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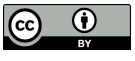
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Enhancing Antioxidant Activity from Aquatic Plant *Cymodocea nodosa* for Cosmetic Formulation Through Optimized Ultrasound-Assisted Extraction Using Response Surface Methodology

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Abstract: This study aimed to enhance antioxidant extraction from the aquatic plant *Cymodocea nodosa* for cosmetic formulation through optimized ultrasound-assisted extraction using response surface methodology. The optimized conditions—30 min of extraction time, 30% ultrasonic power, and 25% hydro-ethanolic solvent—resulted in a high total phenolic content of 113.07 mg EAG/g DM and antioxidant activity of 67.02%. Chromatographic analysis revealed a rich profile of phenolic compounds, including sinapic acid (0.741 mg/g), myricetin (0.62 mg/g), and quercetin-3-O-rutinoside (0.3 mg/g), demonstrating the extract's potent therapeutic properties. While the extract exhibited limited anti-inflammatory activity, it showed no cytotoxic effects on RAW 267.4 cells, ensuring its safety for cosmetic applications. The formulated cream maintained stable pH (6.58 to 6.6), consistent viscosity (5966.38 to 5980.6 cp), and minimal color changes over a 30-day period, indicating robust stability across various temperatures (4 °C, 25 °C, and 40 °C). These results confirm the potential of *C. nodosa* extracts to develop effective, stable, and eco-friendly cosmetic products, offering substantial benefits for skin health and emphasizing the importance of sustainable extraction processes in the cosmetics industry.

Keywords: *Cymodocea nodosa*; green chemistry; antioxidant; cosmetology

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1. Introduction

The demand of consumers for safer, more sustainable, environmentally friendly, organic, and effective cosmetic products has forced the cosmetics industry to increasingly turn to natural bioactive ingredients sourced from a diverse array of natural resources. This shift towards sustainability is not only driven by consumer demand but also by the increasing regulation pressure on the global cosmetics industry [1] to maintain an animal-free cosmetics supply chain and to avoid the problem of toxicity associated with synthetic formulations [2]. While terrestrial plants have traditionally been explored for their bioactive compounds in the cosmetics industry, the search for innovative and potent natural sources has expanded beyond land-based flora to include the rich biodiversity of

marine ecosystems. In fact, marine natural products have attracted significant scientific attention over the past five decades due to their vast potential for yielding novel bioactive compounds [3]. Among these marine resources, seagrasses, a group of marine angiosperms, stand out for their unique properties [4]. Seagrasses are rooted flowering plants that thrive in marine environments and are categorized into four main families: Cymodoceaceae, Hydrocharitaceae, Posidoniaceae, and Zosteraceae [5]. Despite their ecological importance, seagrasses are an underexplored source of bioactive molecules compared to their terrestrial counterparts [4]. Exploring the untapped potential of seagrasses in cosmeceutical production offers a new frontier in the search for sustainable and effective natural ingredients. As research continues to uncover the diverse benefits of these marine plants, seagrasses could play a pivotal role in meeting the growing demand for safe, natural, and effective cosmetic products [6]. Seagrasses have been used in folk medicine for a variety of remedial purposes, such as the treatment of fevers, stomach problems, muscle pain, wounds, and skin diseases; they are also used as a remedy for stings of different kinds of rays and as tranquilizers for babies [7]. Seagrasses are known for their rich content of biomolecules, including various antioxidants that hold significant promise for cosmetic applications. Among these bioactive compounds, polyphenols have garnered particular interest in the cosmetics industry due to their powerful antioxidant properties [8]. Polyphenols act by neutralizing free radicals, which are unstable molecules responsible for oxidative stress and premature skin aging. By protecting the skin against damage caused by free radicals, polyphenols help reduce the appearance of wrinkles, fine lines, and other visible signs of skin aging, while promoting a radiant complexion and even skin texture [9–11]. Additionally, seagrasses produce a variety of secondary metabolites, such as terpenoids and halogenated compounds, which serve as defense mechanisms under stress conditions. These metabolites, produced by several seagrass species, possess anticancer, antifungal, anti-inflammatory, antimicrobial, antiviral, antidiabetic, antimalarial, antioxidant, anti-aging, and cytotoxic properties [3,12], and are effective in preventing human diseases [10]. *C. nodosa*, a species of angiosperm widely distributed in Mediterranean waters, stands out for its high content of polyphenols and other bioactive compounds [13]. This aquatic plant plays a crucial role in the marine ecosystem, as its meadows can store carbon, improve water quality, provide food and habitat, and act as biological indicators [3]. Additionally, it presents promising potential for use in the cosmetics industry due to its beneficial properties for the skin [3,6]. However, to fully exploit the potential of *Cymodocea nodosa* in cosmetic formulations, it is essential to adopt efficient and environmentally friendly extraction methods. The extraction of these valuable compounds is a crucial step in the formulation of effective and natural skincare products. To optimize the extraction process, several innovative and environmentally friendly techniques have been developed, such as the use of ultrasound technology, which has been successfully implemented even at an industrial scale. Ultrasound-assisted extraction has proven to be a method of choice in this context [14,15]. This method uses ultrasonic waves, resulting in the formation of cavitation bubbles [16]. The implosion of these bubbles creates local conditions of high temperature and pressure, which promote the rupture of the plant cell walls and facilitate the release of bioactive compounds [16]. Furthermore, ultrasound-assisted extraction is a fast, efficient, and eco-friendly method that minimizes the use of solvents and reduces extraction times [17]. In this perspective, the main objective of this study is to optimize the ultrasound-assisted extraction process of antioxidants from *Cymodocea nodosa* using response surface methodology. By maximizing the extraction yield of polyphenols while preserving their antioxidant activity, this research aims to provide a solid scientific basis for the development of natural and effective cosmetic formulations. To achieve this, several critical parameters will be optimized, including extraction time, hydro-ethanolic percentage, solvent-to-dry mass ratio, and ultrasonic power. These parameters were selected based on their significant influence on the extraction process, as supported by previous studies. Understanding their effects and interactions is essential for maximizing extraction efficiency and enhancing the quality of the bioactive compounds

obtained. Additionally, this approach will contribute to promoting the sustainable use of marine resources and supporting the conservation of fragile marine ecosystems.

2. Materials and Methods

2.1. Sampling Site and Materials Description

This study was conducted on *Cymodocea nodosa*, a species of magnoliophyte. Sampling was carried out in January 2022 along the Tunisian beach coastlines, specifically in the region of Kheireddine (GPS coordinates: 36°831100 N, 10°319595 E), north of the city of Tunis, in the delegation of Le Kram. *Cymodocea nodosa* (Ucria) (Asch. NCBI Taxonomy ID: txid55448) was identified by Professor Abdessalem Shili (Institut National Agronomique de Tunisie). The shallow waters of the Kheireddine beach have relatively low salinity, creating favorable conditions for the growth of *C. nodosa*.

2.2. Preprocessing of Macrophytic Biomass

The obtained biomass was sorted and washed until salt removal, then dried in a hot air dryer at 40 °C for 12 h. After drying, the seagrass was pulverized into a fine powder of 40 µm using a blade mill (Retsch, GM 200, Haan, Germany). The powder was stored in ziplock bags at −20 °C as in previous studies. This preprocessing ensures the quality and stability of the macrophytic biomass, thereby ensuring homogeneous samples are ready for use in subsequent analyses.

