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Article

Unlocking Pomegranate's Potential: Ultrasonication-Enriched Oil in Nanobeads for Innovative Cosmetic Hydrogels

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Abstract

Pomegranate (*Punica granatum* L.), is renowned for its bioactive compounds, offering significant potential in cosmetic applications due to its antioxidant, anti-inflammatory, and antimicrobial properties. This study presents a sustainably sourced cosmetic ingredient developed by enriching pomegranate seed oil with peel powder using optimized ultrasonication, followed by encapsulation in alginate nanobeads and integration into a minimalist hydrogel formulation. A Box–Behnken experimental design was employed to optimize ultrasonication parameters (15 min, 90% power, 202 mg/mL powder-to-oil ratio), yielding an enriched PSO with significantly enhanced total phenolic content (TPC: 69.23 ± 1.66 mg GAE/g), total flavonoid content (TFC: 61.09 ± 1.66 mg QE/g), and robust DPPH antioxidant activity ($78.63 \pm 3.81\%$). The enriched oil exhibited enhanced oxidative stability (peroxide value: 5.75 ± 0.30 meq O₂/kg vs. 50.95 ± 0.07 meq O₂/kg for neutral oil), improved fatty acid profile, and significant anti-inflammatory (IC₅₀ = 897.25 µg/mL for NO inhibition) and antibacterial activities. Alginate nanobeads (432.46 ± 12.59 nm, zeta potential: -30.74 ± 3.20 mV) ensured bioactivity preservation, while the hydrogel maintained physicochemical and microbial stability over 60 days under accelerated conditions (40 ± 2 °C, $75 \pm 5\%$ RH). This multifunctional formulation, integrating sustainable extraction, advanced encapsulation, and a minimalist delivery system, represents a highly promising natural ingredient for anti-aging and antioxidant cosmetic applications.

Keywords: pomegranate; ultrasonication; Box–Behnken design; alginate nanobeads; hydrogel; biological activity; cosmetic formulation



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1. Introduction

Pomegranate (*Punica granatum* L.) is increasingly recognized as a highly valuable ingredient in cosmetic formulations, primarily due to its abundant array of bioactive compounds. These compounds confer significant antioxidant, anti-inflammatory, and anti-aging benefits,

making pomegranate extracts particularly efficacious for dermatological applications [1]. The fruit's diverse components, notably its seeds and peel, are rich sources of diverse phytochemicals possessing considerable therapeutic potential for skin health. Pomegranate seed oil (PSO), typically obtained through cold-pressing, is distinguished by its unique fatty acid composition. Punicic acid, a conjugated linolenic acid, constitutes the major component, ranging from 65% to 83% of the oil's total fatty acids [2]. This unique fatty acid profile underscores the oil's importance in drug delivery systems, where its lipophilic nature and stability enhance the encapsulation and controlled release of bioactive compounds, making it a promising carrier for pharmaceutical and cosmetic application. This distinctive fatty acid profile, coupled with its lipophilic nature and inherent stability, positions PSO as an excellent candidate for advanced drug and cosmetic delivery systems, facilitating enhanced encapsulation and controlled release of active compounds [3]. Furthermore, PSO contains important minor constituents such as linoleic acid (3–7%), oleic acid (4–9%), and various tocopherols (e.g., α - and γ -tocopherol). These components collectively augment the oil's potent antioxidant capabilities and provide notable photoprotective effects against ultraviolet (UV)-induced skin damage, further solidifying its role in comprehensive skincare formulations [4]. Concurrently, pomegranate peel, often regarded as an underutilized agricultural by-product, represents a remarkably potent and sustainable source of diverse phenolic compounds significantly enhancing its biological activity. This rich composition includes a high concentration of hydrolysable tannins, notably punicalagins, which can constitute up to 20–25% of the peel's dry weight. These punicalagins are metabolically converted into ellagic acid, a compound with well-documented health benefits, alongside other valuable phenolics such as gallic acid, catechin, and quercetin [5]. These synergistic compounds collectively contribute to the peel's strong antioxidant capacity by neutralizing free radicals, chelating metal ions, and inhibiting lipid peroxidation, thereby offering comprehensive protection to skin cells against oxidative stress and environmental damage [6]. Beyond its formidable antioxidant effects, pomegranate peel extracts exhibit anti-inflammatory properties by suppressing pro-inflammatory cytokines (e.g., TNF- α , IL-6) and antimicrobial activity against skin pathogens such as *Staphylococcus aureus*, making it a multifunctional ingredient for acne-prone or sensitive skin [7,8]. The integration of these two components, seed oil and peel phenolics, offers a highly promising and holistic approach to developing advanced multifunctional cosmetic ingredients. This synergistic combination leverages the hydrating and barrier-repairing attributes of the oil with the comprehensive antioxidant, anti-inflammatory, and antimicrobial benefits derived from the peel, leading to enhanced overall bioactivity and efficacy in skincare formulations. Ultrasonication, an eco-friendly extraction technique, has garnered significant interest in its capacity to enhance the recovery of bioactive compounds. This method operates by inducing acoustic cavitation, which effectively disrupts plant cell walls, thereby facilitating the release of intracellular components [9]. It proves particularly advantageous for enriching oils with valuable phenolic compounds, as it significantly improves mass transfer kinetics without the need for elevated temperatures that could potentially compromise the stability and integrity of heat-sensitive bioactives [10]. Nevertheless, achieving optimal extraction efficiency and maximizing the phenolic enrichment and antioxidant activity through ultrasonication necessitates a meticulous optimization of various process parameters. These critical parameters include sonication time, the precise powder-to-oil ratio, and the applied sonication power. Consequently, systematic investigations employing robust experimental design methodologies, such as factorial designs or response surface methodology, are indispensable to thoroughly explore the parameter space and identify the most favorable conditions for superior extraction outcomes [11].

