong antioxidant activity, metal chelating capacity, and significant inhibitory activity of α-amylase, a pancreatic enzyme responsible for starch hydrolysis in the intestine [13]. In *P. orbicularis*, extraction using a methanol:water mixture yielded a TPC of 3.58 mg GAE/g DW and an oxygen radical absorbance capacity (ORAC) value of 9.38 mmol Trolox Equivalents (TE)/g DW [14]. Furthermore, higher antioxidant activity, as measured by the DPPH assay, has been reported when nonpolar solvents such as n-hexane were used instead of methanol, acetone, or dichloromethane [15].

Although macroalgae have emerged as a promising source of polyphenols [14], maximizing the recovery of these compounds requires the identification of optimal extraction conditions that minimize degradation and preserve bioactivity. Temperature is a particularly important factor because many phenolic compounds are susceptible to thermal degradation, making extraction conditions critical for maintaining their functional properties [16–18]. A major limitation of many reported extraction protocols is their insufficient consideration of sustainability, scalability, and consumer safety. Furthermore, there is a lack of comprehensive characterization of macroalgae in general, and *P. orbicularis* in particular, especially regarding their metabolome and intestinal cellular safety. Commonly used solvents such as methanol, acetone, and dichloromethane are not classified as GRAS (Generally Recognized as Safe) [19], restricting their application in products intended for human consumption. Consequently, the development of extraction processes based on GRAS solvents is essential to ensure regulatory compliance and facilitate the incorporation of polyphenol-rich extracts into food and pharmaceutical products. Moreover, the comprehensive characterization via HPLC-MS of these extracts obtained using green methodologies would enable a better understanding of the bioactive compounds extracted.

In this context, the present study focused on the Chilean red macroalga *Pyropia orbicularis* (luche), a species characterized by remarkable tolerance to desiccation. During aerial exposure, *P. orbicularis* can withstand water losses up to 96%, a response associated with enhanced antioxidant defenses, including the accumulation of phenolic compounds [20]. The objective of this work was to optimize extraction conditions to maximize polyphenol

recovery and obtain a bioactive-rich extract with potential applications as a functional ingredient, as well as to characterize using HPLC-MS.

2. Materials and Methods

Pyropia orbicularis was obtained from Matanzas, O'Higgins Region, Chile. Prior to its use in this study, the seaweed was artisanally sun-dried by local seaweed harvesters as part of the customary post-harvest processing of the biomass. The dried seaweed was subsequently ground using a pilot-scale oscillating hammer mill with a lateral hopper, obtaining a particle size of 7 mesh. After grinding, the biomass was immediately packed in airtight, resealable plastic bags, protected from light, and stored under refrigeration until further use.

2.1. Optimization of the Extraction Method for Total Polyphenol Content (TPC) and Antioxidant Capacity Using Conventional Techniques

The extraction process for TPC and antioxidant capacity from *P. orbicularis* was optimized through three sequential stages: (1) response surface methodology (RSM), (2) extraction kinetics, and (3) total solvent fractionation.

2.1.1. Response Surface Methodology

The first stage consisted of solid–liquid extraction experiments aimed at maximizing both TPC and antioxidant capacity, the latter determined by the ferric reducing antioxidant power (FRAP) assay. Ground *P. orbicularis* (2 g) was extracted using an 80% (*v*/*v*) aqueous ethanol solution throughout the RSM optimization experiments. TPC and FRAP were selected as complementary response variables to evaluate phenolic compound recovery and reducing antioxidant capacity, respectively. Individual optimizations for each response, as well as a simultaneous optimization considering both variables, were performed. An axial point central composite design within the RSM framework was employed to optimize these response variables in dehydrated *P. orbicularis* samples. Extraction temperature (T) and algae-to-solvent ratio (Rs) were selected as independent variables. T was evaluated within the range of 25.9–54.1 °C, while Rs ranged from 1:5.9 to 1:34.1 g/mL. The effects of these factors were assessed both individually and in interaction.

Extraction temperature and algae-to-solvent ratio were evaluated first using RSM, while extraction time was subsequently assessed through a kinetic study under the selected temperature and algae-to-solvent ratio conditions (Section 2.1.2), following a sequential optimization approach.

The coded and actual levels of the independent variables are presented in Table 1. A total of 12 experimental runs were performed, including four replicates at the central point (Table 1), and were conducted in a randomized order. The experimental data were fitted to a second-order polynomial model according to the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \varepsilon \quad (1)$$

where Y represents the response variable, X_1 and X_2 correspond to the independent variables (T and Rs, respectively), and the β terms represent the regression coefficients. Two-dimensional contour plots and three-dimensional response surface plots were generated using Statgraphics Centurion 19 software (19.4.01 version) to visualize the effects of the independent variables on the response variables.

Model adequacy was assessed using the coefficient of determination (R^2), with the lack-of-fit test representing a 5% significance level for each response variable. Two-dimensional contour plots and three-dimensional response surface plots were generated to visualize the effects of the variables and their interactions.

Table 1. Independent variables and their coded levels used in the statistical design of Axial Point Central Compound Response Surface Methodology.

Independent Variables	Levels				
	$-\alpha$	-1	0	1	α
T [1]	25.9	30.0	40.0	50.0	54.1
Rs [2]	1:5.9	1:10	1:20	1:30	1:34.1

[1] Temperature (°C); [2] Rs: algae/solvent ratio (g/mL).

To maximize each response variable individually, the desirability function was defined as follows:

$$d = \{0(\frac{x - X_{min}}{X_{max} - X_{min}})1\} \quad x \leq X_{min} X_{min} < x \leq X_{max}, r > 0 x > X_{max}. \tag{2}$$

where x is the predicted response value, X_{min} and X_{max} are the lower and upper target limits, respectively, and r is a weighting factor that determines the shape of the desirability function.

The overall desirability function (D), used to simultaneously optimize TPC and antioxidant capacity (FRAP), was calculated as follows:

$$D = \{d_1 d_2\}^{\frac{1}{2}} \tag{3}$$

where d_1 and d_2 correspond to the desirability values for TPC and FRAP, respectively.

2.1.2. Extraction Kinetics

Extraction kinetics were initially evaluated using the total solvent volume established by the selected algae-to-solvent ratio (Rs). Ground *P. orbicularis* (2 g) was extracted with an 80:20 (v/v) ethanol/water mixture under agitationat 55 °C and 120 rpm using an Rs of 1:29.4 (g/mL), with TPC monitored at 10 min intervals for up to 60 min. Based on these results, sequential solvent fractionation was evaluated at 10-min intervals for up to 40 min.

For sequential extraction, the total solvent volume was maintained constant and divided equally into two, three, or four fractions. After each extraction step, the liquid fraction was separated, and fresh solvent was added to the residual biomass for the subsequent extraction. L1, L2, L3, and L4 correspond to the first, second, third, and fourth successive extraction steps, respectively. TPC was determined for each liquid fraction, and total TPC recovery was calculated as the cumulative TPC recovered after two, three, or four successive extraction steps.

2.2. Analysis of Response Variables

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method [21], with minor modifications. For each determination, 25 µL of extract was mixed with 575 µL of water, followed by the addition of 50 µL of Folin–Ciocalteu reagent. After vortex mixing, the reaction was allowed to proceed for 8 min before adding 150 µL of a 20% (w/v) Na_2CO_3 aqueous solution and 200 µL of water. The samples were subsequently incubated at room temperature for 45 min. An aliquot (200 µL) from each replicate was then transferred to a 96-well microplate, and absorbance was recorded at 760 nm. Gallic acid (GA) solutions ranging from 50 to 500 µg m/L were freshly prepared for each set of analyses and used to construct the calibration curve. All determinations were performed in triplicate. Results are expressed as mg GAE/g of *P. orbicularis* DW.

Antioxidant capacity was assessed using the FRAP assay, according to the method described by Benzie et al. [22], with minor modifications. Three solutions were prepared

for the assay: reagent A, containing 300 mM sodium acetate and 1.6% (*v*/*v*) acetic acid in water (pH 3.6); reagent B, consisting of 10 mM TPTZ prepared in 40 mM aqueous HCl; and reagent C, consisting of 20 mM $FeCl_3$ in water. The FRAP working reagent was freshly prepared by combining reagents A, B, and C at a 10:1:1 (*v*/*v*/*v*) ratio. An aliquot of 250 µL of the working reagent was dispensed into each well of a microplate and pre-incubated at 37 °C for 10 min, after which the initial absorbance was recorded at 593 nm. Subsequently, 40 µL of water, standard solution, or sample was added to the corresponding wells. The plate was then mixed in the microplate reader for 4 min to allow the reaction to develop, and the final absorbance was measured at 593 nm. All determinations were performed in triplicate. Results are expressed in µmol TE per g of *P. orbicularis* DW (µmol TE/g DW).

2.3. Total Polyphenol Content, Antioxidant Capacity, and Flavonoid Content of P. orbicularis Extract Obtained Under Optimal Conditions

The extract obtained under the optimized extraction conditions was concentrated and dried for 24 h using a SpeedVac DDA Concentrator (Savant SC250EXP; Thermo Fisher Scientific, Asheville, NC, USA) at 35 °C under vacuum. The dried residues were stored at −20 °C until analysis. TPC [21] and antioxidant capacity determined by FRAP were measured as previously described [22]. Results are expressed as mg GAE/g of extract DW and µmol TE/g of extract DW, respectively.

