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Extraction of the Phycobiliprotein Pigments from the Red Seaweed *Gelidium pusillum* Using an Ultrasonic Method Combined with Maceration or Homogenization

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A B S T R A C T

Objective: Red algae (Rhodophyta) are a great source of phycobiliproteins like R-phycoerythrin (R-PE) and R-phycocyanin (R-PC). These compounds have notable antioxidant, anti-inflammatory, and fluorescent qualities, making them useful in the food, pharmaceutical, and cosmetic sectors. Because of the resistant polysaccharide cell wall in species like *Gelidium pusillum,* extraction is not efficient. This study aimed to compare the efficiency of two combined extraction methods: ultrasound-assisted homogenization and ultrasound-assisted maceration, for the primary extraction of phycobiliproteins from *G. pusillum* collected from the Oman Sea, and to evaluate the antioxidant activity of the resulting extracts.

Methods: Samples of Gelidium pusillum were collected from the Oman Sea and extracted using two ultrasound-assisted methods: maceration and homogenization. Phycobiliprotein content (R-PE and R-PC), extraction yield, purity, antioxidant activity (DPPH assay), and functional groups (FTIR) were evaluated. All experiments were performed in triplicate, and the data were analyzed using t-tests or one-way ANOVA with Tukey's post-hoc test ($p < 0.05$).

Results: Ultrasound-assisted homogenization significantly outperformed ultrasound-assisted maceration in extracting phycobiliproteins from *Gelidium pusillum*, yielding 36% higher total phycobiliprotein content (0.391 vs. 0.288 mg/g fresh weight; $p < 0.01$) and greater R-PE and R-PC yields. Homogenization also exhibited stronger DPPH radical scavenging activity at 0.5 mg/mL (31.60% vs. 16.81%). FTIR analysis confirmed successful phycobiliprotein extraction by both methods, although lipid contamination was detected in the maceration extract. Despite similar total dry solid yields, both methods produced low-purity extracts, indicating the need for further purification.

Conclusion: Ultrasound-assisted homogenization was more effective than ultrasound-assisted maceration for extracting R-PE and R-PC from *Gelidium pusillum*, resulting in higher phycobiliprotein yield and antioxidant activity. Although the extracts showed promising antioxidant potential, further purification, safety evaluation, and process optimization are required before industrial application.

Keywords: Antioxidant activity, Phycoerythrin, Phycocyanin, *Gelidium pusillum*, Ultrasound-assisted extraction.

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Introduction

Red seaweeds belonging to the phylum Rhodophyta are valuable sources of bioactive compounds that can be exploited in various industries [1, 2]. This group of seaweeds is abundantly found in subtropical and temperate waters and produces photosynthetic accessory pigments that improve light absorption efficiency [3]. Phycobiliproteins, which are known as water-soluble protein-pigment complexes, constitute the major component of this photosynthetic system and include R-phycoerythrin (R-PE), R-phycocyanin (R-PC), and allophycocyanin (Alo-PC). These compounds absorb light in the spectral range of 490–650 nm and transfer energy to chlorophyll a, enhancing the photosynthetic efficiency in low-light marine environments [4, 5]. In addition to their

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biological role, phycobiliproteins have special physicochemical properties such as strong fluorescence, moderate thermal stability, and various biological activities, including antioxidant, anti-inflammatory, antimicrobial, neuroprotective, anticancer, and even antiviral activities, which are attractive for medical and food applications [6, 7]. Recent studies show that R-PE with a maximum absorption of 565 nm has natural antioxidant properties through free radical neutralization, while R-PC has anticancer properties through programmed cell death (apoptosis) induction [8, 9].

Phycobiliproteins have varied applications. These compounds are safe and stable natural colorants in the food sector, replacing synthetic dyes such as tartrazine or brilliant blue and reducing health concerns such as allergenicity and carcinogenicity [10, 11]. In the pharmaceutical field, these proteins are used as fluorescent agents in immunofluorescence diagnostics, photodynamic cancer therapy, and even recombinant vaccines, where their high fluorescence allows precise cellular tracking [3, 12-13]. Because of their antioxidant properties, these compounds are used in the cosmetic industry in anti-aging creams and skin lotions for protection against ultraviolet radiation [6, 13]. In biotechnological applications, phycobiliproteins are used as markers in flow cytometry, fluorescent probes in medical imaging, and even as biosensors for the detection of pollutants [6, 14-15]. The global market for phycobiliproteins, especially R-PE, has experienced impressive growth from an economic perspective. The R-PE market was valued at approximately USD 4.79 million in 2024, USD 5.18 million in 2025, and is projected to reach USD 11.30 million by 2035, with a compound annual growth rate (CAGR) of 8.1% [16]. The growth is majorly attributed to the increasing demand for natural pigments, bioactive compounds, and non-synthetic labeled products in the food, pharmaceutical, and cosmetic industries [17]. Another report says that the phycobiliprotein market across the globe was valued at USD 130 million in 2023 and is estimated to surpass USD 270 million by 2032, growing at a CAGR of about 8.5% during the forecast period [18]. Such market developments have promoted investments in efficient and sustainable extraction technologies and represent important economic opportunities for developing countries with abundant marine resources [7, 19].

The genus Gelidium sp. especially the species *G. pusillum* is one of the major sources of R-PE in tropical red seaweeds, and is found abundantly [20]. The species produces not only high-quality agar but also phycobiliproteins up to 200 mg per 100 g of dry weight, making it a promising candidate for biorefineries [21, 22]. However, the resistant polysaccharide cell wall of these seaweeds, consisting of agar, cellulose, and sulfated galactan, prevents the efficient extraction of phycobiliproteins and necessitates novel methods [23, 24].