2.3. Preliminary Study: Ultrasound-Assisted Extraction of Antioxidant Compounds

Ultrasound-assisted extraction was performed in an ultrasonic bath (Sonorex Digital 10 P, Bandelin, GmbH, Germany) using hydro-ethanolic mixture, at different proportions, as a green solvent. In a glass test tube, a precise amount of previously ground powder was mixed with a determined quantity of solvent. This mixture was then placed in the ultrasound bath to extract the antioxidants. Four parameters, namely extraction time, hydro-ethanolic percentage, solvent-to-dry-mass ratio, and ultrasonic power, were optimized to obtain an antioxidant-enriched extract. Thus, the extraction time was varied from 10 to 30 min, the hydro-ethanolic percentage from 0 to 100%, the solvent-to-dry-mass ratio from 1/50 to 1/2 mL/mg DM, and the ultrasonic wave power from 20 to 80%.

The selection of these parameters was based on their significant influence on the extraction process. Response Surface Methodology (RSM) was employed to analyze the simultaneous effects of these factors and their interactions, allowing the identification of optimal conditions to maximize extraction yield and bioactive compound content. After each extraction, the mixture was centrifuged at 10,000 rpm for 20 min, and the supernatant containing the antioxidants was recovered for subsequent analyses [15].

2.4. Antioxidant Activities

In the evaluation of antioxidant activity, three antioxidant assays were applied. Firstly, the total antioxidant capacity was determined through the reduction of Mo^{6+} to Mo^{5+} in an acidic environment, forming a green phosphate/Mo^{5+} complex. The total antioxidant capacity was expressed as milligrams of gallic acid equivalents per gram of dry matter (mg GAE/g DM) [18]. Secondly, the antiradical activity was assessed using the DPPH test, where the ability of extracts to reduce the stable DPPH radical was measured spectrophotometrically, expressed as percentage inhibition [19]. Furthermore, the reducing power was evaluated by the ferricyanide method, wherein extracts reduced Fe^{3+} to Fe^{2+}, indicative of their antioxidative potential. This method determined the effective concentration (EC_{50}, µg/mL) required for the extract to reach an absorbance of 0.5 at 700 nm [15]. These analyses collectively provided insights into the antioxidant properties of the extracts. All the tests were performed at least in triplicate.

2.5. Assessement of Anti-Inflammatory Properties

For the anti-inflammatory activity, murine macrophage cell line RAW 264.7, sourced from the American Type Culture Collection, was cultured in RPMI 1640 medium supplemented with 10% FBS and antibiotics, maintained at 37 °C with 5% CO_2, and seeded in 24-well plates at 2×10^5 cells/well for 24 h before experimentation [20]. Cytotoxicity of extracts was assessed using the resazurin assay, treating cells with increasing extract concentrations for 24 h followed by fluorescence measurement to determine viability. To evaluate anti-inflammatory activity, RAW 264.7 cells were pretreated with varying extract concentrations for 60 min before lipopolysaccharide (LPS) stimulation. After 24 h, nitrite levels in the culture medium were determined based on the Griess reaction. The absorbance at 540 nm was measured, and the levels of nitric oxide (NO) produced by the murine macrophage-like RAW264.7 cells were determined by comparing the results with a sodium nitrite standard curve (0–50 µM). All assays were conducted in triplicate.

2.6. Colorimetric Quantification of Phenolics

Total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu reagent, where 125 µL of diluted extract was mixed with 500 µL of distilled water and 125 µL of the reagent. After a 3 min rest, 1250 µL of 7% Na_2CO_3 was added, and the mixture was allowed to rest for 90 min at room temperature. Absorbance was measured at 760 nm, and results were expressed as milligrams of gallic acid equivalents per gram of dry matter (mg GAE/g DM) [21]. Total flavonoid content was determined similarly, where 250 µL of diluted extract was mixed with $NaNO_2$ and $AlCl_3$, and absorbance was measured at 510 nm. Total flavonoid content was expressed as milligrams of quercetin equivalents per gram of dry matter (mg QE/g DM) [21]. Additionally, condensed tannins were quantified by their depolymerization in sulfuric acid followed by reaction with vanillin, and absorbance was measured at 500 nm and results were expressed as milligrams of catechin equivalents per gram of dry matter (mg CE/g DM) [21]. All the tests were performed in triplicate.

2.7. Chromatographic Phenolic Composition Assessment

Phenolic compound identification and quantification were performed using an Agilent 1260 system (Agilent Technologies, Waldronn, Germany), a pump, and a photodiode array detector (Agilent Technologies, Waldronn, Germany). Separation of phenolic compounds was achieved on a reverse-phase column (100 mm × 4.6 mm, 5 µm; Zorbax Eclipse XDB C18, Agilent Technologies GmbH, Böblingen, Germany) maintained at 25 °C. The mobile phase consisted of a binary solvent system (A and B). Solvent A comprised HPLC water with 0.1% formic acid, while solvent B consisted of acetonitrile, pumped at a constant flow rate of 0.7 mL/min. The elution gradient was programmed as follows: 90% A/10% B (from 0 to 40 min), 50% A/50% B (from 40 to 41 min), 100% B (from 41 to 50 min), then back to 90% A/10% B (from 50 to 59 min) [22]. The injection volume was 3 µL. Quantification of phenolic compounds from the magnoliophyte *C. nodosa* was performed using standards at a wavelength of 280 nm. The calibration curves of the standards were constructed for the compounds of interest at concentrations ranging from 10 to 1000 µg/mL. Analyses were conducted in triplicate, and mean values were reported in mg/g extract.

2.8. Optimization of Antioxidant Extraction Using Response Surface Methodology

In order to optimize the experimental conditions for the extraction of marine-origin antioxidants and enhance the extraction of these compounds using ultrasound, we utilized the NemrodW (LPRAI, version 2000) program to elaborate an experimental design. This design included 19 experiments grouped in a centered composite model, with 3 independent variables and 2 responses. The variables studied were time (X_1), ultrasonic power (X_2), and ethanol percentage (X_3). Their central values were fixed according to the previous preliminary study, at 30 min, 30%, and 25%, respectively. The chosen variation steps were 10 min for X_1, 10% for X_2, and 5% for X_3. The measured responses were the total polyphenol contents expressed in gallic acid equivalents (GAE) per gram of dry matter

(TPC), represented by Y_{TPC}, and the percentage of DPPH radical inhibition, represented by Y_{PI} (Table 1).

Table 1. Factors and domains of study.

Factors	Levels		
	−1	**0**	**+1**
Extraction time (min), X1	20	30	40
Ultrasonic power (%), X2	20	30	40
Hydro-ethanol percentage (%*v*/*v*), X3	20	25	30

The model used for the analysis of results is a response surface model based on an equation containing 10 coefficients:

$$Y = b_0 + b_1 \times X_1 + b_2 \times X_2 + b_3 \times X_3 + b_{11} \times (X_1 * X_1) + b_{22} \times (X_2 \times X_2) + b_{33} \times (X_3 \times X_3) + b_{12} \times (X_1 \times X_2) + b_{13} \times (X_1 \times X_3) + b_{23} \times (X_2 \times X_3) \quad (1)$$

The coefficients of the equation correspond to the main effects and interactions of the variables studied, namely:

b_0: the intercept or the value of the response when all variables are zero.

b_1, b_2, and b_3: the main effects of variable X_1 (time), variable X_2 (ultrasonic power), and variable X_3 (ethanol percentage), respectively. These coefficients indicate the linear effect of each variable on the studied response.

b_{11}, b_{22}, and b_{33}: the quadratic effects of variable X_1, X_2, and X_3, respectively. These coefficients reflect the curvature of the response concerning each variable, indicating a nonlinear relationship between the variable and the response.

b_{12}, b_{13}, and b_{23}: the interaction effects between pairs of variables, respectively. These coefficients indicate whether the effect of one variable on the response depends on the value of the other variable.