For ORAC analysis, a 75 mM phosphate buffer (pH 7.4) was prepared using KH_2PO_4 and Na_2HPO_4 [23]. This buffer solution was used to prepare 56 nM fluorescein and 133 mM AAPH. The calibration curve was prepared using Trolox® standard (1 mM; Sigma-Aldrich, Søborg, Denmark) diluted in phosphate buffer. For the assay, 210 µL of water, 30 µL blank/standard/sample and 150 µL of fluorescein were loaded into a black 96-well plate. The plate was incubated in the spectrophotometer at 37 °C for 10 min, followed by the addition of 30 µL of AAPH solution. Finally, kinetic measurements were recorded over 90 min, with readings taken every 50 s (λex = 485 nm and λem = 520 nm). Results are expressed in µmol TE per g of extract DW (µmol TE/g DW).

Total flavonoid content (FL) was determined using the aluminum chloride colorimetric method [24]. A quercetin calibration curve was prepared over a concentration range of 10–60 µg m/L. For the assay, 50 µL of sample, quercetin standard, or blank solution (EtOH:H_2O, 70:30, *v*/*v*) was dispensed into a 96-well microplate, followed by the addition of 200 µL of MeOH and 50 µL of 10% (*w*/*v*) $AlCl_3$. The reaction mixture was incubated at room temperature for 5 min, and absorbance was subsequently measured at 425 nm. Results are expressed as quercetin equivalent (QE) per g of extract DW (mg QE/g extract DW).

2.4. HPLC−MS Analysis and Tentative Identification of Major Compounds

2.4.1. HPLC−MS Analysis

All reagents and solvents were of analytical grade. LC-MS grade methanol, acetonitrile, and formic acid were purchased from Fisher-Scientific (Madrid, Spain) and used for mobile phase preparation and sample dilution. Deionized water (18 MΩ·cm), obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA), was used for the aqueous mobile phase (phase A).

The *P. orbicularis* extract was analyzed in duplicate by HPLC−MS to identify its major constituents. Chromatographic separation was performed using an Agilent 1260 HPLC System (Agilent Technologies, Palo Alto, CA, USA) equipped with a Zorbax Eclipse Plus RP-C18 column (4.6 × 150 mm, 1.8 µm, 600 bar; Agilent Technologies, Santa Clara, CA, USA). Mobile phase A consisted of water containing 0.1% formic acid, while mobile phase B consisted of acetonitrile. The chromatographic gradient program was as follows: 5% B at 0 min, 15% B at 15 min, 35% B at 20 min, and 75% B at 28 min. The final mobile-phase composition was maintained for 6 min to re-equilibrate the column before the subsequent

analysis. The injection volume was 5 µL, and the injector needle was rinsed with methanol between injections to minimize carryover. The flow rate was set at 0.4 mL/min, and the column temperature was maintained at 25 °C.

Mass spectrometric detection was performed using an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF mass spectrometer (G6540B; Agilent Technologies, Singapore) equipped with a dual electrospray ionization (ESI) Jet Stream source. Data were acquired in both negative and positive ionization modes over a mass range of *m/z* 50–1700. Ultrapure nitrogen was used as both the nebulizing and drying gas, with temperatures of 325 °C and 350 °C, and flow rates of 10 and 11 L/min, respectively. The source parameters were as follows: capillary voltage, 4 kV; nebulizer pressure, 30 psig; fragmentor (Q1) voltage, 130 V; nozzle voltage, 500 V; and skimmer voltage, 45 V. Continuous internal mass calibration was achieved using the trifluoroacetate anion (*m/z* 112.985587) and the hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine adduct (*m/z* 1033.988109) as reference ions. Data acquisition was performed using MassHunter Workstation software (version B.06.00, Agilent Technologies, Santa Clara, CA, USA).

2.4.2. Tentative Identification of Major Compounds

Major compounds were tentatively identified based on molecular feature detection. Detected peaks were filtered by a 0.2% relative volume threshold and those appearing in the solvent blank. Data processing was carried out using Qualitative Analysis (version B.07.00, Agilent Technologies) within the MassHunter Workstation Software version B.06.00 (Agilent Technologies).

Preliminary compound identification was performed through comparison with published literature on macroalgal metabolites and by consulting databases including CAS SciFinder, (https://www.cas.org/es-es/solutions/cas-scifinder-discovery-platform/cas-scifinder-n; accessed on 27 June 2025), METLIN (http://metlin.scripps.edu; accessed on 27 June 2025), and the Seaweed Metabolite Database (SWMD) (https://www.swmd.co.in/; accessed on 27 June 2025). Compound assignments were based on molecular formulas derived from accurate mass measurements and isotopic distribution patterns, together with retention times and MS/MS fragmentation profiles.

2.5. Cytotoxicity Analysis

The potential cytotoxicity of the dehydrated extract was evaluated in vitro using a lactate dehydrogenase (LDH) release assay (Sigma-Aldrich, St. Louis, MO, USA) in Caco-2 (passages 55 to 57) and HT-29 (passages 71 to 73) human cell lines. Cells were cultured in DMEM/F12 medium supplemented with 10% (*v*/*v*) fetal bovine serum and maintained at 37 °C in a humidified 5% CO_2 atmosphere. The extract was tested at concentrations ranging from 10 µg/mL to 1 mg/mL, while untreated cells (0 µg/mL extract) were used as the negative control, and 7% Triton X-100 was used as the positive control for cytotoxicity. Cells were incubated with the extract for 1 h, after which cytotoxicity was assessed by measuring LDH activity according to the manufacturer's instructions. Absorbance was recorded at 490 nm using 690 nm as the reference wavelength.

2.6. Statistical Analysis

All experiments were performed in triplicate, and results are presented as mean ± standard deviation. Statistical analysis, including Response Surface Methodology and analysis of variance (ANOVA), was conducted using Statgraphics Centurion 19 (version 19.4.01). Differences were considered statistically significant at p-values < 0.05.

3. Results

3.1. Optimization of Conventional Extraction Conditions for TPC from P. orbicularis

3.1.1. Response Surface Methodology Analysis

The experimental conditions generated by the central composite design, along with the corresponding experimental and model-predicted responses for TPC and FRAP, are presented in Table 2. Experimental TPC values ranged from 0.99 to 2.29 mg GAE/g, whereas FRAP values ranged from 2.85 to 11.9 µmol TE/g. The highest experimental TPC was obtained at 54.1 °C and an algae-to-solvent ratio of 1:20 (2.29 mg GAE/g), while the highest FRAP value was observed at 50 °C and a ratio of 1:30 (11.9 µmol TE/g).

Table 2. Statistical design of the Central Composite Design (CCD) with axial points used in the Response Surface Methodology to optimize extraction temperature (T) and algae/solvent ratio (Rs).

Treatments	Independent Variables		Response Variables			
			TPC [3] (mg GAE/g)		FRAP [4] (µmol TE/g)	
	T [1]	Rs [2]	Experimental	Predicted	Experimental	Predicted
T_1	40	1:20	2.02	1.74	4.53	5.70
T_2	50	1:10	1.29	1.03	2.85	2.96
T_3	40	1:20	1.42	1.74	5.91	5.70
T_4	54.1	1:20	2.29	1.20	5.83	6.26
T_5	40	1:5.9	0.99	1.12	3.38	2.82
T_6	25.9	1:20	1.07	1.36	4.79	5.53
T_7	30	1:30	1.88	1.67	11.8	10.3
T_8	40	1:34.1	1.85	1.91	11.2	12.9
T_9	40	1:20	2.04	1.74	5.69	5.70
T_{10}	50	1:10	1.69	1.71	3.32	3.66
T_{11}	40	1:20	1.49	1.74	6.69	5.70
T_{12}	50	1:30	2.17	2.18	11.9	10.6

[1] T: temperature (°C); [2] Rs: algae/solvent ratio (g/mL); [3] TPC: total polyphenolic content (mg GAE/g); [4] FRAP: antioxidant capacity measured by the FRAP assay (µmol TE/g).

The individual analysis of the response variables showed that Rs significantly influenced the antioxidant capacity measured by FRAP ($p < 0.05$), whereas extraction temperature and the interaction between T and Rs did not show significant effects. A relevant quadratic contribution of Rs was also observed, indicating a non-linear response of FRAP to increasing solvent volume. This effect is illustrated in the response surface plots presented in Figure 1.

The regression equation associated with the response variable *TPC* was as follows:

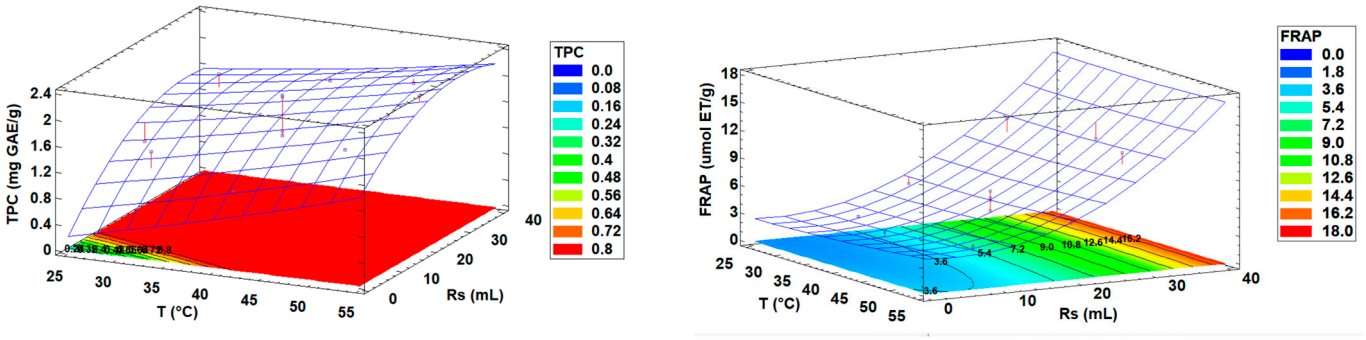
$$TPC = -0.50661 + 0.0236954T + 0.0902519Rs + 0.000183754T^2 - 0.0004325TRs - 0.001128751Rs^2 \quad (4)$$

On the other hand, the regression equation associated with the *FRAP* response variable was as follows:

$$FRAP = 2.67735 - 0.0333912T - 0.0394003Rs + 0.000966263T^2 - 0.000905TRs + 0.0108213Rs^2 \quad (5)$$

The coefficient of determination (R^2) was 71.83% for *TPC* and 91.3% for *FRAP*. Both models were statistically significant ($p < 0.05$), while the lack-of-fit was not significant ($p > 0.05$), indicating an adequate fit of the models to the experimental data. As shown in Figure 1, increasing *T* and *Rs* led to higher *TPC* values within the experimental region evaluated, whereas *FRAP* was predominantly influenced by Rs. Based on the response surface models, the individual optimization of *TPC* predicted optimal conditions of 55 °C

and an *Rs* of 1:29.4 g/mL, while *FRAP* optimization predicted 43.3 °C and an *Rs* of 1:40 g/mL.