Low yields and structural degradation of temperature-sensitive (above 40°C), ultraviolet light, pH, and oxidation-sensitive phycobiliproteins are often associated with conventional extraction techniques such as repeated freeze-thawing [23], simple maceration [24], or mechanical homogenization [24-26]. In the last decade, more attention has been paid to sustainable approaches such as ultrasound-assisted extraction. This technique employs cavitation to rapidly disrupt the cell wall, which increases the yield by two to three times, reduces the processing time, and decreases the energy consumption [20, 27]. Ultrasound-assisted extraction was used for the first time in 2017 in a study of *Gelidium pusillum*, where it was shown that its combination with maceration or homogenization increased the R-PE extraction yield to 77% and R-PC to 93% with optimal parameters such as frequency of 20 kHz, time of 10 to 15 min, and temperature below 30°C [20]. Combined approaches have been a recent advance. Le Guillard and colleagues reported yields above 90% with lower polysaccharide contamination, and they showed the industrial feasibility of this method by combining ultrasound-assisted extraction with enzymatic hydrolysis to extract R-PE from *Grateloupia turuturu* [28]. In a detailed review, Pagels and co-workers found that the best method for macro-algae is ultrasound-assisted extraction, and the combination of ultrasound with homogenization reduces the extraction time by up to 50% and can achieve an initial purity level suitable for food applications [29]. Further research on nanoencapsulation of these compounds is needed for long-term stability and for the development of sustainable technologies for industrial production [30]. In the past decade, reports have shown a shift from traditional methods to combined ultrasound-assisted extraction approaches that have been effective for resistant seaweeds such as Gelidium [20, 21].

Despite the growing body of literature on phycobiliprotein extraction from *Gelidium pusillum*, a critical biogeographic gap remains: to date, all phycobiliprotein-focused investigations on this species have been conducted only on specimens collected from other geographical zones, particularly Tamil Nadu, India [20], with no study reported on populations native to the Oman Sea coast. The geographic origin of red seaweed samples carries substantial scientific weight, since the biochemical makeup and bioactive compound profile of Rhodophyta, encompassing phycobiliprotein content and radical-scavenging capacity, respond dynamically to site-specific environmental drivers

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such as ambient seawater temperature, photon flux density, ionic strength, and seasonal variation [46,47,48,49]. The Iranian Oman Sea shoreline, and specifically the rocky intertidal habitats around Chabahar, constitutes a biogeographically distinct and uninvestigated phycological territory; Kokabi and Yousefzadi [44] have catalogued that macro-algal documentation along the Iranian coastline lags far behind that of the Persian Gulf, owing to chronic under-sampling, making it a high-priority zone for future taxonomic and phytochemical surveys. The bioactive promise of indigenous seaweeds from the Chabahar coast has additionally been highlighted in recent investigations [45], reinforcing the scientific rationale for systematic phytochemical characterization of this regional resource. Most importantly, no prior work has concurrently benchmarked ultrasound-assisted homogenization against ultrasound-assisted maceration on *G. pusillum* collected from the Oman Sea while simultaneously evaluating the antioxidant activity of the resulting extracts, representing a combined methodological and biogeographic knowledge gap that the present study addresses for the first time.

The present study was conducted to compare two combined methods: ultrasound-assisted extraction with maceration and ultrasound-assisted extraction with homogenization, for the initial extraction of phycobiliproteins from the red seaweed *Gelidium pusillum* collected from the coasts of the Oman Sea, which exhibits higher antioxidant properties. This study aims to determine the method with higher yield, stability preservation, purity, and adequate antioxidant potential.

Materials and Methods

Collection and Preparation of Algal Samples

Red seaweed specimens of the species *G. pusillum* were collected during the fall from the Oman Sea in the Chabahar region, from the rocky intertidal shores. To preserve cellular structure and prevent the degradation of bioactive compounds, the samples were stored at -18°C after collection until use [23, 31]. For each extraction, a defined quantity of frozen samples was removed from the freezer and placed at room temperature to thaw slowly, then washed with distilled water [24]. The thawed biomass was spread on clean paper towels for excess water removal, then manually fragmented and mixed with 0.1 M potassium phosphate buffer (pH 6.8) in 1:15 (w/v) ratio to form a suitable medium for phycobiliprotein extraction [20, 32]. The preparation steps were chosen according to standard methods for resistant red seaweeds, such as Gelidium sp., to facilitate the initial cell disruption and to keep the pigment stability.

Combined Extraction Methods

Method 1

Maceration combined with Ultrasound: The fragmented biomass was manually macerated with a porcelain mortar, then mixed with phosphate buffer (pH 6.8) and stirred for 90 minutes on a magnetic stirrer [20, 24]. Ultrasound-assisted extraction was then applied using a FaPaN 400 UPT1 probe sonicator (FaPaN Co., Iran; 400 W, titanium probe, 20 kHz) in an ice bath for 14 minutes at 100% amplitude, pulse mode 9 son/1 s off (duty cycle 90%; active sonication time 756 s; total energy input 302.4 kJ; energy density ~2,016 J/mL). These parameters were adopted from Mittal et al. [20] as a standardized reference, not as an independently optimized protocol. The temperature of the sample was constantly monitored and maintained below 30 °C by an ice bath and a calibrated thermometer [20, 33]. The suspension was centrifuged (9,000 rpm, 4°C, 20 min) and the supernatant containing the phycobiliproteins was collected for quantification.