2.9. Cosmetic Cream Formulation

2.9.1. Preparation of *Cymodocea nodosa* Extract

In order to facilitate the use of hydro-alcoholic extract of *C. nodosa* as a cosmetic additive with anti-aging effects, a preparatory step is required before its incorporation into the cream formula. This preparation involves completely dissolving the lyophilized extract in a specified quantity of vegetable glycerol.

2.9.2. Preparation of *Cymodocea nodosa* Cream

The prepared cream is an oil-in-water (O/W) type emulsion, where the oily phase is dispersed in the aqueous phase [21]. The cream formulation is carried out in two steps. The aqueous phase, accounting for 70.6% of the formulation, is obtained by dissolving the co-emulsifier (xanthan gum) in distilled water. The oily phase is prepared by mixing 17% refined sweet almond oil with 8.5% stearic glyceride, which acts as an emulsifier. These two steps are performed in a water bath at a temperature of about 70 °C while maintaining constant stirring. Subsequently, the two phases are mixed using a helical disperser at a speed of 1300 rpm for 10 min until the temperature returns to 40 °C. Finally, the active ingredient and preservative are added while stirring for an additional 10 min [21].

2.10. Accelerated Stability Assay Assessment

An accelerated stability assessment was performed by storing the active cream at three different temperatures (4 °C, 25 °C, and 40 °C) for a duration of 30 days [22]. Additionally, centrifugation assays were conducted at 3000 rpm for 30 min at room temperature, followed by macroscopic analyses to evaluate appearance and homogeneity characteristics. pH measurements were taken using a pH meter at a controlled temperature of 25 ± 2 °C. Furthermore, color measurements were conducted using a portable colorimeter (PCE-XXM 30, PCE Instruments, Meschede, Germany), while viscosity was assessed using a rotary viscometer (VISCOSIMÈTRE PCE-RVI 2, PCE Instruments, Toulouse, France). A Zetasizer® (Zetasizer Nano-ZS/Malvern Instruments, Worcester, UK) was used to measure the mean droplet diameter and zeta potential after diluting the formulations 200 times with distilled water.

2.11. Sensory Evaluation of Cosmetic Cream

The sensory analysis was conducted on a sample of 60 individuals to assess the cosmetic cream. Several parameters were considered, such as color, fragrance, consistency, spreadability, stickiness, and penetration [23]. Each participant rated these criteria on a scale from 1 to 10. At the end of the analysis, an overall score was given to the cream based on all evaluated parameters. This sensory study thus gathered user impressions and provided an overall assessment of the cosmetic cream.

2.12. Statistical Analysis

Heatmap clustering was conducted utilizing Orange Software (version 3.4.5, University of Ljubljana, Slovenia), whereas experimental design and statistical analysis were executed employing the NemrodW program (version 2000, LPRAI, Marseille, France). The examination of significant differences among the means of independent variables was carried out through analysis of variance (ANOVA) utilizing IBM SPSS Statistics Software (Version 20.0, IBM SPSS Inc., Armonk, NY, USA), followed by Duncan's multiple range test at a significance level of $p < 0.05$.

3. Results

3.1. Preliminary Study

The preliminary study conducted to optimize the extraction of antioxidants from Cymodocea nodosa using ultrasound revealed significant variations in DPPH radical inhibition percentages depending on the extraction conditions. Analysis of the results matrix (Figure 1) highlighted the significant influence of ultrasound power on antioxidant activity. An ultrasound power of 20% resulted in significant antioxidant activity, with inhibition percentages ranging from 42.60 to 68.33% for extraction durations of 10 to 30 min. However, increasing ultrasound power (40%, 60%, and 80%) did not lead to improved antioxidant activity. Additionally, increasing ethanol concentration in the solvent mixture had a significant effect on the results. DPPH radical inhibition percentages varied from 30.98 to 74.27% for ethanol concentrations ranging from 0% to 50%. Beyond these concentrations, inhibition percentages decreased. These results allowed us to define the study domain for these three factors as follows: for ethanol–water percentage, we decided to limit the study domain from 20 to 30% ($EtOH/H_2O$; $\%v/v$); for extraction time, we chose to work from 20 to 40 min; and for ultrasound bath power, we delimited the domain from 20 to 40%.

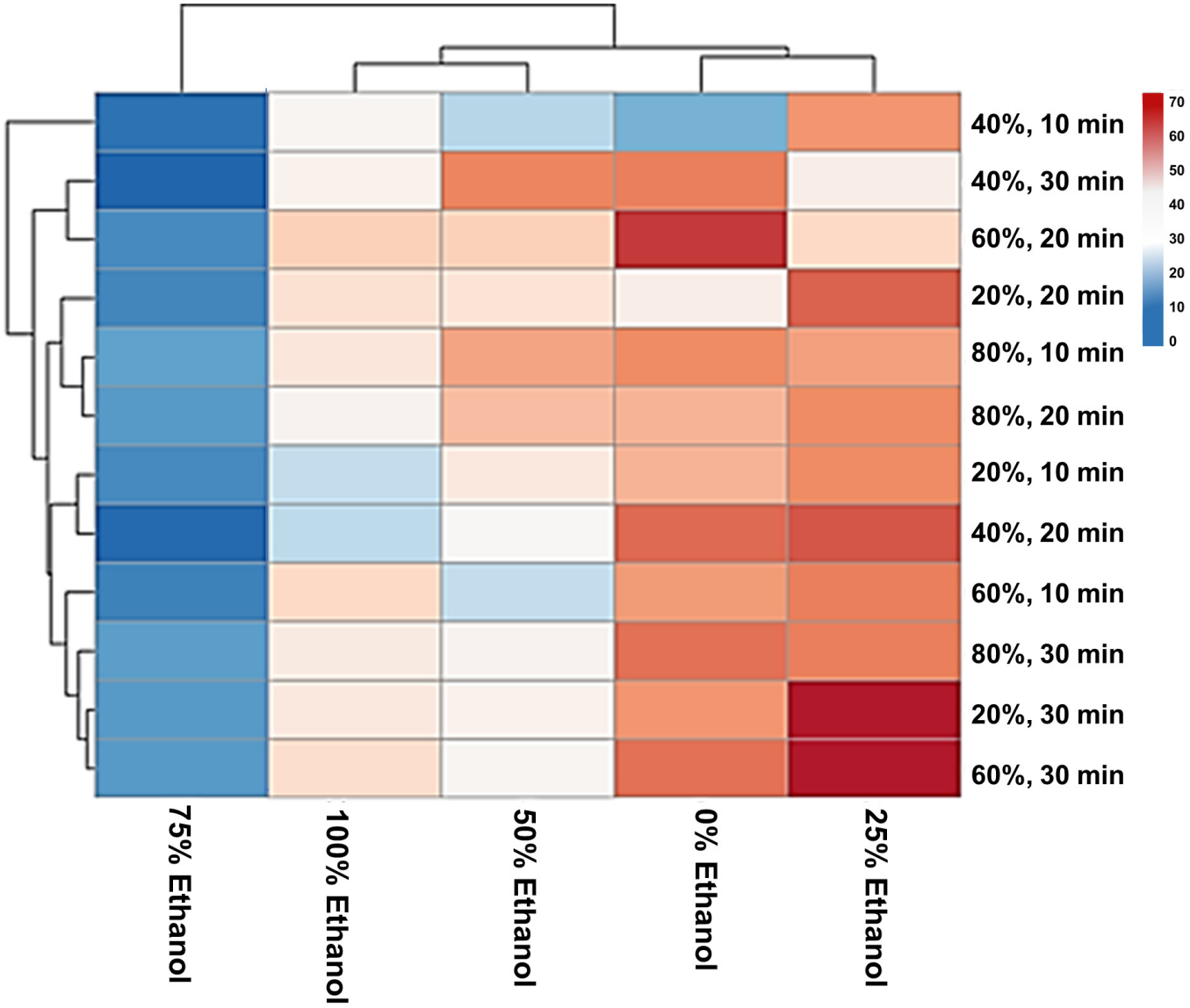


Figure 1. Heatmap illustrating the impact of extraction parameters on the free radical scavenging activity of *Cymodocea nodosa* extracts expressed in DPPH radical inhibition percentage.