(**a**) (**b**)

Figure 1. Response surface and contour plots illustrating the effect of extraction temperature (*T*, in °C) and algae-to-solvent ratio (*Rs*) on the extraction performance of dehydrated *P. orbicularis*. (**a**) Total phenolic content (TPC, in mg GAE/g); (**b**) Antioxidant capacity determined by the FRAP assay (µmol TE/g).

The simultaneous optimization of *TPC* and antioxidant capacity was evaluated using a desirability function approach. The response surface and contour plots are presented in Figure 2. Higher extraction *T* and *Rs* promoted the maximization of both *TPC* and *FRAP* values. The overall desirability reached the maximum value of 1.0, indicating that the selected conditions successfully optimized all response variables.

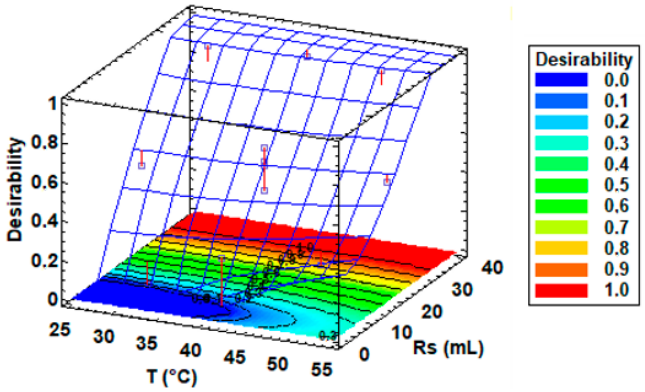


Figure 2. Response surface and contour plots of the overall desirability function for the simultaneous optimization of *TPC* (mg GAE/g) and antioxidant capacity measured by the *FRAP* assay (µmol TE/g), in *P. orbicularis* extract. The plots illustrate the combined effects of extraction temperature (*T*, in °C) and algae-to-solvent ratio (*Rs*, in mL for 1 g of seaweed). The experimental domain evaluated ranged from 25.9 to 54.1 °C for *T* and from 1:5.9 to 1:34.1 for *Rs*.

The optimized factor levels are presented in Table 2, while the individual and simultaneous optimizations of TPC and FRAP yielded different predicted optimal extraction conditions (Table 3). Therefore, the conditions predicted for each optimization criterion were experimentally evaluated. Among them, the conditions predicted for *TPC* optimization (*T*: 55 °C and *Rs*: 1:29.4 g/mL) resulted in significantly higher *TPC* (1.30 ± 0.03 mg GAE/g) and *FRAP*(0.61 ± 0.04 µmol TE/g) values than those obtained under the conditions predicted for *FRAP* optimization and simultaneous *TPC*–*FRAP* optimization ($p < 0.05$). Although the predicted temperature of 55 °C was slightly above the upper axial point of the experimental design (54.1 °C), this condition was evaluated experimentally before selection.

Consequently, 55 °C and an algae-to-solvent ratio of 1:29.4 g/mL were selected for the subsequent extraction stages.

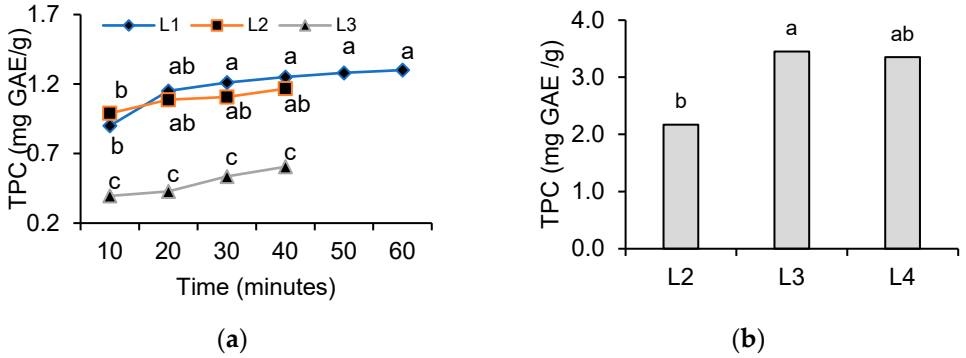
Table 3. Predicted and experimental values of *TPC* and antioxidant capacity obtained under the optimized extraction conditions determined by response surface methodology (RSM) using Central Composite Design with axial points.

Response Variable	Optimal Conditions	*TPC* [1] (mg GAE/g)	*FRAP* [2] (μmol TE/g)
TPC Single Variable Optimization	*T*: 55.0 °C; *Rs*: 1:29.4 g/mL	1.30 ± 0.03 a	0.61 ± 0.04 a
FRAP Single Variable Optimization	*T*: 43.3 °C *Rs*: 1:40 g/mL	0.84 ± 0.04 b	0.48 ± 0.02 b
TPC + FRAP Combined Variable Optimization	*T*: 42.37 °C *Rs*: 1:36.1 g/mL	0.85 ± 0.04 b	0.50 ± 0.01 b

[1] TPC: total polyphenol content expressed as gallic acid equivalent; [2] FRAP: Antioxidant capacity measured by FRAP assay. $n = 3$. Lowercase letters show significant differences between treatments for each column, evaluated by Tukey's HSD test ($p < 0.05$).

3.1.2. Extraction Kinetics and Solvent Fractionation

The extraction kinetics and the effect of sequential solvent fractionation on TPC recovery are shown in Figure 3. During the first extraction step (L1), TPC increased during the initial extraction period, reaching values at 30 min that did not differ significantly from those obtained at longer extraction times (40–60 min; $p > 0.05$) (Figure 3a). Based on this behavior, subsequent extraction steps were evaluated within a shorter time range. Renewal of the solvent allowed additional phenolic compounds to be recovered from the previously extracted biomass. During the second extraction step (L2), TPC increased with extraction time, while during the third extraction step (L3), the highest recovery was observed at 40 min, which was significantly higher than that obtained at the preceding evaluated times ($p < 0.05$).



(**a**) (**b**)

Figure 3. Effect of extraction time and sequential solvent fractionation on total phenolic content (TPC) recovery from *P. orbicularis*. (**a**) TPC recovered during each successive extraction step using fresh solvent: first (L1), second (L2), and third (L3) extraction. (**b**) Cumulative TPC recovered after two (L2), three (L3), or four (L4) successive extraction steps. The total solvent volume was divided equally among the corresponding number of extraction steps. $n = 3$. Lowercase letters indicate significant differences between treatments, as evaluated by Tukey's HSD test ($p < 0.05$).

The cumulative effect of sequential solvent fractionation is shown in Figure 3b. The total TPC recovered after three successive extraction steps was significantly higher than that obtained after two extraction steps ($p < 0.05$). However, increasing the number of extraction steps from three to four did not produce a significant additional increase in

total TPC ($p > 0.05$). Therefore, three sequential extraction steps were selected as the most suitable fractionation strategy.

3.2. Total Polyphenol Content, Flavonoid Content, and Antioxidant Capacity of the Optimized P. orbicularis Extract

The extract obtained under the optimized extraction conditions was dehydrated and subsequently characterized. TPC is expressed as mg GAE/g, while total flavonoid content is expressed as mg QE/g. Antioxidant capacity was determined using both FRAP and ORAC assays. The results are presented in Table 4.

Table 4. Total polyphenol content (TPC), flavonoid content (FL), and antioxidant activity of optimized *P. orbicularis* extract.

Parameter	TPC [1] (mg GAE/g)	FL [2] (mg QE/g)	FRAP [3] (µmol TE/g)	ORAC [4] (µmol TE/g)
Dried *P. orbicularis* extract	106.9 ± 3.5	12.4 ± 0.3	50.0 ± 2.2	500.1 ± 8.9

[1] TPC: total polyphenol content measured in gallic acid equivalent; [2] FL: total flavonoids measured in quercetin equivalent; [3] FRAP: Antioxidant capacity measured under FRAP methodology; [4] ORAC: Antioxidant capacity measured under ORAC methodology. $n = 3$.

3.3. Qualitative Characterization of the Extract by HPLC−MS

Qualitative characterization was performed to tentatively identify the major compounds present in the *P. orbicularis* extract. The extract obtained under the optimized extraction conditions was analyzed by HPLC-MS at a concentration of 30 mg/mL. The total compound chromatograms (TCC) acquired in both ionization modes are presented in Figure 4.