Despite the bioactivity of pomegranate-derived ingredients, their direct incorporation into cosmetic formulations often presents significant challenges. These hurdles primarily stem from their inherent susceptibility to oxidative degradation and, in some cases, poor solubility, which can compromise their efficacy and shelf-life [12,13]. To overcome these limitations and fully exploit the potential of these valuable compounds, encapsulation within biocompatible carriers such as alginate nanobeads has proven to be a highly effective and promising strategy. This approach offers multifaceted advantages, including the provision of controlled release mechanisms, substantial improvements in ingredient stability, and enhanced bioavailability within the skin [14]. Alginate, a naturally occurring polysaccharide extracted from brown algae, is particularly favored in cosmetic and pharmaceutical applications due to its excellent biocompatibility, biodegradability, and remarkable ability to form stable gel-like structures through ionic cross-linking with divalent cations like calcium ions [15,16]. This makes alginate an ideal material for creating protective nanocarriers that safeguard sensitive bioactive compounds while facilitating their targeted and sustained delivery.

In modern cosmetic science, hydrogels are increasingly recognized as effective carriers for sensitive bioactive compounds, thanks to their hydrophilic, porous structure and excellent skin compatibility. Nanoencapsulation represents a key strategy to protect fragile actives, improve their bioavailability, and extend their duration of action [17]. Alginate nanobeads, made from a natural, biocompatible, and biodegradable polysaccharide, allow gentle encapsulation of thermosensitive compounds such as pomegranate oil, preserving their bioactive polyphenols and essential fatty acids. Thus, integrating pomegranate oil-loaded nanobeads into a simple hydrogel offers multiple benefits: alginate's gentle nature ensures skin compatibility, the hydrogel protects the oil from degradation, and together they enable controlled, sustained release for improved skin efficacy [18]. This innovative approach aligns with cosmetic market trends favoring natural, sustainable, and eco-friendly solutions. Using marine-derived alginate and food-industry byproduct pomegranate oil supports circular economy principles. Additionally, the minimalist formulation minimizes irritants, meeting the growing demand for clean beauty products [19].

This study proposes a novel and sustainable strategy to develop multifunctional cosmetic ingredients by combining eco-friendly extraction, active oil enrichment, nanoencapsulation, and minimalist formulation. Specifically, it aims to: (1) optimize the enrichment of pomegranate seed oil with peel-derived polyphenols via ultrasonication using a Box–Behnken design; (2) characterize the enriched oil for TPC, TFC, fatty acid profile, and bioactivities (antioxidant, anti-inflammatory, and antimicrobial); (3) encapsulate the oil in alginate nanobeads to enhance stability and bioavailability; and (4) incorporate these nanobeads into a minimalist hydrogel designed to reduce irritation risk while ensuring effective delivery. This integrative approach, grounded in circular economy principles and aligned with clean beauty standards, supports the development of next-generation cosmetic products with enhanced efficacy and sustainability.