3.2. Experimental Design

Based on observations from the preliminary study, the experimental ranges for each factor (extraction time (min), ultrasonic power (%) and hydroethanol percentage (%*v*/*v*)) were defined (Table 1).

In order to reduce the number of experiments required to evaluate the factors and their interactions, we opted to use a Box–Behnken design, which is a response surface design. This design enabled us to analyze the effect of each factor and their interactions on the two desired responses, namely total polyphenol content (Y_{TPC}) and DPPH radical inhibition percentage (Y_{PI}), and to determine the optimum conditions for extracting polyphenols from C. nodosa with good yield and high antioxidant activity. Table 2 shows the 19 trials carried out, including five replicates at the central point. These trials were carried out by combining different levels of the three factors studied according to the Box–Behnken design.

Table 2. Experimental conditions and responses obtained using the Box–Behnken design.

Exp	Independent Variables			Responses	
	Time (min)	Ultrasonic Power (%)	Hydroethanol Percentage (%*v*/*v*)	Y_{TPC} (mg GAE/g DM)	Y_{PI} (%)
1	20	20	20	137.69	61.61
2	40	20	20	112.69	59.12
3	20	40	20	142.61	60.03
4	40	40	20	105.87	58.15
5	20	20	30	97.54	54.24
6	40	20	30	120.27	57.27
7	20	40	30	120.27	60.30
8	40	40	30	127.46	63.21
9	20	30	25	127.08	64.03
10	40	30	25	120.28	62.75
11	30	20	25	117.64	65.24
12	30	40	25	124.43	67.51
13	30	30	20	124.05	62.57
14	30	30	30	119.13	62.6
15	30	30	25	129.13	66.45
16	30	30	25	129.55	66.30
17	30	30	25	129.99	66.45
18	30	30	25	129.36	66.97
19	30	30	25	122.92	66.30

3.3. Validity of Models Through ANOVA Analysis

ANOVA analysis verifies whether the independent variables involved in the model have an effect on the studied responses. Table 3 provides a summary of the analysis of variance for the two proposed models to describe the sought-after responses (Y_{TPC}, Y_{PI}). To verify the validity of the regression models describing the effect of significant factors on Y_{TPC} and Y_{PI}, Fisher's *F* test was used. According to Fisher's *F* test, the observed values of the F-ratio, which corresponds to the ratio between the mean square of the regression and the mean square of the residual, are higher than the tabulated values for the degree of freedom ([$F_{Observed}$ = 20.71] TPC > $F_{Tabulated(9, 5, 0.05)}$ = 3.33; [$F_{Observed}$ = 131.97] PI > $F_{Tabulated(9, 5, 0.05}$ = 3.33). Furthermore, the ratio between the mean square of validity and the estimation of the experimental variance is lower than the tabulated value, confirming the results of Fisher's test and the validity of the proposed model. The determination coefficients R^2 for the two responses Y_{TPC} and Y_{PI} are approximately 0.954 and 0.992, respectively, demonstrating a good correlation between the experimental and predicted values by the models.

Table 3. Analysis of variance (ANOVA) of second-order polynomial models for the two responses (Y_{TPC}, Y_{PI}).

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	Fisher's *F*-Test	Significance
			Y_{TPC}		
Regression	1.8354	9	2.0393	20.7245	***
Validity	5.3478	5	1.0695	1.2171	43.6%
R^2 = 0.954			$F_{Obs}(20.72) > F_{tab}(3.33)$		
			Y_{PI}		
Regression	250.72	9	27.8579	131.9728	***
Validity	1.5965	5	0.3193	4.2115	9.5%
R^2 = 0.992			$F_{Obs}(131.97) > F_{tab}(3.33)$		

The significance levels are represented as follows: ***: Very significant.

3.4. Interpretation of Coefficients

The significance of the coefficients in the second-degree polynomial model corresponding to each of the two responses studied (Y_{TPC}, Y_{PI}) was assessed using Student's *t*-test with a confidence interval of 95% (α = 5%). A coefficient is considered significant only when the probability is lower than the set threshold (5%). Table 4 summarizes the coefficient values of the two proposed models. The results indicate that the three factors (extraction time (X_1), ultrasonic power (X_2), and hydro-ethanolic percentage (X_3)) exhibit a significant linear effect ($p < 5\%$) on the total polyphenol content (Y_{TPC}). However, only factors X_2 and X_3 show a significant linear effect on the radical scavenging activity (Y_{PI}). Additionally, it is observed that only for the DPPH test (Y_{PI}) does the extraction time (X_2) demonstrate a significant quadratic effect ($p < 0.05$). Regarding the interaction effect between factors on the two studied responses, it is noted that the interaction between extraction time (X_1) and hydro-ethanolic percentage (X_3), as well as that between ultrasonic power (X_2) and hydro-ethanolic percentage (X_3), are deemed significant ($p < 0.05$). Moreover, the higher the coefficient value, the more significant the factor. The interaction between extraction time (X_1) and ultrasonic power (X_2) only has a significant effect on TPC.

Table 4. Regression coefficients of the second-order polynomial models predicted for the two responses studied (TPC and PI of the DPPH radical).

Terms	TPC		DPPH	
	Coefficient	**Significance %**	**Coefficient**	**Significance %**
b 0	126.438	***	66.515	***
		Linear Effect		
b 1	−3.862	**	0.029	83.80%
b 2	3.482	**	1.172	***
b 3	−3.826	**	−0.386	*
		Quadratic Effect		
b 11	−0.567	76.80%	−3.149	***
b 22	−3.211	12.20%	−0.165	57.40%
b 33	−2.658	19.30%	−3.954	***
		Interaction Effect		
b 12	−3.409	*	0.061	71.60%
b 13	11.458	***	1.288	***
b 23	3.977	**	1.818	***

The significance levels are represented as follows: ***: Very significant, **: Significant, *: Marginally significant. A percentage value denotes that the corresponding term is not statistically significant.

3.5. Analysis of Response Surface Curves

The response surface curves depicted in two and three dimensions were used to graphically visualize the mathematical models describing the effect of significant factors on the total phenolic content (TPC) and the radical scavenging activity (PI) of the extract. Figure 2A illustrates the effects of interactions between significant independent variables (extraction time (X_1) and hydro-ethanolic percentage (X_2)) on the total phenolic content of the *C. nodosa* extract. By fixing the ultrasonic power at 30%, the total polyphenol contents can reach values higher than 134.42 mg EAG/g DM for extraction durations ranging from 20 to 30 min and hydro-ethanolic percentages between 20 and 25%. Regarding the DPPH test, Figure 2B illustrates the effects of interactions between significant independent variables (extraction time (X_1) and hydro-ethanolic percentage (X_2)) on the extract's ability to stabilize the DPPH radical. Thus, by fixing the ultrasonic power at 30%, the percentage inhibition of the DPPH radical can reach values lower than 62.68% for extraction durations ranging from 20 to 30 min and hydro-ethanolic percentages between 20 and 25%.