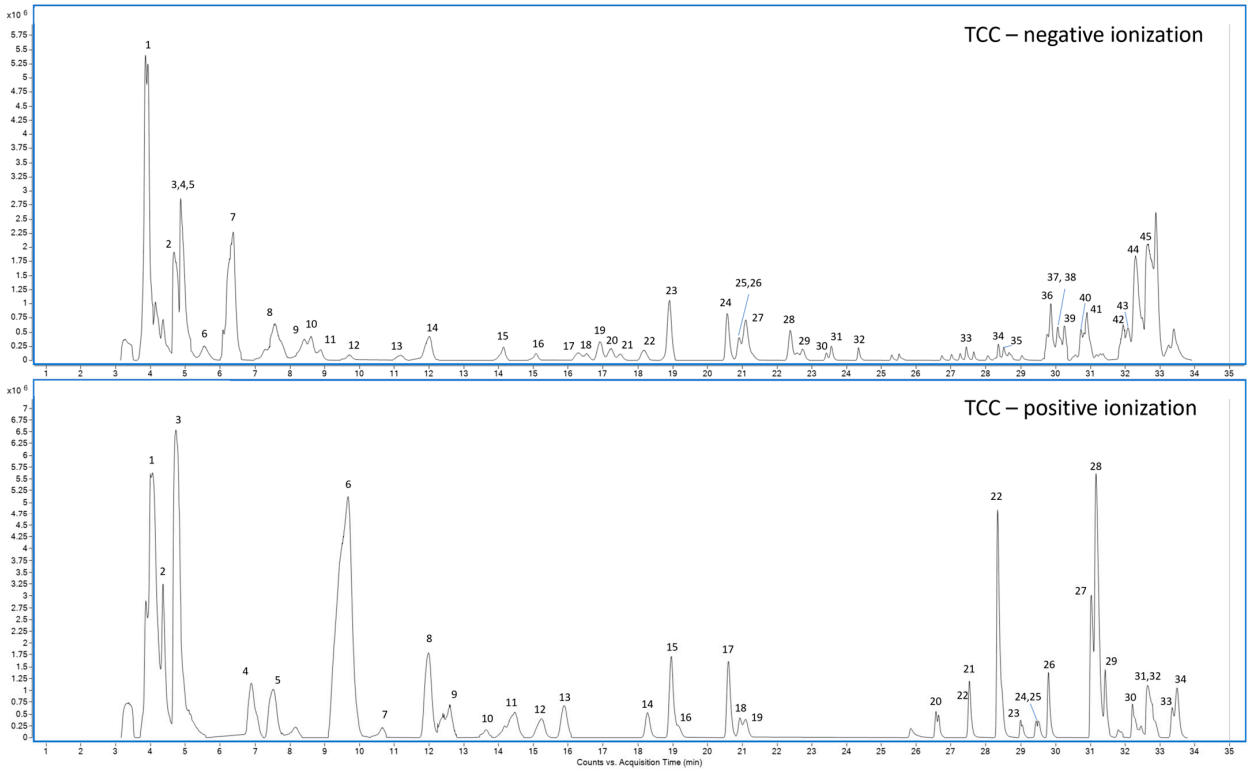


Figure 4. TCC (Total compound chromatograms) of *P. orbicularis* extract at a concentration of 30 mg/mL in both polarities. The numbers indicated on the peaks of the chromatogram correspond to the compounds in Tables 5 and 6.

Table 5. Compounds tentatively identified in the *P. orbicularis* extract by HPLC−MS in negative ionization.

Peak	Proposed Compound	RT (min)	Molecular Formula	*m/z*	Mass	Relative Amount (%)	Family
1	-	3.98	-	290.0776	291.0854	8.84	-
2	Gibberellic acid	4.70	$C_{19}H_{22}O_6$	345.1312	346.1384	3.42	Terpenoid
3	Citric acid derivative	4.88	$C_{10}H_{16}O_{16}$	391.0335	392.0412	1.81	Organic acid
4	Citric acid	4.90	$C_6H_8O_7$	191.0202	192.0275	4.13	Organic acid
5	Porphyra 334	5.16	$C_{14}H_{22}N_2O_8$	345.1306	346.1378	0.38	Micosporine aminoacid
6	Carbohydrate	5.54	$C_{17}H_{12}O_{11}$	391.0332	392.0405	0.6	Carbohydrate
7	Citric acid	6.32	$C_6H_8O_7$	191.0200	192.0273	7.64	Organic acid
8	Succinic acid	7.70	$C_4H_6O_4$	117.0196	118.0269	1.5	Organic acid
9	Mannosyl glycerate	8.39	$C_9H_{16}O_9$	267.0737	268.0809	1.1	Organic acid
10	Glycosidic derivative	8.60	$C_{12}H_{24}O_{10}$	327.1313	328.139	1.46	Glycosidic derivative
11	Unknown	8.87	$C_{11}H_{17}N_3O_5$	270.1094	271.1166	0.7	Unknown
12	Amino acid derivative	9.71	$C_{12}H_{25}NO_7$	294.1558	295.1631	0.3	Amino acid
13	Glutaric acid	11.17	$C_5H_8O_4$	131.0348	132.0421	0.31	Organic acid
14	Phenyl-alanine	11.96	$C_9H_{11}NO_2$	164.0718	165.0791	0.59	Amino acid
15	Succinyl adenosine	14.13	$C_{14}H_{17}N_5O_8$	382.1008	383.1079	0.62	Amino acid
16	Protein/Peptide sequence	15.07	$C_{22}H_{31}N_5O_9S$	540.177	541.1838	0.29	Protein
17	Furoic acid	16.28	$C_5H_4O_3$	111.0090	112.0162	0.37	Carboxilic acid
18	Protein/Peptide sequence	16.52	$C_{22}H_{31}N_5O_9S$	540.1768	541.1836	0.36	Protein
19	Pyroglutamyl valine	16.91	$C_{10}H_{16}N_2O_4$	227.1040	228.1112	0.71	Protein
20	Ethylcitrate/Dimetilcitrate	17.22	$C_8H_{12}O_7$	219.0513	220.0586	0.61	Organic compound
21	10-hydroxylimonene-7-oic acid-10-O-β-glucopyranoside	17.50	$C_{15}H_{23}O_6$	298.141	299.1482	0.28	Monoterpene
22	Tryptophane	18.20	$C_{11}H_{12}N_2O_2$	203.0829	204.0902	0.3	Aminoacid
23	Epigallocatechin derivative 1	18.91	$C_{23}H_{33}N_5O_9S$	554.1933	555.1999	2.48	Phenolic compound
24	Epigallocatechin derivative 2	20.57	$C_{23}H_{33}N_5O_9S$	554.1931	555.1997	1.86	Phenolic compound
25	Epigallocatechin derivative 3	20.91	$C_{23}H_{33}N_5O_9S$	554.1926	555.1993	0.57	Phenolic compound
26	1-ethyl-2-methoxy malate	20.94	$C_7H_{12}O_5$	175.0612	176.0685	0.35	
27	Hariganetin	21.10	$C_{18}H_{18}O_4$	297.1096	298.1168	1.84	Phenolic compound
28	Carboxilic acid Derivative of Diorcinol D	22.38	$C_{19}H_{20}O_5$	327.1204	328.1275	1.14	Diphenyl ether
29	Unknown	22.74	$C_{15}H_{30}O_{10}$	369.1779	370.1850	0.15	-
30	Unknown	23.42	$C_{16}H_{25}O_6$	312.1565	313.1637	0.18	-
31	Bicycloamid/3,7-Dihydroxy-cis,cis-1,8-nonadiene-1, 9-dicarboxylic acid diamide	23.56	$C_{11}H_{18}N_2O_4$	241.1195	242.1267	0.39	Amide derivative
32	Bicycloamid/3,7-Dihydroxy-cis,cis-1,8-nonadiene-1, 9-dicarboxylic acid diamide	24.34	$C_{11}H_{18}N_2O_4$	241.1196	242.1269	0.29	Amide derivative
33	Azelaic acid	27.44	$C_9H_{16}O_4$	187.0979	188.1052	0.27	Carboxilic acid
34	Decurrentetraol	28.36	$C_{20}H_{32}O_6$	367.2129	368.2189	0.36	Diterpene
35	Yuanhuadin	28.52	$C_{32}H_{42}O_{10}$	585.2721	586.2792	0.28	Diterpene
36	Prostaglandin E2 isomer 1	29.87	$C_{20}H_{32}O_5$	351.2185	352.226	0.99	Fatty acid
37	Prostaglandin E2 isomer 2	30.07	$C_{20}H_{32}O_5$	351.2186	352.2257	1.14	Fatty acid
38	Orthosiphols R	30.17	$C_{36}H_{42}O_{10}$	633.268	634.2754	0.2	Diterpene
39	Prostaglandin E2 isomer 3	30.26	$C_{20}H_{32}O_5$	351.2186	352.2259	0.86	Fatty acid
40	THETA (Trihydroxyeicosa-trienoic acid)	30.76	$C_{20}H_{34}O_5$	353.2338	354.2411	0.87	Lipid

Table 5. *Cont.*

Peak	Proposed Compound	RT (min)	Molecular Formula	*m/z*	Mass	Relative Amount (%)	Family
41	Palmitic oleic dimer hexoside isomer 1	30.91	$C_{32}H_{60}O_{16}$	699.3811	700.3882	0.61	Fatty acid
42	1-Linoleoylglycerol	31.94	$C_{20}H_{34}O_5$	353.2337	354.241	0.34	Fatty acid
43	9,15-Dihidroxikaur-16-en-19-oic acid	32.00	$C_{20}H_{30}O_4$	333.2074	334.2148	1.15	Diterpene
44	Gibberellin A12	32.09	$C_{20}H_{28}O_4$	331.1917	332.1989	0.3	Diterpene
45	Palmitic oleic dimer hexoside isomer 2	32.29	$C_{32}H_{60}O_{16}$	699.3818	700.3889	2.83	Fatty acid

Table 6. Compounds tentatively identified in the *P. orbicularis* extract by HPLC−MS in positive ionization.