Method 2

Homogenization with Ultrasound: The protocol involved the addition of a defined amount of fragmented *Gelidium pusillum* biomass to 0.1 M potassium phosphate buffer (pH 6.8) at a ratio of 1:15 (w/v) and then homogenization for 4 minutes at up to 20,000 rpm using a stand-mounted rotor-stator homogenizer (Dragon Lab, China). Following that, ultrasound extraction was performed under the same conditions, (14 min, 100% amplitude, 9 s/1 s pulse, 90% duty cycle, 302.4 kJ energy input, 2,016 J/mL energy density)with the same sonicator, FaPaN 400 UPT1 [20] as a standardized reference. The temperature was kept by an ice bath (kept below 30 ◦C and continuously monitored) [20, 33]. The suspension was centrifuged (9,000 rpm, 4°C, 20 min), and the supernatant was collected for quantification.

Phycobiliprotein concentration measurement

Phycobiliprotein concentrations were determined by measuring absorbance at 280, 564, 618, and 730 nm in a UV-Vis spectrophotometer. The optical absorbance of the supernatant was recorded immediately after extraction and centrifugation. Initially, all absorbance values were corrected by baseline correction at 730 nm to eliminate the effects of turbidity and light scattering [21, 34]. The correction equation was as in Equation 1.

(Equation 1)

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$$A_{\lambda,\text{corr}} = A_{\lambda} - A_{730}$$

Where: A_{λ}: raw absorbance measured at the target wavelength, A_{730}: absorbance at 730 nm (baseline), $A_{\lambda,\text{corr}}$: corrected absorbance (representing only the true absorbance due to phycobiliproteins)

The concentrations of R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) were calculated based on the spectrophotometric equations proposed by Castejón et al. [21] (Equations 2 and 3):

$$\text{R-PE (mg/mL)} = 0.1247 \times [(A_{564,\text{corr}}) - 0.4583 \times (A_{618,\text{corr}})] \quad \text{(Equation 2)}$$

Where 0.1247 is the corrected extinction coefficient for R-PE, and 0.4583 is the correction factor for R-PC absorbance interference at 564 nm.

$$\text{R-PC (mg/mL)} = 0.154 \times (A_{618,\text{corr}}) \quad \text{(Equation 3)}$$

Where 0.154 is the specific extinction coefficient for R-PC at 618 nm. The total phycobiliprotein concentration was obtained from the sum of R-PE and R-PC concentrations.

The purity ratio for assessing the quality of the crude extract was calculated using Equation 4:

$$\text{Purity Ratio} = A_{564,\text{corr}} / A_{280} \quad \text{(Equation 4)}$$

Where absorbance at 280 nm indicates total protein (including impurities), and a ratio greater than 4 indicates high purity for R-PE [20, 35].

Extraction yield was determined according to Equation 5 [20]:

$$\text{Yield (mg/g fresh weight)} = (\text{Concentration (mg/mL)} \times \text{Volume of supernatant (mL)}) / \text{Fresh biomass weight (g)} \quad \text{(Equation 5)}$$

Concentration: calculated concentration of phycobiliprotein (R-PE, R-PC, or total). Volume of supernatant: volume of supernatant collected after centrifugation (mL). Fresh biomass weight: exact weight of fresh seaweed used (g). All experiments were performed in triplicate, and data are expressed as mean ± standard deviation.

Total Dry Solid Yield

After extraction and centrifugation, a given volume of the clear supernatant (20 mL) was pipetted onto pre-weighed glass plates. The initial weight of the plates was determined by weighing the plate in an analytical balance with an accuracy of ± 0.001 g. To achieve complete freezing, the samples were frozen at -18°C for 24 hours in a freezer. Then, the plates with the frozen samples were moved to a freeze dryer (model JFD 2L) and subjected to vacuum conditions (pressure < 0.1 mbar) at -40°C for 36-48 hours. At the end of the drying process, the samples were immediately placed in a desiccator containing silica gel for equilibration at room temperature (25±2°C) and to avoid moisture reabsorption. The final weight of the plates with the dried material was measured on the same analytical balance [14, 29]. Three independent replicates (n = 3) were performed for each measurement.

The net dry weight of the sample was computed using Equation 6:

$$\text{Net dry weight (g)} = \text{Final weight (plate + dried sample)} - \text{Initial weight (empty plate)} \quad \text{(Equation 6)}$$

Given that 20 mL of the total final supernatant volume was used for this determination, and the total supernatant volume was 120 mL in the

homogenization

method and 135 mL in the maceration method, the total weight of dry solids present in the entire extract was estimated using Equation 7:

$$\text{Total dry solids weight (g)} = (\text{Net dry weight} \times \text{Total supernatant volume}) / 20 \quad \text{(Equation 7)}$$

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The extraction yield of dry solids, based on the initial fresh biomass weight (10 g), was calculated using Equation 8 and expressed as a weight percentage:

Yield (g per 100 g fresh weight) = (Total dry solids weight / Fresh biomass weight) × 100 [32, 35-36] (Equation 8)

FTIR Analysis

FTIR–ATR spectroscopy was used to investigate the structural properties of the two different extracts. The scanning was performed using the Spectrum-RXI FT-IR model in the range of 4000-400 cm^{-1}. The samples were used directly on the FTIR–ATR mode and the transmittance was measured.