2. Materials and Methods

2.1. Materials

All reagents and solvents used in the preparation of pomegranate extract and nanobeads, including ethanol and sodium alginate, were of analytical grade and obtained from Sigma-Aldrich (Deisenhofen, Germany). All materials were used as received without further purification. Dried pomegranate peel powder (*Punica granatum* L.), certified organic by ECOCERT (TN-BIO-001), was provided by Nopal Tunisie SA (ZI Route de Tala 1200, Kasserine, Tunisia), with a production date of November 2024. The cold-pressed

pomegranate seed oil, from the same harvest and also ECOCERT certified (batch no. HPG032024), was used as the lipidic phase.

2.2. Enrichment of Pomegranate Seed Oil

2.2.1. Preliminary Study

A preliminary study was conducted to evaluate ultrasonication parameters for enriching pomegranate seed oil with peel powder. Pomegranate seed oil (10 mL) was mixed with pomegranate peel (PP) powder at ratios of 100 mg/mL, 200 mg/mL, and 300 mg/mL (*w/v*) in a 50 mL sterile beaker. Ultrasonication was performed using ultrasound bath (SONOREX DIGIPLUS, Berlin, Germany) at power levels of 40%, 60%, and 80% (equivalent to 80, 120, and 160 W) for durations of 5, 10, 15, and 20 min. After ultrasonication, samples were centrifuged at 10,000 rpm for 10 min, and the supernatant was filtered through a 0.45 µm nylon membrane to obtain enriched oil. The antioxidant activity of the enriched oil was assessed using the DPPH assay, with the percentage inhibition relative to the control serving as the response variable to determine optimal conditions.

2.2.2. Experimental Design

The formulation of enriched PSO was optimized using a Box–Behnken experimental design, implemented through NemrodW software (LPRAI, version 2000). This statistical approach was applied to evaluate the effects and interactions of three independent variables: ultrasonication power (X_1 , %), ultrasonication time (X_2 , minutes), and powder-to-oil ratio (X_3 , mg/mL). The experimental domain was defined with central values of 70% for power, 10 min for time, and 200 mg/mL for the powder-to-oil ratio, each varied by a step of ± 10 , ± 5 , and ± 10 mg/mL, respectively. A total of 17 experimental runs were conducted, allowing for the estimation of 10 regression coefficients in the second-order polynomial model

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

The model was constructed to optimize three dependent responses: Total Phenolic Content (Y_{TPC} , mg GAE/g), DPPH antioxidant activity (Y_{DPPH} , % inhibition), and Total Flavonoid Content (Y_{TFC} , mg QE/g), which are critical indicators of the enriched oil's bioactivity and suitability for cosmetic applications. This design enabled an efficient exploration of the ultrasonication parameter space and facilitated the identification of optimal conditions for maximizing phenolic and flavonoid enrichment and antioxidant potential.

2.3. Characterization of Enriched Oil

2.3.1. Total Phenolic Content and Total Flavonoid Content

The total phenolic content (TPC) of the enriched oil was determined using the Folin–Ciocalteu assay. Briefly, 0.125 mL of enriched oil was mixed with 1.25 mL of 10% (*v/v*) Folin–Ciocalteu reagent and 1 mL of 7% (*w/v*) sodium carbonate solution. After incubation for 30 min at room temperature in the dark, the absorbance of the mixture was measured at 760 nm using an Agilent Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Gallic acid was used to generate a standard calibration curve ($R^2 = 0.99$), and the results were expressed as milligrams of gallic acid equivalents (mg GAE) per gram of oil.