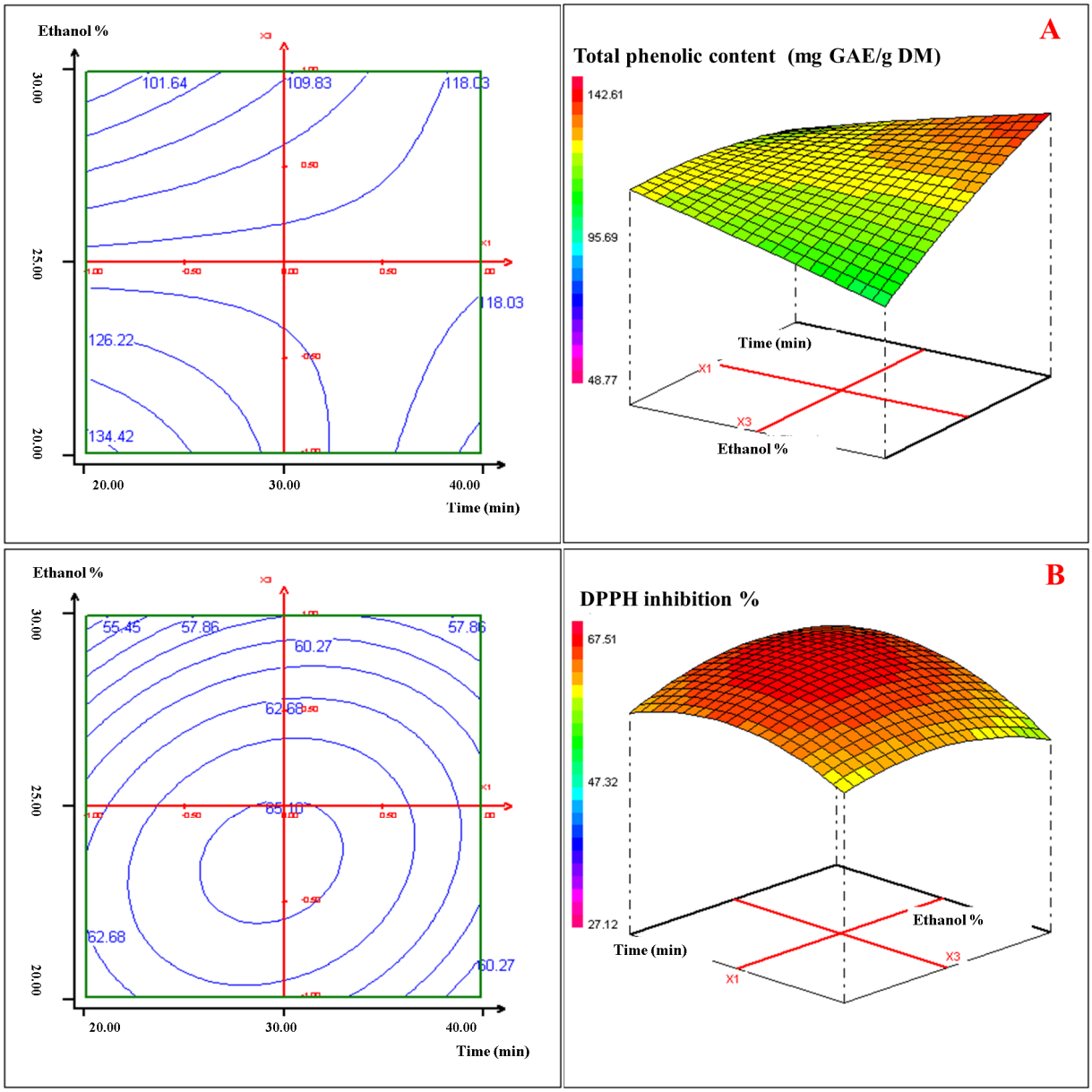


Figure 2. (**A**) 2D and 3D response surface curves illustrating the effect of the interaction between time (X_1) and hydro-ethanolic percentage (X_2) on total phenolic contents. (**B**) 2D and 3D response surface curves illustrating the effect of the interaction between time (X_1) and hydroethanolic percentage (X_2) on the percentage of DPPH radical inhibition.

3.6. Determination of Optimal Extraction Conditions for Antioxidants from C. nodosa

The objective of using the experimental design methodology, particularly response surface designs, is to describe the link between factors (independent parameters) and response (dependent parameters) and to evaluate the influence of these factors and their interactions on the response. This approach aims to optimize the process to obtain a high-quality eco-extract enriched in phenolic compounds with maximum antioxidant activity from the magnoliophyta *C. nodosa*. The optimum corresponds to the following experimental extraction conditions: an extraction time of 30 min, an ultrasonic power of 30%, and a hydro-ethanolic percentage of 25%. The proposed models were validated using these optimal conditions. The experimental values obtained for the TPC and PI responses are 113.07 mg EAG/g DM and 67.02%, respectively. These results are consistent with those obtained from the proposed models, which were 126.44 mg EAG/g DM for the TPC response and 66.51% for the PI response (Table 5).

Table 5. Predicted and experimental values of the studied responses under optimal conditions.

Factor			Experimental Value		Predicted Value	
Time (min)	**Ultrasound Power (%)**	**Hydro-Ethanolic Percentage (%*v*/*v*)**	**TPC (mgEAG/gDM)**	**PI (%)**	**TPC (mgEAG/gDM)**	**PI (%)**
30	30	25	113.07 ± 1.45	67.02 ± 0.02	126.44	66.51

3.7. Phenolic Compounds Content in C. nodosa

After optimizing the extraction conditions and determining the optimal levels of experimental factors to enhance the phenolic content and antioxidant capacity, the optimum extraction conditions (30 min extraction time, 30% ultrasonic power, and 25% hydro-ethanolic solution) were applied for all subsequent analyses in this study. Based on the colorimetric analysis, the results given in Table 6 revealed the richness of *C. nodosa* extract in phenolic compounds with potent antioxidant activities. Indeed, the quantification of total polyphenols (TPC) in *C. nodosa* showed that it exhibited a high concentration of these metabolites, with a content of around 113.07 mg EAG/g MS in the hydro-ethanolic extract. As for flavonoids, *C. nodosa* species expressed a high concentration of approximately 303.94 mg EC/g MS under the same extraction conditions. Additionally, the amounts of condensed tannins measured in *C. nodosa* extracts were around 172.85 mg EC/g MS.

Table 6. Spectrometric evaluation of phenolic composition and antioxidant activities of *C. nodosa* eco-extract.

Assay	Values
Total phenolic content (mg EAG/g DM)	113.07 ± 1.45
Total flavonoids content (mg EC/g MS)	303.94 ± 1.45
Total condensed tannins (mg EC/g MS)	172.85 ± 1.25
Total antioxidant activity (mg/mL)	218.75 ± 1.87
Radical scavenging activity (DPPH) (µg/mL)	CI_{50} = 1.9 ± 1.26
Reduction power (µg/mL)	CE_{50} = 1.75 ± 0.07

3.8. Phytochemicals Identification by RP-HPLC

Under optimal extraction conditions, a 25% hydro-ethanolic extract of *C. nodosa* showed a higher content of total polyphenols and flavonoids, along with effective free radical scavenging activity. To separate and identify the main bioactive compounds of this extract, RP-HPLC analysis was conducted and the results are displayed in Table 7. Seven compounds were successfully identified, namely, ascorbic acid, rutin, quercetin-3-O-rutinoside, sinapic acid, ferulic acid, myricetin, and trans-cinnamic acid. The most abundant compound is sinapic acid (0.741 mg/g), followed by myricetin (0.62 mg/g) and quercetin-3-O-rutinoside (0.3 mg/g) (Figure 3), highlighting the potential antioxidant and therapeutic properties of *C. nodosa* hydro-ethanolic (25%) extract.