DPPH Assay

The antioxidant activity of phycobiliprotein extracts obtained from *G. pusillum* was studied by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging method. The method is based on the reduction of the optical absorbance of DPPH at 517 nm and is one of the most common methods to determine the antioxidant activity of marine extracts [37]. A stock solution of DPPH (0.1 mM) was prepared in 95% methanol and stored in the dark. The phycobiliprotein extracts were diluted in methanol at different concentrations (1, 0.5, 0.1, and 0.01 mg/mL). For each concentration, 100 µL of sample was mixed with 900 µL of DPPH solution and considered as the sample (A_{sample}). The sample blank comprised 100 µL of sample and 900 µL of pure methanol ($A_{sample\ blank}$). The control comprised 100 µL of methanol and 900 µL of DPPH solution ($A_{control}$). Butylated hydroxytoluene (BHT) at the same concentrations was used as a positive standard. The mixtures were incubated for 30 minutes in the dark at room temperature. Optical absorbance at 517 nm was then measured using a spectrophotometer. The inhibition percentage of DPPH radical was calculated by the following Equation 9:

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100 \quad \text{(Equation 9)}$$

Where: $A_{control}$: mean optical absorbance of the DPPH solution, A_{sample}: corrected optical absorbance of the sample.

Statistical analysis

Each sample was tested in three independent replicates (n = 3) for accuracy and reproducibility, and results are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). The Shapiro-Wilk test assessed normality. Independent-samples t-test was used for between-method comparisons, and one-way ANOVA with Tukey's post-hoc test for within-treatment concentration comparisons. Statistical significance was set at $p<0.01$.

Results

Concentration and Extraction Yield of Phycobiliproteins

The results of pairwise comparisons of phycoerythrin, phycocyanin, and total phycobiliprotein levels between the ultrasound-assisted extraction method combined with homogenization and that combined with maceration are presented in Table 1. Phycoerythrin levels in the ultrasound-homogenization combined method showed a significant difference compared to the maceration method ($p < 0.05$). Phycocyanin and total phycobiliprotein levels in the ultrasound-homogenization method exhibited a significant difference compared to the ultrasound-maceration method ($p < 0.01$). In all comparisons, the ultrasound homogenization method yielded higher extraction values.

Table 1. Concentration of Phycobiliproteins Extracted Using Ultrasound Combined With Homogenization and Maceration (mg/mL)

Bioactive compound	Maceration	Homogenization
Phycoerithrin	0.0095 ± 0.001	0.054 ± 0.002 *

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Phycocyanin	0.0118 ± 0.001	0.0172 ± 0.001 **
Phycobiliproteins	0.0213 ± 0.002	0.0326 ± 0.003 **

The results are reported as mean ± standard deviation, n = 3. The stars in each row represent a meaningful difference. * ($p<0.05$) and ** ($p<0.01$)

The extraction yields of phycobiliprotein compounds obtained using the ultrasound homogenization and ultrasound maceration methods are presented in Table 2. According to the results, the extraction yields of phycoerythrin, phycocyanin, and total phycobiliproteins in the ultrasound-homogenization method showed a significant difference compared to the ultrasound-maceration method ($p < 0.01$). These results reveal the higher yield of the ultrasound-homogenization method at a high level of statistical significance.

Table 2. Phycobiliprotein Extraction Yields Using Ultrasound-Assisted Method Combined With Homogenization and Maceration (mg/g Wet Weight)

Compound	Maceration	Homogenization
Phycoerithrin	0.129 ±0.010	0.184 ± 0.015 **
Phycocyanin	0.159 ±0.012	0.206 ±0.012 **
Phycobiliproteins	0.288 ±0.024	0.391 ± 0.029 **

The results are reported as mean ± standard deviation, n = 3. The asterisk in each row represents a meaningful difference. ** ($p<0.01$)

Total Dry Solids Extraction Yield

The results of the statistical comparison of total dry solid extraction yields between the two combined extraction methods, ultrasound-homogenization and ultrasound-maceration, are presented in Table 3. According to the findings, the total dry solid extraction yield in the ultrasound-homogenization method did not show a significant difference compared to the ultrasound-maceration method, although the homogenization method exhibited a slightly higher yield (approximately 0.46% increase).

Table 3. Total Dry Solids Yield From Ultrasound-Assisted Extraction Combined With Homogenization and Maceration (mg/g Wet Weight)

Parameters	Maceration	Homogenization
Supernatant volume	135	120
Total dry biomass weight	2.390	2.436

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Yield (g/100g wet)	23.90	24.36

FTIR Results

The FTIR results of the extracts obtained by the maceration and homogenization methods are shown in Figure 1. Both products are highly similar. A broad peak in the region of 3249–3400 cm^{-1} was observed in both spectra. An absorption peak around 2900 cm^{-1} was seen in the maceration spectrum, but this absorption peak was absent in the homogenization spectrum. A large absorption peak at 1634 cm^{-1} and another absorption peak at 1075 cm^{-1} were observed in both spectra. Two weaker absorption peaks at 1145 and 988 cm^{-1} were observed in both spectra.