The total flavonoid content (TFC) was determined according to the aluminum chloride colorimetric method. In brief, 0.25 mL of enriched oil extract was mixed with 0.15 mL of 10% aluminum chloride (AlCl_3), 0.5 mL of 1 M sodium hydroxide, and 2.5 mL of distilled water. The mixture was incubated at room temperature for 30 min. Absorbance was recorded at

510 nm. Quercetin was used to construct the standard calibration curve ($R^2 = 0.98$), and TFC values were expressed as milligrams of quercetin equivalents (mg QE) per gram of oil.

2.3.2. Fatty Acid Profile

According to Yeddes et al. [20], the fatty acid profile of the enriched PSO was determined by methylating fatty acids using sodium methoxide (CH_3ONa) for subsequent analysis: a 10 mg sample of enriched oil was weighed into a reaction vial, mixed with 2 mL of hexane, followed by the addition of 0.5 mL of sodium methoxide (3% (v/v) in methanol), agitated vigorously for 1 min with a vortex mixer, decanted for 2 min to separate phases, neutralized with 0.2 mL of 1 N sulfuric acid (H_2SO_4), and mixed with 1.5 mL of distilled water with further agitation; the fatty acid methyl esters (FAMES) were then extracted into the hexane layer, separated, and concentrated by evaporation under a gentle nitrogen stream for chromatographic analysis using a SCION 436-GC system (Bruker, Billerica, MA, USA) controlled by Compass CDS software (Version 3.0.1), with conditions including nitrogen (U grade) as the carrier gas at 1.5 mL/min, split mode with a 20:1 ratio, injector temperature of 250 °C, detector temperature of 280 °C, and an oven temperature program starting isothermally at 180 °C for 1 min, ramping to 200 °C at 15 °C/min, then to 250 °C at 3 °C/min, and holding at 250 °C for 10 min; fatty acids were identified by comparing retention times with pure reference standards (18918-1AMP SUPELCO F.A.M.E. Mix, C8-C24, Sigma-Aldrich, St. Louis, MO, USA) under identical conditions.

2.3.3. Antioxidant Activity

To evaluate the antioxidant potential of the enriched PSO, two complementary *in vitro* assays were employed. Total antioxidant capacity (TAC) was assessed using the phosphomolybdenum method, based on the reduction of Mo(VI) to Mo(V) in an acidic environment, forming a green phosphate/Mo(V) complex. Enriched oil (0.1 mL) was mixed with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95 °C for 90 min. After cooling, absorbance was measured at 695 nm. Results were expressed as milligrams of gallic acid equivalents per gram of oil (mg GAE/g) as described by Prieto et al. (1999) [21]. Free radical scavenging activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, in which the decrease in absorbance of the stable DPPH radical was measured at 517 nm. Enriched oil (1 mL) was mixed with 0.25 mL of 0.1 mM DPPH in methanol, incubated in the dark for 30 min, and absorbance was recorded. Results were expressed as percentage inhibition relative to the control [22].

2.3.4. Anti-Inflammatory Activity

The anti-inflammatory activity of the enriched PSO was evaluated by measuring nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages using the Griess reaction assay [23]. The RAW 264.7 macrophage cell line used in our study was obtained from the American Type Culture Collection (ATCC, accession number TIB-71, Manassas, VA, USA). The cell line was cultured in RPMI 1640 medium (Gibco Bio-cult, Glasgow, UK) supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL of penicillin, 100 mg/mL of streptomycin (Sigma-Aldrich, Deisenhofen, Germany).

RAW 264.7 cells were seeded at 2×10^5 cells/well in 96-well plates, and allowed to adhere overnight for 24 h, at 37 °C under a humidified 5% CO_2 atmosphere. After that, macrophages were treated with varying concentrations of oil. After 1 h of incubation inflammation was induced by adding lipopolysaccharide (LPS; 1 $\mu\text{g}/\text{mL}$). The cells were further incubated for 24 h under the same conditions. Post-incubation, 100 μL of cell culture supernatant from each well was mixed with 100 μL of Griess reagent. Absorbance was measured at 570 nm using a BioTek Synergy H(T) microplate reader. NO concentration

was quantified against a sodium nitrite (NaNO_2) standard curve, with nitrite accumulation serving as an indicator of NO production. The IC_{50} value (concentration required for 50% inhibition of NO production) was calculated. N-nitro-L-arginine methyl ester (L-NAME) was used as positive control. All measurements were performed in triplicate.