Peak	Proposed Compound	RT (min)	Molecular Formula	*m/z*	Mass	Relative Amount (%)	Family
1	Unknown	4.00	$C_8H_{17}NO_3$	176.1285	175.1212	1.2	-
2	Acidic amino acid	4.37	$C_7H_{11}NO_4$	174.0767	173.0694	1.7	Aminoacid
3	Gibberellic acid	4.40	$C_{19}H_{22}O_6$	347.146	346.1386	0.2	Terpenoid
4	Leucine/Isoleucine	6.88	$C_6H_{13}NO_2$	132.1014	131.0941	1.28	Amino acid
5	Leucine/Isoleucine	7.51	$C_6H_{13}NO_2$	132.1026	131.0953	1.81	Amino acid
6	Unknown	9.60	$C_9H_{19}NO_2$	174.1493	173.142	20.06	-
7	Palythene	10.65	$C_{13}H_{20}N_2O_5$	285.1448	284.1375	0.37	Mycosporine amino acid
8	Phenylalanine	11.97	$C_9H_{11}NO_2$	166.0866	165.0794	2.38	Amino acid
9	Hydroxylated derivative of trichodermolide B/Caulerpenyne	12.58	$C_{21}H_{26}O_6$	375.1768	374.1694	1.5	Terpenoid
10	Succinoadenosine	13.63	$C_{14}H_{17}N_5O_8$	345.1662	344.1591	0.29	Amino acid
11	Unknown	14.43	$C_{12}H_{17}NO_2$	208.1335	207.1261	1.5	-
12	Unknown	15.22	$C_8H_{11}N_3O_3$	198.0876	197.0803	0.54	-
13	Unknown	15.88	$C_{10}H_{21}NO_2$	188.165	187.1577	1.3	-
14	Indole acrylic acid	18.28	$C_{11}H_9NO_2$	188.0708	187.0635	0.28	Carboxilic acid
15	Epigallocatechin derivative isomer 1	18.96	$C_{23}H_{33}N_5O_9S$	556.2081	555.2009	1.58	Phenolic compound
16	N-Acetyl-4-hydroxyvalinelactone	19.16	$C_7H_{11}NO_3$	158.0815	157.0742	0,.4	Amino acid derivative
17	Epigallocatechin derivative isomer 2	20.60	$C_{23}H_{33}N_5O_9S$	556.2079	555.2006	1.24	Phenolic compound
18	Epigallocatechin derivative isomer 3	20.94	$C_{23}H_{33}N_5O_9S$	556.2077	555.2006	0.44	Phenolic compound
19	Trimethoxy-chalcone	21.10	$C_{18}H_{18}O_4$	299.1242	298.1168	0.49	Flavonoid
20	Loliolide isomer 1	26.57	$C_{11}H_{16}O_3$	197.1166	196.1093	0.41	Monoterpene
21	Loliolide isomer 2	27.53	$C_{11}H_{16}O_3$	197.1159	196.1086	0.98	Monoterpene
22	Unknown	28.34	$C_{21}H_{28}N_2O$	325.2279	324.2206	3.85	-
23	Unknown	29.01	$C_{34}H_{46}O_{11}$	631.3118	630.3044	0.33	-
24	3-hydroxy-β-damascone	29.45	$C_{13}H_{20}O_2$	209.1529	208.1456	0.31	Terpenoid
25	Unknown	29.53	$C_{13}H_{18}O$	191.1412	190.1339	1.08	-
26	Yuanhuadin	29.80	$C_{32}H_{42}O_{10}$	587.2872	586.2799	1.08	Diterpenoid
27	Unknown	31.04	$C_{14}H_{31}N$	214.2528	213.2455	3.11	-
28	3-epi-xestoaminol C	31.18	$C_{14}H_{31}NO$	230.2467	229.2396	6.71	Lipid
29	Unknown	31.43	$C_{19}H_{33}N$	276.2684	275.2611	0.88	-
30	Diterpenoid derivative	32.46	$C_{20}H_{26}O_3$	315.195	314.1877	0.29	Diterpenoid
31	GSL Derivative	32.70	$C_{40}H_{78}NO_5$	653.5961	652.5888	1.37	Lipid
32	4-((2,4-dihydroxy-6-nonylbenzoyl)oxy)-2hydroxy-6-nonylbenzoicacid	33.36	$C_{32}H_{45}O_7$	542.3261	541.3185	0.62	Lipid
33	Unknown	33.48	$C_{18}H_{26}O_2$	275.2011	274.1938	0.36	-
34	Unknown	33.50	$C_{34}H_{42}O_{12}$	643.2773	642.2669	0.26	Unknown

All detected compounds, together with their mass spectrometric data and tentative identifications, are listed in Tables 5 and 6 for negative and positive ionization modes, respectively. The major identified compounds are highlighted in Figure 4. In all cases, the mass accuracy error was below 10 ppm.

In the negative ionization mode, 45 compounds were detected, of which 42 were tentatively identified. Organic acids represent the predominant chemical family based on relative abundance, followed by amino acid/protein derivatives and more apolar molecules, including terpenoid derivatives and fatty acids. The most abundant compounds detected under this ionization mode were citric acid, gibberellic acid, palmitoyl oleoyl hexoside, and an epigallocatechin derivative.

In the positive ionization mode, 33 compounds were detected, and 22 were tentatively identified. The most abundant compound corresponded to peak 6 (Figure 4, Table 6) with the molecular formula $C_9H_{19}NO_2$. Other identified constituents included amino acid and protein derivatives, terpenoid derivatives and lipids.

3.4. Cytotoxic Potential of P. orbicularis Extract

The *P. orbicularis* extract did not induce significant cytotoxicity in either Caco-2 or HT-29 cells at concentrations ranging from 10 to 1000 µg/mL after 1 h of exposure (Figure 5). Cytotoxicity levels at all tested extract concentrations were not significantly different from the negative control (0 µg/mL; $p > 0.05$), whereas the positive control (7% Triton X-100) showed significantly higher cytotoxicity than all extract treatments ($p < 0.05$).

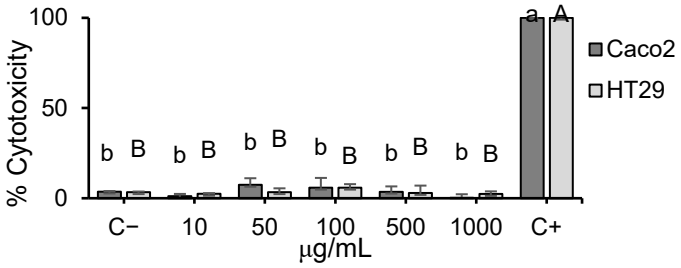


Figure 5. Cytotoxic activity of the *P. orbicularis* extract in Caco-2 and HT-29 cells, determined using the LDH assay. Extract concentrations ranged from 10 to 1000 µg/mL. C- indicates the negative cytotoxicity control (0 µg/mL); C+ indicates the positive cytotoxicity control (7% Triton X-100). Data are presented as mean ± standard deviation ($n = 3$). Different lowercase and uppercase letters indicate significant differences among treatments in Caco-2 and HT-29 cells, respectively, as determined by one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

4. Discussion

Extraction temperature influences the transfer of bioactive compounds from the algal matrix to the solvent and their chemical stability [17,18]. Moderate increases in temperature may enhance phenolic recovery by improving solubility and mass transfer [24], whereas excessive temperatures can promote the degradation or oxidation of thermolabile compounds [25,26]. In the present study, neither temperature nor the algae-to-solvent ratio had a significant individual effect on TPC ($p > 0.05$), although an increasing trend was observed at higher levels of both variables. In contrast, the algae-to-solvent ratio significantly affected FRAP ($p < 0.05$), whereas temperature and its interaction were not significant. Greater solvent availability may favor mass transfer by maintaining the concentration gradient between the solid matrix and the extraction medium [25,27], which may explain its stronger influence on FRAP.

The differing responses of TPC and FRAP may reflect variations in the chemical profiles of the recovered compounds, since antioxidant capacity depends not only on phenolic content but also on the chemical structure and assay-specific responses of individual

compounds [28]. Moreover, the relationship between TPC and antioxidant activity in seaweed extracts is not always consistent [10], supporting the use of both responses as complementary indicators during optimization. Although the individual effects on TPC were not significant, the fitted model was significant and showed no significant lack of fit. The predicted optima were therefore experimentally validated, and the TPC-predicted condition (55 °C; Rs 1:29.4 g/mL) yielded significantly higher TPC and FRAP values than the other validated optima, supporting its selection for subsequent experiments.