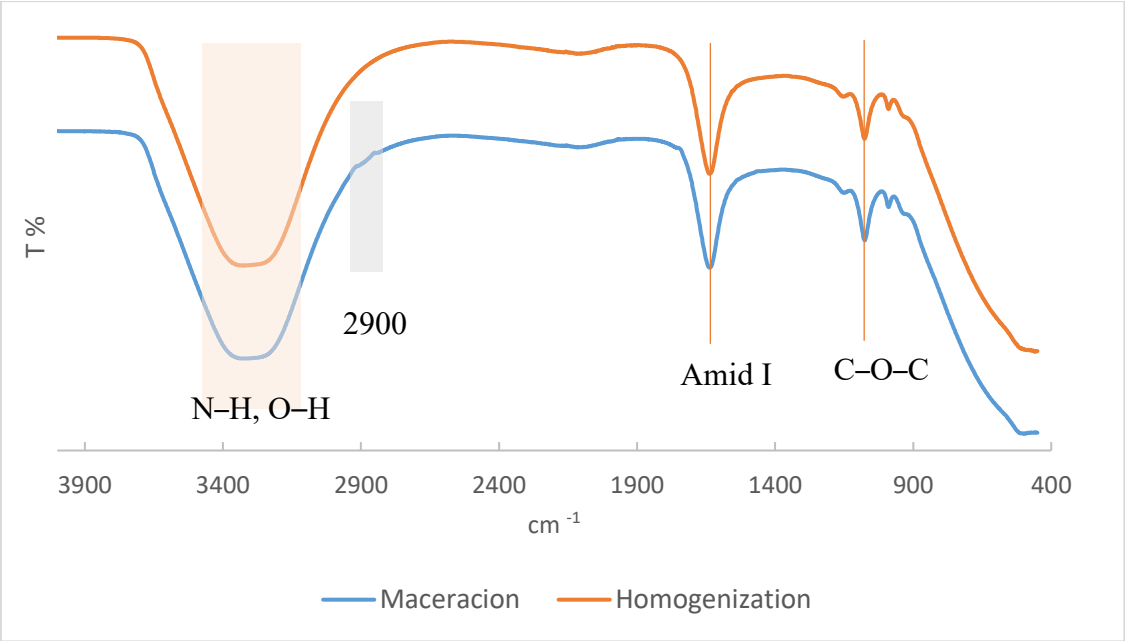


Fig 1. FTIR spectrum of the products from maceration and homogenization extract methods from *Gelidium pusillum.*

Antioxidant Activity Assessed by the DPPH Method

A comparison of DPPH radical scavenging capacity between extracts obtained via ultrasound-homogenization and ultrasound-maceration extraction methods is presented in Table 4. At concentrations of 1 and 0.5 mg/mL, the extract obtained from the ultrasound-homogenization method exhibited higher free radical scavenging activity, which was significant at the concentration of 0.5 mg/mL ($p < 0.05$) but not significant at 1 mg/mL ($p > 0.05$). This lack of significance at 1 mg/mL was attributed to the high standard deviation in the results for the ultrasound-maceration extract at this concentration (SD = 11.35%), which was driven by one deviating replicate (22.44%) relative to the other two replicates (42.94% and 41.14%); this intra-group variability is consistent with protein aggregation phenomena documented in crude phycobiliprotein extracts at higher concentrations, where co-extracted polysaccharides and proteins can reduce DPPH accessibility [48]. The IC50 values for DPPH radical scavenging, estimated by linear interpolation (homogenization) and linear extrapolation (maceration), were 0.906 mg/mL and 1.388 mg/mL, respectively, indicating that the homogenization-derived extract requires a lower concentration to achieve 50% radical inhibition. At concentrations of 0.1 and 0.01 mg/mL, no significant differences were observed.

Table 4. DPPH Radical Scavenging Activity of Phycobiliprotein Extracts from *Gelidium pusillum* Obtained Using Ultrasound Combined with Homogenization and Maceration (%)

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Concentration(mg/mL)	Maceration	Homogenization
1	35.50 ± 11.35	54.34 ± 3.78
0.5	16.81 ± 6.07	31.60 ± 4.82 *
0.1	6.51 ± 1.05	6.62 ± 3.73
0.01	3.55 ± 0.29	1.30 ± 1.23
Butylated Hydroxytoluene	94.04 ± 0.71	93.70 ± 0.25

The results are reported as mean ± standard deviation, n = 3. The asterisk in each row represents a meaningful difference. * ($p<0.05$)

The effect of extract concentration on DPPH scavenging activity within each extraction method was analyzed separately, as presented in Figure 2. In the ultrasound-homogenization method, as concentration decreased, DPPH free radical scavenging activity also decreased, with the highest activity observed at a concentration of 1 mg/mL, which showed a significant difference compared to the other concentrations ($p < 0.0001$). No significant difference was observed between the performance at 0.1 and 0.01 mg/mL in this method ($p > 0.05$). In the ultrasound-maceration method, free radical scavenging activity was also dose dependent and decreased with decreasing concentration. The highest activity was observed at a concentration of 1 mg/mL, which showed a significant difference compared to the three other concentrations ($p < 0.05$), but no significant difference was observed among the three lower concentrations ($p > 0.05$). All treatments in both methods showed significant differences compared to BHT as the positive control.