All in vitro experiments involving cell lines were performed in strict compliance with the applicable institutional guidelines and ethical regulations, with rigorous adherence to established protocols for the safe handling and appropriate disposal of all biological materials.

2.3.5. Cytotoxicity Assay

The cytotoxic effect of the enriched PSO was evaluated on the murine macrophage cell line RAW 264.7 under the same experimental conditions used for the anti-inflammatory activity assay. After adherence, cells were treated with either untreated culture medium (control) or medium containing PSO at concentrations ranging from 62.5 to 4000 $\mu\text{g/mL}$. Cell viability was assessed using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Following treatment, 100 μL of MTT solution (0.5 mg/mL) was added to each well and the plates were incubated for 4 h at 37 °C. The supernatant was then carefully removed, and the resultant formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader [24]. The selectivity index (SI), representing the therapeutic potential of the oil, was calculated as:

$$\text{SI} = \text{CC}_{50} / \text{CC}_{50} \quad (2)$$

where CC_{50} denotes the concentration causing 50% cell death.

2.3.6. Enriched Pomegranate Seed Oil Stability

The evaluation of oil stability was performed by measuring free acidity using the protocol outlined by the International Olive Council [25], expressed as a weight percentage of oleic acid (% oleic acid, w/w); the peroxide level was determined following the IOOC method [26], with outcomes reported in milliequivalents of active oxygen per kilogram of oil ($\text{meq O}_2/\text{kg oil}$); specific extinction coefficients (K_{232} and K_{270}) were calculated using the IOOC 2019 approach [27], involving the preparation of a 1% oil solution by dissolving 100 mg of oils in 10 mL of cyclohexane, followed by absorbance readings at 232 and 270 nm with a UV-Vis spectrophotometer, and each sample was analyzed in triplicate to ensure accuracy.

2.3.7. Antibacterial Activity

The antibacterial potential of the pomegranate extracts and enriched oils was assessed using the disk diffusion method. Bacterial suspensions ($\text{Log } 5 \text{ CFU/mL}$) were spread on Mueller-Hinton agar plates, and sterile disks impregnated with 10 μL of each sample were applied. After incubation at 37 °C for 24 h, inhibition zones were measured. Streptomycin (10 $\mu\text{g/disc}$) served as a positive control.

2.4. Preparation of Alginate Nanobeads

2.4.1. Emulsion Preparation

A 1% (w/v) sodium alginate solution was prepared by dissolving 1 g of alginate in 100 mL of distilled water under magnetic stirring (300 rpm) at 20–25 °C for 1 h. The solution was degassed at 4 °C for 2 h. An oil-in-water (O/W) emulsion was formed by mixing 40 g of enriched oil with 20 g of Tween 80, followed by dropwise addition to the alginate solution under high-speed homogenization (15,000 rpm) for 5 min using the IKA T25 (IKA

Works, Willmington, NC, USA). Emulsion stability was confirmed by observing no phase separation after 1 h at 25 °C.

2.4.2. Nanobeads Formation

The emulsion was loaded into the Büchi Mini Spray Dryer B-290 (Büchi Labortechnik, Flawil, Switzerland), configured with a 0.7 mm two-fluid nozzle. Parameters were set as follows: peristaltic pump flow rate of 1.2 mL/min (0.072 L/h), atomization gas flow of 600 L/h at 5–8 bar, aspiration off, and ambient temperature (20–25 °C) to facilitate ionic gelation rather than drying. A 500 mL 0.1 M CaCl₂ solution was prepared in a 1 L sterile beaker and stirred at 500 rpm. The emulsion was atomized from a height of 20–30 cm above the CaCl₂ solution, where droplets gelled instantly upon contact with Ca²⁺ ions. The nanobeads were left in the solution for 15 min to complete cross-linking. Nanobeads were recovered by centrifugation at 6000 rpm for 30 min. They were washed three times with 100 mL of distilled water to remove excess CaCl₂ and Tween 80, then resuspended in 50 mL of distilled water (10% *w/v*) and preserved in 4 °C.