Table 7. HPLC analysis of optimal *C. nodosa* extract.

	Identified Compounds	mg/g DW	Calibration Curve	R^2
1	Ascorbic acid	0.046 ± 0.01	Y = 0.0255X + 0.0036	0.989
2	Quercetin-3-o-rutinoside	0.300 ± 0.05	Y = 1128.4X + 21.114	0.999
3	Sinapic acid	0.741 ± 0.08	Y = 7658.1X − 412.41	0.998
4	Rutin	0.044 ± 0.01	Y = 16860X + 5320.60	0.998
5	Ferulic acid	0.324 ± 0.03	Y = 3674.9X + 56.235	0.998
6	Myrecitin	0.620 ± 0.05	Y = 995.4X − 23.982	0.998
7	Trans cinnamic acid	0.001 ± 0.00	Y = 4236.6X − 36.756	0.998

Results are presented as the means of at least three replicates ± standard deviation.

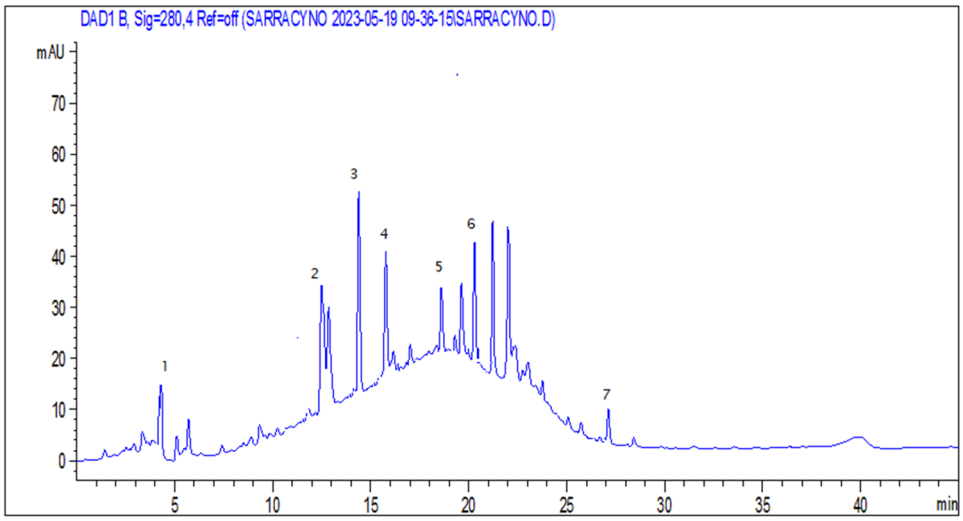


Figure 3. HPLC-DAD profiling of the optimal eco-extract of *C. nodosa*. 1. Ascorbic acid, 2. Quercetin-3-o-rutiniside, 3. Sinapis acid, 4. Rutin, 5. Ferulic acid, 6. Myrectin, 7. Trans-cinnamic acid.

3.9. Evaluation of Anti-Inflammatory Activity

3.9.1. Evaluation of Cytotoxicity of *C. nodosa* Eco-Extract

Firstly, the cytotoxicity of the hydro-ethanolic extract of *C. nodosa* was assessed using the resazurin assay, a molecule that can be converted into fluorescent resorufin during mitochondrial metabolism and by cytoplasmic enzymes. A decrease in mitochondrial metabolism or enzymatic activity indicates inhibition of cell growth. RAW 267.4 cells were treated with increasing concentrations of *C. nodosa* eco-extract (from 25 to 400 µg/mL). Thus, Figure 4 showed that no toxicity was detected for the hydro-ethanolic extract at concentrations ranging from 25 to 400 µg/mL, with a cell viability percentage exceeding 100%.

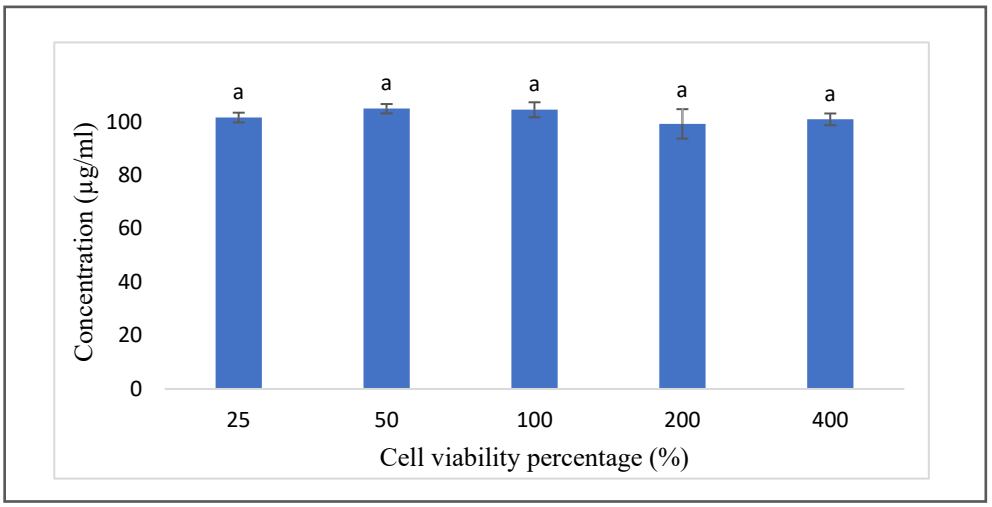


Figure 4. The viability of Raw 264.7 murine macrophage cells treated with increasing concentrations (25, 50, 100, 200, and 400 µg/mL) of the eco-extract of *C. nodosa*.

3.9.2. Measurement of Nitrite Production (NO)

The anti-inflammatory activity of *C. nodosa* extracts was assessed by measuring their ability to inhibit the production of nitric oxide (NO) by RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS). The results obtained indicate that the *C. nodosa* extract exhibits weak anti-inflammatory activity. Indeed, increasing concentrations of 25, 50, 100, and 200 µg/mL of *C. nodosa* eco-extract did not cause inhibition of NO production. However, slight inhibition of approximately 7.8% was observed at very high concentrations, reaching 400 µg/mL.

3.10. Valorization of C. nodosa Eco-Extract for the Formulation of a Cosmetic Cream

Using innovative ultrasound-assisted extraction and experimental design methodology, we obtained an antioxidant-rich extract from *C. nodosa* in just 30 min, using only 25% ethanol as the solvent. This eco-extract showed exceptional antiradical activity (1.9 µg/mL), surpassing synthetic antioxidants like Trolox (65 µg/mL), with notably, no toxicity even at high concentrations (400 µg/mL). Encouraged by these findings, we formulated an innovative anti-aging cosmetic product, featuring *C. nodosa*'s active eco-extract as its key ingredient, meeting the rising demand for natural anti-aging solutions.

3.11. Characterization and Stability of Cosmetic Cream

The cream's stability was meticulously tracked over a 30-day period, revealing intriguing insights into its performance. For instance, the pH remained remarkably stable, hovering around 6.58 to 6.6 throughout the observation period, indicating consistent acidity critical levels for product efficacy (Table 8). Viscosity measurements showed minimal variation, with values ranging from 5966.38 to 5980.6 cp, suggesting that the cream maintained its desired texture and consistency over time. Additionally, color analysis indicated subtle changes, with the cream exhibiting slight shifts towards yellow (b* values increasing from 4.4 to 4.6), although the overall color difference (ΔE) remained low, indicating negligible perceptible changes. Importantly, the cream demonstrated stability under various centrifuge conditions and temperatures, remaining stable at 4 °C, 25 °C, and 40 °C throughout the testing period (Table 8).