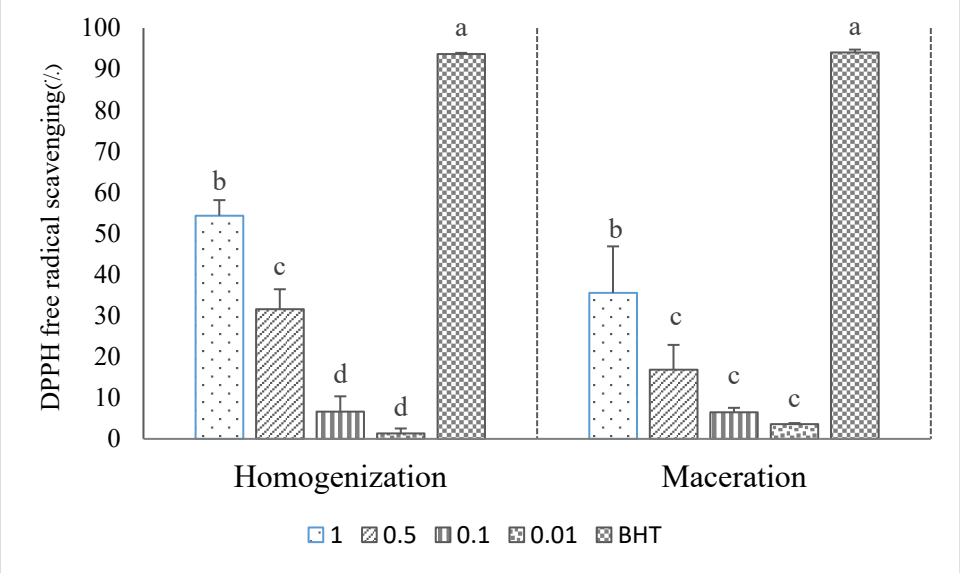


Figure 2: Influence of extract concentration on DPPH free radical scavenging activity in ultrasound-homogenization and ultrasound-maceration methods. Different letters (a, b, c, d) above bars in each series denote statistically significant differences ($p <0.001$ for the homogenization method and $p < 0.05$ for the maceration method).

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Discussion

The findings of the present study show that the combined ultrasound-homogenization extraction method, compared to the combined ultrasound-maceration method, leads to a significant increase in the extraction yield of the phycobiliproteins R-PE and R-PC from the red seaweed *Gelidium pusillum*. Specifically, the total phycobiliprotein yield obtained using the ultrasound-homogenization method was 0.391 ± 0.029 mg/g wet weight, whereas that obtained using the ultrasound-maceration method was 0.288 ± 0.024 mg/g wet weight, representing an approximately 36% increase in extraction yield. These results are consistent with the reports of Mittal et al. [20], who reported R-PE and R-PC extraction yields from the same species using similar combined methods, and confirm that the combination of mechanical techniques with ultrasonic extraction can improve the disruption of the resistant cell wall of agarophytic seaweeds. The advantage of the homogenization method over maceration can be attributed to differences in cell disruption mechanisms. High-speed mechanical homogenization (20,000 rpm) produces intense shear forces that more efficiently break down the resistant polysaccharide cell wall of Gelidium, which is composed mainly of agar, cellulose, and sulfated galactan [23, 24]. This initial disruption increases the contact surface between the solvent and the intracellular contents and facilitates the permeability of the phosphate buffer into the cells. In contrast, maceration only causes superficial and non-uniform disruption, which, due to the compact and resistant structure of the Gelidium cell wall, results in lower yield [24, 32].

Both techniques include ultrasonic cavitation, which is effective only when the cells have been initially disrupted. Ultrasound waves at a frequency of 20 kHz form microscopic bubbles in the solution that, upon abrupt collapse, give rise to pressure waves and localized shear forces [20, 38]. This phenomenon is effective in samples where the cells were previously broken by homogenization because the cavitation can traverse the gaps existing in the cell wall and obtain the release of phycobiliproteins [33, 39]. The study by Le Guillard et al. [28] on *Grateloupia turuturu* showed that the combination of ultrasonic extraction and enzymatic hydrolysis produced R-PE in excess of 90%. Comparable results have been reported.

Based on the FTIR results, the absorption at 3249–3400 cm^{-1} corresponds to the stretching vibrations of O–H (water and phenols) and N–H (amid groups) (55). The lack of difference between the two methods shows that both extraction methods have well preserved the compounds containing hydroxyl and amine groups. The region around 2900 cm^{-1} is related to the C–H stretching vibrations of methyl and methylene groups derived from membrane lipids and fatty acid residues [56]. This region was observed only in the maceration method. This suggests that the homogenization method either effectively removes or does not extract lipid compounds. The band in the 1634 cm^{-1} region corresponds to amide I. The presence of this band in both methods indicates significant preservation of the secondary protein structure (including α-helices and β-sheets) in the phycobiliproteins. In FTIR studies on phycobiliproteins and phytochromes, the amide I band is recognized as the key indicator of conformational changes in proteins. For example, in a study of the cyanobacterial phytochrome Cph1, the Pfr-Pr differential FTIR spectrum was dominated by the amide I band at 1653 cm^{-1} (+) and 1631 cm^{-1} (–) [57]. Also, in a study of the plant phytochrome phyA, the amide I band showed protein structural changes during photoconversion (Schwinte et al., 2008). The bands in the 900–1200 cm^{-1} region are characteristic of algal sulfated polysaccharides, attributed to the ν (C–O–C) vibrations of polysaccharides [59]. In fact, sulfated polysaccharides have been extracted along with phycobiliproteins in both methods. It could be said that the homogenization method is more efficient due to the absence of fats and methyl/methylene compounds derived from membrane lipids and fatty acid residues in the extracted composition.