Extraction time and solvent renewal further influenced phenolic recovery after selecting the temperature and algae-to-solvent ratio. During the first extraction cycle, TPC initially increased and then plateaued at approximately 30 min, with no significant differences at longer extraction times. This behavior is consistent with solid–liquid extraction kinetics, in which an initially high concentration gradient between the solid matrix and the extraction medium promotes mass transfer, whereas the extraction rate progressively decreases as the system approaches equilibrium [25,27,29]. Sequential extraction with fresh solvent enabled the recovery of additional phenolic compounds from the previously extracted biomass. Repeated extraction cycles involving solvent renewal have also been employed to enhance the recovery of bioactive compounds from algae [30]. In the present study, three sequential extraction cycles resulted in significantly higher cumulative TPC than two cycles, whereas a fourth cycle did not provide a significant additional increase. Therefore, three extraction cycles were selected as an appropriate compromise between phenolic recovery and process intensity.

TPC varies considerably among seaweed species and extraction conditions. In a study involving six red algae species (*Phacelocarpus peperocarpos*, *Gelidium asperum*, *Areschougia congesta*, *Callophyllis* sp., *Hymenena australis*, and *Dicranema revolutum*), extraction with 70% ethanol at a Rs of 1 g:20 mL, 10 °C, and 16 h of agitation yielded TPC values ranging from 0.13 to 4.67 mg GAE/g, with *H. australis* and *P. peperocarpos* exhibiting the lowest and highest values, respectively [9]. Similarly, extracts from *Palmaria palmata*, *Porphyra purpurea*, *Chondrus crispus*, *Mastocarpus stellatus*, *Polysiphonia fucoides*, and *Gracilaria vermiculophyllum* obtained using 96% ethanol at a 1:10 Rs and room temperature exhibited TPC values ranging from 0.50 to 3.47 mg GAE/g [8]. The TPC obtained in the present study (3.1 mg GAE/g) was within the range reported for these red algae (0.13–4.67 mg GAE/g). Differences among studies may reflect both biological variability and differences in extraction and biomass-processing conditions [31].

Previous studies have reported considerable variability in the phenolic content and antioxidant capacity of *Pyropia* extracts. Recently, an aqueous extract of *P. spiralis*, obtained by repeated water extraction followed by freeze-drying, showed TPC values ranging from 234.33 to 548.17 mg GAE/g DW [31]. For Chilean *Pyropia* spp., aqueous and acetone/water extracts exhibited TPC values of 6.21 ± 0.04 and 3.1 ± 0.1 mg GAE/g, respectively, with corresponding ORAC values of 26.95 ± 0.83 and 30.7 ± 2.9 µmol TE/g [32,33]. In the present study, the dehydrated optimized extract of *P. orbicularis* exhibited a TPC of 106.9 mg GAE/g extract DW and an ORAC value of 500.1 µmol TE/g extract DW. The variability among the reported values is consistent with previous studies showing that phenolic recovery from seaweeds is strongly dependent on the extraction procedure, including solvent-to-solid ratio, extraction technique, and sample pretreatment [10].

For the six red algae previously described, flavonoid content ranged from 0.02 to 0.23 mg QE/g, with *Rhodophyllis* sp. and *P. peperocarpos* exhibiting the lowest and highest values, respectively [10]. More recently, an aqueous freeze-dried extract of *P. spiralis* was reported to contain 59.9 mg QE/g DW [31]. In the present study, the optimized *P. orbicularis* extract exhibited a flavonoid content of 12.4 mg QE/g extract DW. The variability in flavonoid content reported among seaweeds may be associated with biological

factors, including species, geographical origin, and harvesting season [10,34]. In addition, processing and extraction conditions can influence flavonoid recovery, as solvent composition, temperature, extraction time, and extraction method affect their recovery and stability [35,36].

Optimization of the extraction process increased TPC recovery by 41.4% while reducing extraction time by 33.4%. Shorter extraction times are technologically advantageous because prolonged exposure to extraction conditions may promote structural changes in polyphenols and reduce their antioxidant activity [17,37]. Thus, the optimized conditions improved phenolic recovery while reducing processing time, potentially contributing to a more resource-efficient extraction process. The average TPC reported for red algal extracts is approximately 12 mg GAE/g [10], and the values obtained in this study exceeded that benchmark.

The principal compounds tentatively identified in the optimized extract included amino acid derivatives, lipids, and other organic molecules (Tables 5 and 6). The chemical profile differed from those reported in previous studies [38–40], likely because different algal species were investigated. Since relatively few studies have focused on *P. orbicularis*, direct comparisons remain limited. Previous analyses identified quercetin in methanol/water extracts, while aqueous extracts contained adenosine, dexamethasone, minoxidil, phenolic compounds, and the alkaloid vincamine. Acetonitrile extracts were reported to contain 4-pyridoxic acid, acetyl-CoA, chrysoeriol, colchicine, and emetine [15].

The optimized extract analyzed in this study contained some compounds detected in both negative and positive ionization modes, such as gibberellic acid, epigallocatechin derivatives or yuanhuadin, whereas others were observed exclusively in one polarity. For example, in terpenoid detection, loliolide was detected only in positive ionization mode, whereas ortospirhols R and gibberellin A12 were detected in negative ionization mode (Tables 5 and 6). In addition to essential and non-essential amino acids, peptides, and protein-derived compounds, several metabolites not widely reported in the literature were identified. Notably, mycosporine-like amino acid (MAA) derivatives were detected. In negative ionization mode, a compound with molecular formula $C_{14}H_{22}N_2O_8$ was tentatively identified as porphyra 334, while a compound with molecular formula $C_{13}H_{20}N_2O_5$ detected in positive mode was assigned as palythene. These MAAs are characteristic of red algae and function as natural photoprotective compounds with strong antioxidant properties. Although both metabolites have been previously reported in macroalgae from Latin-American coastal regions [41,42], this is the first report of their occurrence in *P. orbicularis*.

Only two phenolics were confidently detected: a trimethoxychalcone in positive ionization mode and an epigallocatechin derivative detected in both ionization modes. Trimethoxychalcones belong to the chalcone family, compounds structurally related to flavonoids that contribute to pigmentation and antioxidant activity. Their bioavailability and interactions with other biomolecules remain active areas of investigation [43].

A compound with molecular mass 555.2006 Da, detected at *m/z* 554.1931 in negative mode and *m/z* 556.2077 in positive mode, was tentatively assigned as an epigallocatechin derivative, based on its mass spectrum, extracted ion chromatogram (EIC) and previously reported literature. Similar compounds have been identified in red algae and other macroalgal species [12,44]. The EICs corresponding to m/z 495.12 and m/z 554.19 are presented in Supplementary Figure S1. The mass spectrum at 20.6 min showed the molecular ion at *m/z* 554.1926 and two additional peaks at *m/z* 495.1185 and 1109.3924. The similar chromatographic behavior of these ions suggests a common origin. The ion at *m/z* 495.1185 was tentatively assigned to an adduct formed between epigallocatechin and formic acid from the mobile phase, whereas the ion at *m/z* 1109.3924 likely represents a dimeric species. Con-

sequently, compounds with molecular mass 555.2 and molecular formula $C_{23}H_{33}N_5O_9S$ were classified as epigallocatechin derivatives.

Several terpenoids were also tentatively identified, including decurrentetraol, yuanhuadin, gibberellin A, and loliolide. Terpenoids constitute one of the most abundant classes of natural compounds in red algae, although many occur at relatively low concentrations [45]. To the best of our knowledge, no previous reports have described these compounds in *P. orbicularis*. Their identification was based on spectral matching with scientific literature and available databases. Decurrentetraol has been reported in the brown alga *Turbinaria decurrens* [45], yuanhuadine in *Daphne tangutica* [46], gibberellin A in red algae [47], and loliolide in representatives of all three major macroalgal groups [40,48]. Further studies are required to confirm these assignments and better characterize the terpenoid profile of *P. orbicularis*.

The remaining compounds identified consisted primarily of lipids including fatty acids, protein-derived metabolites, and common organic acids such as glutaric acid, detected at 11.17 min in negative ionization mode and tentatively attributed to cell wall degradation products. Because the extraction process was optimized based on TPC and antioxidant activity, phenolic compounds were expected to predominate. However, HPLC-MS analysis revealed that relatively few phenolics were extracted under the optimized conditions. Compounds such as bromophenols, lignans, or flavonoid-related metabolites, which have previously been reported in macroalgae [11,41], were not detected. This finding may be attributable to the degradation of phenolic compounds during the prolonged traditional sun-drying process. Although elevated temperatures are known to promote phenolic degradation, information regarding the stability of naturally occurring bromophenols remains limited [30,49].

Most studies characterizing ethanolic seaweed extracts by HPLC−MS use either fresh or freeze-dried biomass to preserve phenolic compounds more effectively [40,50–52]. For example, Cruces et al. (2017) showed that freeze-drying improved TPC in *Lessonia spicata*, compared with freezing, silica-drying, oven-drying, or air-drying [2]. Nevertheless, freeze-drying is energy-intensive and presents challenges for large-scale industrial implementation [26].

The optimized *P. orbicularis* extract showed no cytotoxicity in HT-29 and Caco-2 cells at concentrations ranging from 10 to 1000 μg/mL. These findings are consistent with previous evidence for *Pyropia*-derived extracts. Extracts from Chilean *Pyropia* sp. did not reduce HT-29 cell viability below the threshold considered indicative of cytotoxicity at concentrations up to 1000 μg/mL, although a slight decrease in viability was observed at higher concentrations [30]. Similarly, recent evidence for *P. haitanensis* showed no significant reduction in Caco-2 cell viability at concentrations of 0.5–2 mg/mL [53]. Together, these findings indicate a consistent absence of cytotoxic effects of Pyropia-derived extracts within the concentration ranges evaluated.