Table 8. Evaluation of pH values, viscosity, mean droplet diameter (Z-average), zeta potential (ZP), and color of preliminary stability tests of cream containing phenolic-enriched *C. nodosa* extract over 30 days.

	Day 0	**Day 10**	**Day 20**	**Day 30**
pH	6.58 ^{a} ± 0.01	6.58 ^{a} ± 0.00	6.59 ^{a} ± 0.00	6.6 ^{a} ± 0.02
Viscosity (cp)	5966.38 ^{a} ± 0.54	5972.55 ^{a} ± 0.48	5975.75 ^{a} ± 0.49	5980.60 ^{a} ± 0.02
Z-average (d.nm)	326.26 ^{a} ± 15.22	331.99 ^{a} ± 10.15	342.51 ^{a} ± 16.01	322.85 ^{a} ± 12.48
Zeta potential (mV)	−38.25 ^{a} ± 2.25	−37.44 ^{a} ± 3.66	−37.58 ^{a} ± 1.26	−36.27 ^{a} ± 4.26
L*	80.3	80.3	80.3	80.3
a*	0.1	0.2	0.2	0.3
b*	4.4	4.5	4.5	0.6
Color difference ΔE	-	0.14	0.14	0.28
Centrifuge stability 4 °C	stable	Stable	stable	stable
Centrifuge stability 25 °C	Stable	Stable	stable	stable
Centrifuge stability 40 °C	Stable	Stable	stable	stable

Results are presented as means of at least three replicate ± standard deviation, followed by different letters (a, b) show significant differences at $p \leq 0.05$.

3.12. Sensory Analysis of the Cosmetic Cream

The results were extremely positive, as can be seen from the radar graph (Figure 5) showing the scores for each criterion. The cream was rated very positively in all areas, reflecting the general satisfaction of the participants. Color and fragrance scored a high average of 9.45, demonstrating the success of our efforts to offer a pleasing aesthetic and fragrance. Consistency (average of 9.06), spreadability (average of 9.26), tackiness (average of 9.18), and penetration (average of 9.5) also scored highly, underlining the quality of our product in a number of areas that are important to our users. Finally, the overall score of 9.49 reflects the participants' general satisfaction with our cosmetic cream.

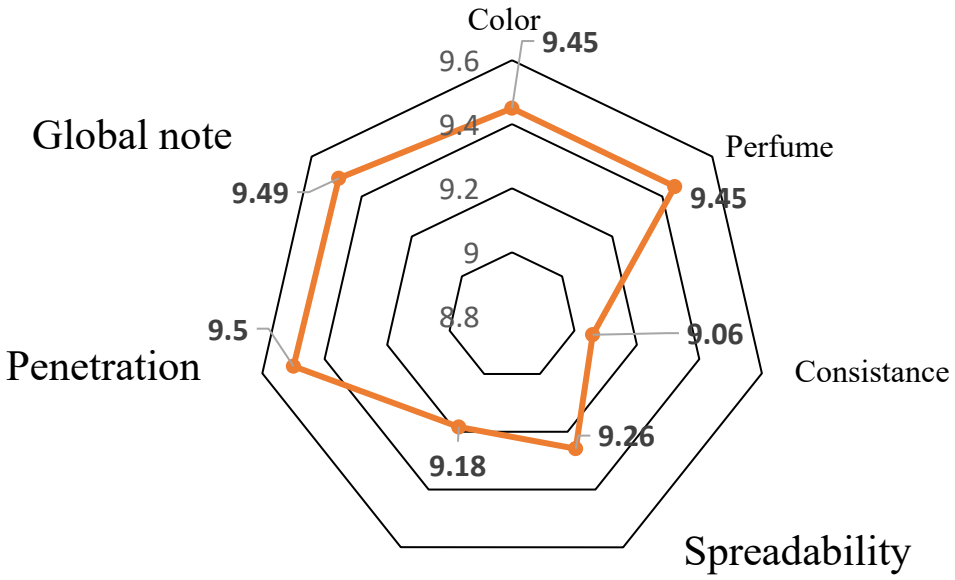


Figure 5. Sensory analysis of *C. nodosa* cosmetic cream.

4. Discussion

Ultrasound enhances antioxidant extraction efficiency by improving the recovery of bioactive compounds from various sources [17]. While the use of this innovative technique for the extraction of antioxidants from marine sources has been reported previously, to the best of our knowledge, there is no work on optimizing the green extraction of phenolics from *C. nodosa* using ultrasound-assisted extraction (UAE). Susilo et al. [24] demonstrated the effectiveness of UAE in enhancing the extraction of antioxidants from Indonesian seagrass *Syringodium isoetifolium*, resulting in increased phenolic contents and promising antioxidant activity. Similarly, Rashad et al. [25] reported the effectiveness of this green technique in the extraction of phenolics from green seaweed *Ulva lactuca*. The use of ultrasound helps in breaking down cell walls, facilitating the release of intracellular components, and enhancing mass transfer processes during extraction. These results in higher total polyphenol recovery, improved antioxidant activity of the extracts, and reduced energy consumption compared to conventional extraction methods. Additionally, ultrasound not only enhances extraction efficiency but also optimizes key parameters such as solvent consumption, temperature, extraction time, solvent concentration, solvent polarity, and ultrasound power, leading to more efficient and sustainable extraction processes [17,26].

The optimization of ultrasound-assisted extraction (UAE) using response surface methodology (RSM) was carefully designed to maximize the yield of bioactive compounds from *Cymodocea nodosa*. Key parameters affecting extraction efficiency were selected. The solvent concentration was chosen to optimize the solubility of the desired compounds while minimizing impurities, thereby enhancing the extraction yield and quality. In addition, the temperature was adjusted to improve the solubility and release of bioactive compounds without degrading heat-sensitive components. Then, extraction time was optimized to allow adequate diffusion of compounds while preventing degradation, ensuring maximum recovery of antioxidants. Additionally, varying ultrasound power was essential for enhancing cavitation effects, promoting cell wall disruption, and improving extraction efficiency while reducing energy consumption. This systematic approach effectively addresses the complex interactions between these parameters to achieve optimal extraction results for *C. nodosa*. Conventionally, scientists assess each parameter separately to evaluate its impact on the extraction process, which requires a significant number of experiments and is time-consuming. This approach also fails to account for the interactions between factors and to quantify their combined effects on extraction efficiency. Hence, the design of experiments is a valuable statistical approach for identifying significant variables in the ultrasound-assisted extraction process and studying the relationship between factors (input