The antioxidant activity results showed that extracts obtained using the ultrasound-homogenization method exhibited greater DPPH radical scavenging activity at higher concentrations (0.5 and 1 mg/mL) compared to the ultrasound-maceration method. At a concentration of 0.5 mg/mL, the DPPH scavenging percentage was 31.60 ± 4.82% for the homogenization method and 16.81 ± 6.07% for the maceration method ($p < 0.05$). This difference is due to the high concentration of extracted phycobiliproteins in the homogenization method, which is in accordance with the results shown in Tables 1 and 2. The phycobilin chromophores and polyene conjugated groups of phycobiliproteins, in particular R-PE and R-PC, enable them to neutralize free radicals by donating hydrogen or passing electrons [40, 41]. Ismail et al. [42] studies also showed significant antioxidant activity of red macro-algae, which is in line with the present study findings. However, at a concentration of 1 mg/mL, no significant difference was observed between the two methods ($p > 0.05$), probably due to the high variance in samples of the maceration method (35.50 ± 11.35%), which indicated non-uniformity in the maceration extraction process and decreased

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reproducibility [20]. The antioxidant activity was significantly reduced at lower concentrations (0.1 and 0.01 mg/mL), and no significant difference was observed between the methods, indicating a dose-dependent effect of phycobiliproteins on free radical scavenging. This result is consistent with the reports of Sonani et al. [41], who proved a direct correlation between the concentration of phycobiliproteins and antioxidant activity. The high intra-group variability found in the maceration extract at 1 mg/mL (SD = 11.35%) requires a specific mechanistic explanation. Analysis of individual replicate values shows that two of the three replicates have similar scavenging percentages, 42.94% and 41.14%, while the third replicate has a distinctly different value, 22.44%. Such a pattern is an indication of a deviation in a single replicate rather than systematic random error and is best explained by the well-documented phenomenon of protein aggregation in concentrated crude algal extracts wherein, at elevated concentrations of protein, co-extracted polysaccharides, especially agar type sulfated polysaccharides that are abundant in the cell walls of Gelidium, can interact with phycobiliproteins and limit their accessibility to DPPH radicals resulting in artificially depressed scavenging readings in individual replicates [54]. This effect is stochastic at the macro-scale and is exacerbated by the lack of a purification step, and has been similarly noted in crude red algal extracts by Pereira et al. [32]. The estimated IC_{50} values for DPPH radical scavenging were 0.906 mg/mL for the extract obtained by homogenization (calculated by linear interpolation between the data points 0.5 and 1 mg/mL) and 1.388 mg/mL for the extract obtained by maceration (calculated by linear extrapolation beyond the tested concentration range, because the threshold of 50% inhibition was not achieved in the experimental range). These values are in line with the range reported for crude phycobiliprotein extracts from red macro-algae without purification, which typically show moderate DPPH activity with IC_{50} values in the sub-milligram to low-milligram range [3, 54]. By contrast, highly purified phycoerythrin from *Kappaphycus alvarezii* has been reported to show much lower IC_{50} values (0.043 mg/mL) [54], highlighting that the moderate activity observed in this study is an expected feature of crude primary extracts and a baseline for further purification-guided optimization. In this study, the DPPH assay was selected as the main method for antioxidant evaluation because it is widely accepted as a rapid, reliable and reproducible screening tool for phycobiliprotein-containing extracts [41, 42]; however, it is known that the DPPH assay only measures hydrogen-atom-transfer and single-electron-transfer mechanisms, and does not reflect metal-chelation or oxygen-radical-absorbance capacity. Complementary assays, such as ABTS, FRAP, or ORAC, will provide in future studies a more complete mechanistic profile of the antioxidant capacity of *G. pusillum* phycobiliprotein extracts from the Iranian Oman Sea coast.

The total dry solid yields of the two extraction methods were similar, being 24.36 g in 100 g wet weight for the homogenization method and 23.90 g in 100 g wet weight for the maceration method. The results show that the two methods have similar yields in the extraction of total soluble compounds such as proteins, polysaccharides, and lipids, although the selectivity and efficiency to release specific phycobiliproteins may vary between methods. Le Guillard et al. [28] demonstrated that the combination of methods, such as enzymatic hydrolysis with ultrasound, can improve the purity and recovery of R-phycoerythrin while the total yield is not significantly affected.

The purity ratios obtained in the current study (0.0057 for ultrasound-homogenization and 0.0145 for ultrasound-maceration) are in the range of what can be expected for unprocessed crude primary extracts that have not undergone any of the downstream purification steps such as ammonium sulfate fractionation, dialysis, or chromatographic separation. A widely referenced classification framework proposed by Rito-Palomares et al. [51], defines food-grade phycobiliprotein preparations as those with $A_{amax}/A_{230} \geq 0.7$, reactive-grade at $A_{amax}/A_{230} = 3.9$, and analytical-grade at $A_{amax}/A_{230} \geq 4.0$; in this context, the values recorded here fall below the food-grade benchmark and are fully consistent with the purity levels typical of single-step primary extracts from macro-algae, as has been similarly reported in studies applying analogous non-purified extraction protocols to *Gracilaria gracilis* and other Rhodophyta without purification [28, 29]. The markedly lower purity ratio of the homogenization-derived extract compared to the maceration-derived extract (0.0057 vs. 0.0145) is mechanistically explicable: high-speed mechanical homogenization (20,000 rpm) causes more extensive disruption of the algal cell matrix relative to manual maceration, releasing a substantially greater quantity of co-extractable non-phycobiliprotein compounds including other structural proteins, agar-type polysaccharides, lipids, and photosynthetic pigments such as chlorophylls, all of which absorb at 280 nm and inflate the denominator of the purity ratio [20, 28]. This trade-off between extraction yield and extract purity is well-documented in phycobiliprotein literature and represents a fundamental characteristic of aggressive mechanical disruption methods applied to polysaccharide-rich red algal species [29, 51,52,53]. To minimize potential interference from co-extracted pigments, especially chlorophylls,

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baseline correction at 730 nm was performed during the spectrophotometric measurements, to reduce the contribution of scattering and non-specific absorption to the absorbance readings of phycobiliproteins, as recommended by Beer and Eshel [35] and adopted in the quantification equations of Castejón et al. [21]. It must be acknowledged, however, that this correction does not eliminate chlorophyll interference, and the absence of formal analytical validation parameters, including a limit of detection (LOD), limit of quantification (LOQ), and linearity range, constitutes a methodological limitation of the present study that should be addressed in future work through the application of validated spectrophotometric or chromatographic protocols.