Differences in cellular responses among seaweed extracts may nevertheless depend on species, extract composition, extraction conditions, concentration, exposure time, and the cellular model employed. Therefore, the absence of cytotoxicity observed for *P. orbicularis* under the present in vitro conditions should not be considered definitive evidence of safety, but rather provides preliminary support for further toxicological and biological evaluation of the extract for food-related applications.

5. Conclusions

Optimization of the extraction variables enabled the development of a suitable methodology for recovering bioactive compounds from *P. orbicularis* using a food-compatible hydroethanolic extraction system. The selected conditions (80% ethanol, 55 °C, an algae-

to-solvent ratio of 1:29.4 g/mL, and 40 min) resulted in a dried extract with a TPC of 106.9 mg GAE/g, a flavonoid content of 12.4 mg QE/g, and antioxidant capacities of 50.0 μmol TE/g by FRAP and 500.1 μmol TE/g by ORAC. Furthermore, this study expands current knowledge of the chemical composition of *P. orbicularis* by tentatively identifying several bioactive compounds using HPLC-MS. No cytotoxicity was observed in Caco-2 or HT-29 cells at concentrations up to 1000 μg/mL, supporting further investigation of the extract for food-related applications.

Overall, these findings contribute to the valorization of *P. orbicularis* as an underutilized marine resource and provide a basis for developing natural bioactive ingredients derived from marine biomass. Potential future applications may include functional foods, bioactive coatings, innovative packaging materials, and nutraceutical products; however, their technological performance in these systems remains to be experimentally demonstrated. Further studies should also confirm the identity of the tentatively identified compounds and evaluate the biological activity, stability, and safety of the extract before its practical application in food systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applbiosci5040085/s1, Figure S1. EIC (m/z: 554.19 and 495.12) and spectrum at 20.55 min.

Author Contributions: Conceptualization, C.P.M.-G., I.A.C.-P. and M.G.; methodology, C.P.M.-G., I.A.C.-P. and I.M.-R.; experimental work, C.P.M.-G., D.M.-B. and I.M.-R.; statistical analysis, C.P.M.-G. and I.A.C.-P.; writing—review and editing validation, C.P.M.-G., I.A.C.-P., D.M.-B., I.M.-R., M.G. and V.V.; project administration, C.P.M.-G. and I.A.C.-P.; funding acquisition, C.P.M.-G., M.G. and I.A.C.-P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Agency for Research and Development, Chile, grant number TDP220007 and by the Chilean Economic Development Agency (CORFO), project number 20SN-139578/2021.

Data Availability Statement: The original contributions presented in this study are included in the article and Supplementary Material. Further inquiries can be directed to the corresponding authors.

Acknowledgments: Carla P. Morales-Guzmán is grateful to the National Agency for Research and Development, the National PhD Scholarship, the Mobility Scholarship between Andalusian and Latin American Universities, and CIDAF for the research stay grant.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

TPC	Total Phenolic Content
FRAP	Ferric reducing antioxidant power
ORAC	Oxygen radical absorbance capacity
GAE	Gallic acid equivalent
TE	Trolox equivalent
T	Extraction temperature
Rs	Algae-to-solvent ratio
FL	Total flavonoid content
QE	Quercetin equivalent
HPLC-MS	High-performance liquid chromatography–mass spectrometry

References

1. Baweja, P.; Kumar, S. Biology of seaweeds. In *Seaweed in Health and Disease Prevention*; Fleurence, J., Levine, I., Eds.; Academic Press: London, UK, 2016; pp. 41–106. [CrossRef]
2. Cruces, E.; Rautenberger, R.; Rojas-Lillo, Y.; Cubillos, V.M.; Arancibia-Miranda, N.; Ramírez-Kushel, E.; Gómez, I. Physiological acclimation of *Lessonia spicata* to diurnal changing PAR and UV radiation: Differential regulation among down-regulation of photochemistry, ROS scavenging activity and phlorotannins as major photoprotective mechanisms. *Photosynth. Res.* **2017**, *131*, 145–157. [CrossRef] [PubMed]
3. Hermund, D.B. Antioxidant properties of seaweed-derived substances. In *Bioactive Seaweeds for Food Applications*; Qin, Y., Ed.; Academic Press: London, UK, 2018; pp. 201–221. [CrossRef]
4. Ren, J.; Li, Y.; Lin, Q.; Li, Z.; Zhang, G. Development of biomaterials based on plasticized polylactic acid and tea polyphenols for active-packaging application. *Int. J. Biol. Macromol.* **2022**, *217*, 814–823. [CrossRef] [PubMed]
5. Wrona, M.; Nerín, C.; Alfonso, M.J.; Caballero, M.Á. Antioxidant packaging with encapsulated green tea for fresh minced meat. *Innov. Food Sci. Emerg. Technol.* **2017**, *41*, 307–313. [CrossRef]
6. Zhu, F. Polysaccharide based films and coatings for food packaging: Effect of added polyphenols. *Food Chem.* **2021**, *359*, 129871. [CrossRef] [PubMed]
7. Wang, Y.; Jia, W.; Wang, X.; Aslam, M.M.; Li, W.; Shao, Y. Tea polyphenols coating improves physiological properties, microstructure and chemical composition of cuticle to suppress quality deterioration of passion fruit during cold storage. *Food Chem.* **2025**, *463*, 141524. [CrossRef] [PubMed]
8. Pu, Y.; Jiang, H.; Zhang, Y.; Cao, J.; Jiang, W. Advances in propolis and propolis functionalized coatings and films for fruits and vegetables preservation. *Food Chem.* **2023**, *414*, 135662. [CrossRef] [PubMed]
9. Qin, Y. Seaweed bioresources. In *Bioactive Seaweeds for Food Applications*; Qin, Y., Ed.; Academic Press: London, UK, 2018; pp. 3–24. [CrossRef]
10. Torres, P.; Osaki, S.; Silveira, E.; Santos, D.Y.A.C.; Chow, F. Comprehensive evaluation of Folin-Ciocalteu assay for total phenolic quantification in algae (Chlorophyta, Phaeophyceae, and Rhodophyta). *Algal Res.* **2024**, *80*, 103503. [CrossRef]
11. Sabeena Farvin, K.H.; Jacobsen, C. Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. *Food Chem.* **2013**, *138*, 1670–1681. [CrossRef] [PubMed]
12. Ebrahimi, F.; Subbiah, V.; Agar, O.T.; Bringloe, T.T.; Legione, A.R.; Suleria, H.A.R. LC-ESI-QTOF-MS/MS characterization of phenolic compounds from Victorian shorebound red seaweeds and their antioxidant capacity. *Algal Res.* **2024**, *82*, 103609. [CrossRef]
13. Sapatinha, M.; Oliveira, A.; Costa, S.; Pedro, S.; Gonçalves, A.; Mendes, R.; Bandarra, N.M.; Pires, C. Red and brown seaweeds extracts: A source of biologically active compounds. *Food Chem.* **2022**, *393*, 133453. [CrossRef] [PubMed]
14. Uribe, E.; Vega-Gálvez, A.; García, V.; Pastén, A.; Rodríguez, K.; López, J.; Di Scala, K. Evaluation of physicochemical composition and bioactivity of a red seaweed (*Pyropia orbicularis*) as affected by different drying technologies. *Dry. Technol.* **2020**, *38*, 1218–1230. [CrossRef]
15. Namvar, F.; Mohamed, S.; Ghasemi Fard, S.; Behravan, J.; Mustapha, N.M.; Alitheen, N.B.M.; Othman, F. Polyphenol-rich seaweed (*Eucheuma cottonii*) extract suppresses breast tumour via hormone modulation and apoptosis induction. *Food Chem.* **2012**, *130*, 376–382. [CrossRef]
16. Latorre, N.; Castañeda, F.; Meynard, A.; Rivas, J.; Contreras-Porcia, L. First approach of characterization of bioactive compound in *Pyropia orbicularis* during the daily tidal cycle. *Lat. Am. J. Aquat. Res.* **2019**, *47*, 826–840. [CrossRef]
17. Iylia Arina, M.Z.; Harisun, Y. Effect of extraction temperatures on tannin content and antioxidant activity of *Quercus infectoria* (Manjakani). *Biocatal. Agric. Biotechnol.* **2019**, *19*, 101104. [CrossRef]
18. Zheng, H.; Du, H.; Ye, E.; Xu, X.; Wang, X.; Jiang, X.; Min, Z.; Zhuang, L.; Li, S.; Guo, L. Optimized extraction of polyphenols with antioxidant and anti-biofilm activities and LC-MS/MS-based characterization of phlorotannins from *Sargassum muticum*. *LWT* **2024**, *198*, 116069. [CrossRef]
19. Manchanda, S.; Chandra, A.; Bandopadhyay, S.; Deb, P.K.; Tekade, R.K. Formulation additives used in pharmaceutical products: Emphasis on regulatory perspectives and GRAS. In *Dosage Form Design Considerations*; Tekade, R.K., Ed.; Academic Press: Cambridge, MA, USA, 2018; pp. 773–831. [CrossRef]
20. Fierro, C.; López-Cristoffanini, C.; Meynard, A.; Lovazzano, C.; Castañeda, F.; Guajardo, E.; Contreras-Porcia, L. Expression profile of desiccation tolerance factors in intertidal seaweed species during the tidal cycle. *Planta* **2017**, *245*, 1149–1164. [CrossRef] [PubMed]
21. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158. [CrossRef]
22. Benzie, I.F.F.; Strain, J.J. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* **1999**, *299*, 15–27. [CrossRef] [PubMed]