variables) and responses (output variables), offering the advantage of acquiring extensive information and reaching optimal conditions with minimal time and fewer experiments. This structured approach has been successfully used to optimize the extraction of antioxidants and bioactive compounds from various sources, including plants [14], agricultural residues, byproducts [25,26], algal biomass [25], and plant biomass [17]. Surface response methodology (RSM) is a useful tool for optimizing extraction processes; it is an effective mathematical and statistical method for analysis of empirical models that describes the effect of independent variables and their interactions on response variables [27,28]. Studies have shown that RSM helps in determining the optimal conditions for extraction, leading to higher yields of bioactive compounds like total phenolic content and total flavonoids [29]. By utilizing RSM in conjunction with UAE, researchers can identify the ideal combination of factors such as solvent concentration, temperature, and extraction time to maximize the extraction efficiency of antioxidants [30]. Additionally, the use of RSM aids in the development of sustainable and eco-friendly extraction techniques [31], making it a valuable tool for industries seeking to harness bioactive compounds for various applications in the food, pharmaceutical, and cosmetics sectors. The integration of UAE with RSM in this study has yielded promising results in the context of enhancing antioxidant extraction from *C. nodosa*. By leveraging the synergistic benefits of these techniques, we have effectively optimized the extraction process, resulting in a significant increase in the recovery of bioactive compounds. Our findings confirm that the application of UAE under optimized conditions substantially improves the yield of antioxidant compounds. This combination allowed for the efficient disruption of plant cell walls, leading to an enhanced release of phenolic compounds and other antioxidants. The models proposed through the RSM were validated using these optimal conditions. The experimental values obtained for total phenolic content (TPC) and antioxidant activity index (PI) were 113.07 mg EAG/g DM and 67.02%, respectively. These experimental results closely aligned with the values predicted by the models, which were 126.44 mg EAG/g DM for TPC and 66.51% for PI. The consistency between the experimental and predicted values underscores the robustness and accuracy of the RSM in optimizing the UAE process. The optimal extraction time and ultrasound frequency for maximum antioxidant extraction from marine plants depend on the species, the rigidity of the cell wall, and the nature of the target metabolites [14]. Susilo et al. [24] reported that for the extraction of antioxidants from the seagrass *Syringodium isoetifolium*, the optimal extraction time was found to be 5 min with an ultrasound frequency of 20 kHz and 50% ethanol as the solvent. Additionally, for the extraction of antioxidants from *Ulva lactuca*, a sea lettuce macroalgae, the optimal extraction time was found to be 1 h with an ultrasound frequency of 40 kHz [32]. The RP-HPLC profile of the 25% hydro-ethanolic extract of *C. nodosa* revealed the presence of several key compounds, namely, ascorbic acid, rutin, quercetin-3-O-rutinoside, sinapic acid, ferulic acid, myricetin, and trans-cinnamic acid. The most abundant compound identified was sinapic acid, followed by myricetin and quercetin-3-O-rutinoside. Sinapic acid is known for its strong antioxidant, anti-inflammatory, and antimicrobial properties, making it highly valuable for cosmetic formulations aimed at protecting and rejuvenating the skin [33,34]. Myricetin, another potent antioxidant, offers additional anti-inflammatory and photoprotective benefits [35,36], while quercetin-3-O-rutinoside, commonly known as rutin, provides cytoprotective protective effects and enhances skin elasticity and firmness [37,38]. Ascorbic acid, a well-known antioxidant, plays a crucial role in collagen synthesis and skin brightening, further enhancing the cosmetic value of the extract [39].

The anti-inflammatory potential of *C. nodosa* eco-extract was evaluated through a series of in vitro assays. The assessment focused on cytotoxicity and the extract's ability to inhibit nitric oxide (NO) production, which is a common indicator of anti-inflammatory activity [40]. *C. nodosa*, a type of seagrass, has not been directly studied for its anti-inflammatory properties in previous research.

The high total phenolic content and significant antioxidant activity of the *C. nodosa* extract underscore its potential as a valuable ingredient in cosmetics aimed at combating oxidative stress and inflammation. Thus, integrating such extracts enhances the efficacy

of cosmetic formulations in providing skin protection and rejuvenation, making them appealing options for consumers seeking effective skincare solutions.

However, related studies on marine organisms like starfish (*Protoreaster nodosus*) [41], seaweeds (*Cystoseira crinita, Cystoseira sedoides, Cystoseira compressa*) [42], and plants (*Clusia nemorosa, Lippia nodiflora*) have shown promising anti-inflammatory effects [43]. These findings suggest that marine organisms and plant extracts can contain bioactive compounds with anti-inflammatory potential, indicating that further research on *C. nodosa* extract may reveal similar beneficial effects in combating inflammation. Algae extracts, particularly from microalgae and cyanobacteria species like *Chlorella vulgaris*, *Trachydiscus minutus*, and *Synechococcales cyanobacteria*, have shown significant potential for cosmetic applications due to their antioxidant, anti-aging, and photoprotective properties [44–46]. These extracts have been found to possess high total antioxidant capacity, inhibit enzymes associated with skin aging, and protect against UVA-induced cell damage, making them valuable ingredients in skincare products [47–49]. Additionally, algae-derived compounds are utilized in cosmetics as moisturizers, anti-inflammatory agents, and regenerative components, contributing to the rise of "blue cosmetics" that emphasize natural active ingredients and sustainability in the industry [50]. Phenolic compounds, well-known for their potent antioxidant activity, play an essential role in promoting skin health by neutralizing free radicals, thereby reducing oxidative stress, a key factor in skin aging and multiple dermatological conditions [51]. By incorporating phenolic-rich ingredients into cosmetic formulations, several skin-related benefits can be achieved, including anti-aging effects, as these antioxidants help reduce the appearance of wrinkles and fine lines, contributing to a more youthful complexion [8]. Additionally, the anti-inflammatory properties of many phenolic compounds can soothe skin irritation and alleviate redness, enhancing overall skin comfort [52]. Moreover, certain phenolics offer photoprotective benefits by absorbing ultraviolet (UV) radiation, thus shielding the skin from UV-induced damage [53].

The sensory analysis of our cosmetic cream revealed overwhelmingly positive feedback from participants, with strong ratings across all key attributes. Color and fragrance achieved an impressive average score of 9.45, highlighting the success of our formulation in creating a visually appealing and pleasantly scented product, both critical factors in consumer preference for cosmetic items. The cream's consistency, rated at 9.06, was also well-received, with participants appreciating the smooth, uniform texture that enhanced the product's usability and overall experience. In terms of spreadability, the cream scored an average of 9.26, indicating that it was easy to apply and distribute evenly on the skin, a crucial factor for user satisfaction in cosmetic applications, as it directly affects how quickly and comfortably the product can be used. Tackiness, often a concern with cosmetic creams, was minimal, earning a score of 9.18. This suggests that the product left little to no sticky residue, which is important for ensuring a pleasant post-application feel on the skin. The cream's penetration ability was rated at 9.5, demonstrating its effectiveness in quickly absorbing into the skin without leaving an oily or greasy layer, which is a significant determinant of long-term comfort and product performance. Finally, the cream achieved an overall satisfaction score of 9.49, underscoring the high level of consumer approval across all sensory categories. This strong feedback suggests that the cream not only meets but exceeds consumer expectations in terms of texture, ease of application, sensory feel, and overall experience. These results confirm the product's potential to be highly competitive in the cosmetic market, and further trials could be conducted to reinforce these findings on a broader scale.

This study highlights the successful optimization of ultrasound-assisted extraction (UAE) from *C. nodosa*, but several limitations warrant attention. Notably, the research primarily focused on short-term extraction parameters without assessing the long-term stability or shelf-life of the extracted antioxidants, an area that should be explored in future studies. Additionally, while the sensory analysis yielded positive feedback, the sample size was limited, necessitating broader demographic studies for generalizability. Preliminary in vitro anti-inflammatory assays call for further in vivo investigations to validate

observed benefits. Future research should also explore the potential synergistic effects of the identified bioactive compounds to deepen our understanding of their mechanisms and enhance their applications in the cosmetics industry.

5. Conclusions

In conclusion, this study successfully optimized the ultrasound-assisted extraction of antioxidants from *Cymodocea nodosa*, yielding a potent extract rich in phenolic compounds with significant antioxidant activity and non-toxicity to RAW 267.4 cells, confirming its safety for cosmetic applications. The formulated cream exhibited excellent stability under various conditions, further supporting the extract's potential as a valuable ingredient in eco-friendly cosmetic products. These findings underscore the effectiveness of the optimized extraction method and the stability of the cream, highlighting *C. nodosa*'s potential as a key component in the development of eco-friendly, effective skincare products.

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