From the industrial point of view, the results show that the ultrasound-homogenization method is appropriate for small to medium-scale extraction of phycobiliproteins from *Gelidium pusillum*. However, the scalability of the process needs industrial-scale ultrasonic equipment and high-capacity and precise control homogenizers to avoid protein denaturation [20, 43]. An economic evaluation of the process is essential, as energy consumption for homogenization and ultrasonication would be considerable on an industrial scale. Finally, phycobiliproteins extracted from *G. pusillum* demonstrate preliminary physicochemical and antioxidant properties that, following downstream purification, cytotoxicity evaluation, and stability testing, may support their development as natural colorants in the food industry, as antioxidant and anti-inflammatory candidates in the pharmaceutical industry, and as bioactive ingredients in cosmetic products [13, 41]; however, given the crude and unrefined nature of the current extracts, direct industrial applicability claims are premature at this stage and require substantiation through additional experimental evidence. Given the rich algal resources along the coasts of Iran, the sustainable exploitation of these resources could create substantial economic opportunities for the development of marine biotechnology industries, provided that full characterization and safety evaluation are conducted.

Several limitations should be acknowledged. First, ultrasound parameters were adopted from Mittal et al. [20] without independent optimization; RSM or CCD-based optimization remains a priority for future work. Second, since both methods shared an identical ultrasound step, the independent contribution of the mechanical pre-treatment cannot be fully decoupled without factorial controls. Third, the small sample size (n = 3) limits statistical power. Despite these constraints, this study provides a well-controlled comparative baseline for the primary extraction of phycobiliproteins from a geographically distinct and previously uninvestigated population of *G. pusillum* from the Iranian Oman Sea coast, and establishes a scientifically grounded foundation for subsequent optimization and scale-up studies.

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Conclusion

The results showed that the combined ultrasound-homogenization extraction method is a more effective approach for the initial extraction of R-PE and R-PC phycobiliproteins from the red seaweed *Gelidium pusillum*. This method increased the total phycobiliprotein yield by about 36% compared to the ultrasound-maceration method and had higher antioxidant activity at moderate concentrations (0.5 mg/mL). The homogenization method gave better results because it is more effective at breaking the resistant polysaccharide cell wall of Gelidium, improving the penetration and cavitation effects of the following ultrasound step. The IC_{50} value for DPPH radical scavenging was estimated at 0.906 mg/mL for the homogenization extract and 1.388 mg/mL for the maceration extract, confirming the higher antioxidant potential of the preparation obtained by homogenization. From an applied perspective, the extracts exhibited initial bioactive features in accordance with the phycobiliproteins from Rhodophyta [13, 41], but with purity ratios of 0.0057 (homogenization) and 0.0145 (maceration), they are raw preliminary preparations without testing for cytotoxicity and stability assessment. Therefore, the results should be seen as a proof-of-concept baseline and not as evidence of market-readiness. Further purification (ammonium sulfate fractionation, dialysis, SEC chromatography), MTT cytotoxicity assays, encapsulation studies, and complementary antioxidant assays (ABTS, FRAP) are important to support the applied potential. The rich algal resources along the coasts of the Oman Sea and the Persian

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Gulf can significantly contribute to the development and localization of efficient and sustainable extraction technologies, which can lead to the creation of added value and the strengthening of Iran's position in the marine biotechnology value chain.

Based on the results of this study, several research directions are proposed for future investigation. First, the simultaneous optimization of ultrasound process parameters (frequency, time, and amplitude) and mechanical homogenization parameters (speed and duration) by using experimental design methods such as the response surface method. 2. Full characterization of the purity ratio and structural identification of the phycobiliproteins by using advanced chromatographic and spectroscopic techniques. Third, the study of the long-term stability of the extracted phycobiliproteins under different storage conditions and the development of encapsulation strategies to improve their functional robustness. Fourth, study the process scalability in the industrial context and conduct an economic analysis to assess commercial feasibility. Finally, the study of other biological activities of the extracts, such as anti-inflammatory, antimicrobial, and anti-cancer activity, could provide a more complete picture of the application potential of these compounds. To sum up, this research provides an efficient approach to the sustainable development of phycobiliproteins extraction from native Iranian marine resources and can be a foundation for further extensive application-oriented research in marine biotechnology.

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Authors' contributions

Ali Taheri: Proposed the idea, supervised the research, reviewed and edited the manuscript; Maryam Bameri gathered the data, conducted the experiment, and wrote the initial draft of the manuscript; Salim Sharifian: Advised the research and analyzed the data.

Conflict of interests

The authors declare that they have no conflicts of interest.

Ethical approval

Not applicable. This study involved the collection and biochemical analysis of marine macro-algae and did not involve human participants, vertebrate animals, or endangered species; therefore, formal ethics approval was not required.

Consent to Participate

Not applicable.

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