23. Dávalos, A.; Gómez-Cordovés, C.; Bartolomé, B. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *J. Agric. Food Chem.* **2004**, *52*, 48–54. [CrossRef] [PubMed]
24. Cumbao, J.L.T.; Alvarez, P.L.J.; Belina-Aldemita, M.D.; Micor, J.R.L.; Angelia, M.R.N.; Manila-Fajardo, A.C.; Cervancia, C.R. Total phenolics, total flavonoids, antioxidant activity and antibacterial property of propolis produced by the stingless bee, *Tetragonula biroi* (Friese), from Laguna and Quezon, Philippines. *Philipp. Entomol.* **2016**, *30*, 63–74. [CrossRef]
25. Ajila, C.M.; Brar, S.K.; Verma, M.; Tyagi, R.D.; Godbout, S.; Valéro, J.R. Extraction and analysis of polyphenols: Recent trends. *Crit. Rev. Biotechnol.* **2011**, *31*, 227–249. [CrossRef] [PubMed]
26. Wang, J.; Zhang, M.; Fang, Z. Recent development in efficient processing technology for edible algae: A review. *Trends Food Sci. Technol.* **2019**, *88*, 251–259. [CrossRef]
27. Chemat, F.; Rombaut, N.; Sicaire, A.G.; Meullemiestre, A.; Fabiano-Tixier, A.S.; Abert-Vian, M. Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrason. Sonochem.* **2017**, *34*, 540–560. [CrossRef] [PubMed]
28. Csepregi, K.; Neugart, S.; Schreiner, M.; Hideg, É. Comparative evaluation of total antioxidant capacities of plant polyphenols. *Molecules* **2016**, *21*, 208. [CrossRef] [PubMed]
29. Bucić-Kojić, A.; Planinić, M.; Tomas, S.; Bilić, M.; Velić, D. Study of solid–liquid extraction kinetics of total polyphenols from grape seeds. *J. Food Eng.* **2007**, *81*, 236–242. [CrossRef]
30. Vo, T.P.; Nguyen, D.Q.; Ho, T.A.T.; Nguyen, T.M.; Ha, N.M.H.; Vo, P.H.N. Novel extraction of bioactive compounds from algae using green solvent: Principles, applications, and future perspectives. *J. Agric. Food Res.* **2024**, *18*, 101535. [CrossRef]
31. Duan, X.; Subbiah, V.; Agar, O.T.; Barrow, C.J.; Ashokkumar, M.; Dunshea, F.R.; Suleria, H.A.R. Optimizing extraction methods by a comprehensive experimental approach and characterizing polyphenol compositions of *Ecklonia radiata*. *Food Chem.* **2024**, *455*, 139926. [CrossRef] [PubMed]
32. O'Sullivan, A.M.; O'Callaghan, Y.C.; O'Grady, M.N.; Queguineur, B.; Hanniffy, D.; Troy, D.J.; Kerry, J.P.; O'Brien, N.M. In vitro and cellular antioxidant activities of seaweed extracts prepared from five brown seaweeds harvested in spring from the west coast of Ireland. *Food Chem.* **2011**, *126*, 1064–1070. [CrossRef]
33. Mokrani, A.; Madani, K. Effect of solvent, time and temperature on the extraction of phenolic compounds and antioxidant capacity of peach (*Prunus persica* L.) fruit. *Sep. Purif. Technol.* **2016**, *162*, 68–76. [CrossRef]
34. Cotas, J.; Leandro, A.; Monteiro, P.; Pacheco, D.; Figueirinha, A.; Gonçalves, A.M.M.; da Silva, G.J.; Pereira, L. Seaweed phenolics: From extraction to applications. *Mar. Drugs* **2020**, *18*, 384. [CrossRef] [PubMed]
35. Han, Y.-S.; Chen, Y.; Ho, C.-T.; Tung, Y.-C. Drying temperature and ethanol extraction parameters of calamondin peel and their effects on flavonoid composition, in vitro antioxidant capacity and anti-lipid accumulation efficacy. *S. Afr. J. Bot.* **2026**, *193*, 269–279. [CrossRef]
36. Moovendhan, M. Polysaccharides and flavonoids from seaweeds: A natural arsenal against drug-resistant malaria. *J. Funct. Foods* **2025**, *134*, 107050. [CrossRef]
37. Urrea-Victoria, V.; Furlan, C.M.; Santos, D.Y.A.C.; Chow, F. Antioxidant potential of two Brazilian seaweeds in response to temperature: *Pyropia spiralis* (red alga) and *Sargassum stenophyllum* (brown alga). *J. Exp. Mar. Biol. Ecol.* **2022**, *549*, 151706. [CrossRef]
38. Figueroa, V.; Holdt, S.L.; Jacobsen, C.; Aguilera, J.M. Effects of cooking on the composition of volatiles, total phenolic compounds, and antioxidant capacity of three Chilean seaweeds. *J. Appl. Phycol.* **2023**, *35*, 3057–3068. [CrossRef]
39. Pacheco, L.V.; Parada, J.; Pérez-Correa, J.R. Bioactive polyphenols from Southern Chile seaweed as inhibitors of enzymes for starch digestion. *Mar. Drugs* **2020**, *18*, 353. [CrossRef] [PubMed]
40. Nørskov, N.P.; Bruhn, A.; Cole, A.; Nielsen, M.O. Targeted and untargeted metabolic profiling to discover bioactive compounds in seaweeds and hemp using gas and liquid chromatography-mass spectrometry. *Metabolites* **2021**, *11*, 259. [CrossRef] [PubMed]
41. Angulo-Nuñez, E.; Rincón-Díaz, N.; Puyana, M.; Costa, G.; Urrea-Victoria, V.; Castellanos, L.; Ramos, F.A. Photoprotective potential of mycosporine-like amino acids in red algae (Rhodophyta) from San Andrés and Providencia Islands, Colombian Caribbean. *Rev. Bras. Farmacogn.* **2025**, *35*, 754–762. [CrossRef]
42. Orfanoudaki, M.; Hartmann, A.; Karsten, U.; Ganzera, M. Chemical profiling of mycosporine-like amino acids in twenty-three red algal species. *J. Phycol.* **2019**, *55*, 393–403. [CrossRef] [PubMed]
43. Samota, M.K.; Yadav, D.K.; Koli, P.; Kaur, M.; Kaur, M.; Rani, H.; Selvan, S.S.; Mahala, P.; Tripathi, K.; Kumar, S. Exploring natural chalcones: Innovative extraction techniques, bioactivities, and health potential. *Sustain. Food Technol.* **2024**, *2*, 1456–1468. [CrossRef]
44. Yoshie, Y.; Wang, W.; Petillo, D.; Suzuki, T. Distribution of catechins in Japanese seaweeds. *Fish. Sci.* **2000**, *66*, 998–1000. [CrossRef]
45. Peixoto, W.F.S.; Pereira, R.C.; Azevedo, E.S.S.S.; dos Santos, F.M.; Coutinho, R.; de Oliveira, L.S. The molecular complexity of terpene biosynthesis in red algae: Current state and future perspectives. *Nat. Prod. Rep.* **2025**, *42*, 965–981. [CrossRef] [PubMed]

46. Otsuki, K.; Zhang, M.; Tan, L.; Komaki, M.; Shimada, A.; Kikuchi, T.; Zhou, D.; Li, N.; Li, W. Isomer differentiation by UHPLC-Q-Exactive-Orbitrap MS led to enhanced identification of daphnane diterpenoids in *Daphne tangutica*. *Phytochem. Anal.* **2025**, *36*, 1053–1062. [CrossRef] [PubMed]
47. Jennings, R.C.; McComb, A.J. Gibberellins in the red alga *Hypnea musciformis* (Wulf.) Lamour. *Nature* **1967**, *215*, 872–873. [CrossRef] [PubMed]
48. Percot, A.; Yalçın, A.; Aysel, V.; Erduğan, H.; Dural, B.; Güven, K.C. Loliolide in marine algae. *Nat. Prod. Res.* **2009**, *23*, 460–465. [CrossRef] [PubMed]
49. Barciela, P.; Carpena, M.; Perez-Vazquez, A.; Silva, A.; Jorge, A.O.S.; Prieto, M.A. Bromophenols in red algae: Exploring the chemistry and uncovering biological benefits of these unknown compounds. *Biol. Life Sci. Forum* **2024**, *35*, 11. [CrossRef]
50. Zhong, B.; Robinson, N.A.; Warner, R.D.; Barrow, C.J.; Dunshea, F.R.; Suleria, H.A.R. LC-ESI-QTOF-MS/MS characterization of seaweed phenolics and their antioxidant potential. *Mar. Drugs* **2020**, *18*, 331. [CrossRef] [PubMed]
51. Santos, S.A.O.; Félix, R.; Pais, A.C.S.; Rocha, S.M.; Silvestre, A.J.D. The quest for phenolic compounds from macroalgae: A review of extraction and identification methodologies. *Biomolecules* **2019**, *9*, 847. [CrossRef] [PubMed]
52. Kumar, M.; Kuzhiumparambil, U.; Pernice, M.; Jiang, Z.; Ralph, P.J. Metabolomics: An emerging frontier of systems biology in marine macrophytes. *Algal Res.* **2016**, *16*, 76–92. [CrossRef]
53. Du, X.; Luo, X.; Chen, X.; Chen, L.; Chen, Z.; Zheng, M.; Zhu, Y.; Jiang, Z.; Li, Q.; Ni, H.; et al. Mechanism of seaweed polyphenols interacting with intestinal flora to regulate blood glucose. *Food Chem.* **2026**, *525*, 150323. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.