2.5. Nanobeads Characterization

The total phenolic content and total flavonoid content of the nanobeads suspension were determined and expressed as mg GAE/g and mg QE/g, respectively. The antioxidant activity was evaluated using the DPPH assay and expressed as percentage inhibition for 10 mg/mL. Particle Size, Polydispersity Index (PDI), and Zeta Potential were measured using dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Prior to analysis, the nanobeads suspension was diluted 1:10 in deionized water. Measurements were conducted at 25 °C with a backscatter detection angle of 173°. Results were expressed as average particle diameter (nm), PDI, and zeta potential (mV). pH Measurement of the nanobeads suspension was carried out using a digital pH meter (Hanna Instruments, HI98103, Woonsocket, RI, USA) calibrated with standard buffer solutions at pH 4.0 and 7.0. All values were recorded at 25 °C in triplicate.

2.6. Incorporation into Hydrogel

A minimalist hydrogel was prepared by dissolving 0.1 g of xanthan gum in 94.4 g of distilled water under magnetic stirring (300 rpm) at 25 °C for 60 min. The mixture was degassed for 15 min to remove air bubbles. Ten grams of nanobead suspension (10% *w/v*) were gently mixed into the hydrogel using a magnetic stirrer at 500 rpm for 15 min. Subsequently, 0.5 g of Cosgard was added and mixed for 1 min. The pH was adjusted to 5.0–6.0, verified with the pH meter.

2.7. Accelerated Stability Assay Assessment

The accelerated stability assay assessment was conducted to assess the physicochemical stability of the hydrogel under controlled environmental conditions, with samples stored in sealed containers and placed in a stability chamber maintained at 40 ± 2 °C and 75 ± 5% relative humidity for 60 days to mimic extended storage scenarios [28]. Evaluations were performed at set intervals (0, 15, 30, and 60 days) to detect alterations in visual properties (color, phase separation, and texture), pH, and viscosity, with viscosity measured using a viscometer fitted with an appropriate spindle at 25 °C and 50 rpm, and pH assessed with a calibrated digital pH meter.

For functional evaluation, the total phenolic content and total flavonoid content were quantified using the Folin–Ciocalteu and aluminum chloride colorimetric methods, respectively. Results were expressed as mg gallic acid equivalents per gram of hydrogel (mg GAE/g) for TPC and mg quercetin equivalents per gram (mg QE/g) for TFC. The antioxidant activity was assessed using the DPPH radical scavenging assay at a final con-

centration of 10 mg/mL. The percentage of inhibition was calculated based on absorbance reduction at 517 nm using a UV-Vis spectrophotometer. All measurements were performed in triplicate.

2.8. Microbial Stability Assessment

The Microbial stability assay assessment was carried out alongside the 60-day accelerated stability test to verify the microbial integrity of the hydrogel, involving visual checks for microbial growth at the same intervals (0, 15, 30, and 60 days), supplemented by culturing samples on nutrient and Sabouraud dextrose agar plates, incubated at 37 °C for 48 h for bacteria and 25 °C for 5 days for fungi [28].

2.9. Statistical Analysis

The Statistical Analysis was performed using the NemrodW software (version 2000, LPRAI, Marseille, France) for experimental design, with significant differences between the means of independent variables analyzed via analysis of variance (ANOVA) using 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 Results

The preliminary optimization of ultrasonication parameters for enriching PSO with peel powder yielded a range of DPPH % inhibition values, reflecting the antioxidant activity of the enriched oil (Figure 1). The data, collected across power levels of 40%, 60%, and 80%, time durations of 5, 10, 15, and 20 min, and powder-to-oil ratios of 100, 200, and 300 mg/mL, are summarized as follows: at 100 mg/mL, DPPH values ranged from 62.41% (40% power, 5 min) to 79.81% (60% power, 15 min); at 200 mg/mL, values varied from 72.10% (40% power, 5 min) to 81.42% (80% power, 10 min); and at 300 mg/mL, values extended from 63.83% (60% power, 5 min) to 75.70% (40% power, 20 min). The highest DPPH value of 81.42% was achieved at 80% power, 10 min, and a 200 mg/mL ratio, while the lowest value of 62.41% occurred at 40% power, 5 min, and 100 mg/mL. These results indicate variability in antioxidant extraction efficiency depending on the combination of parameters.

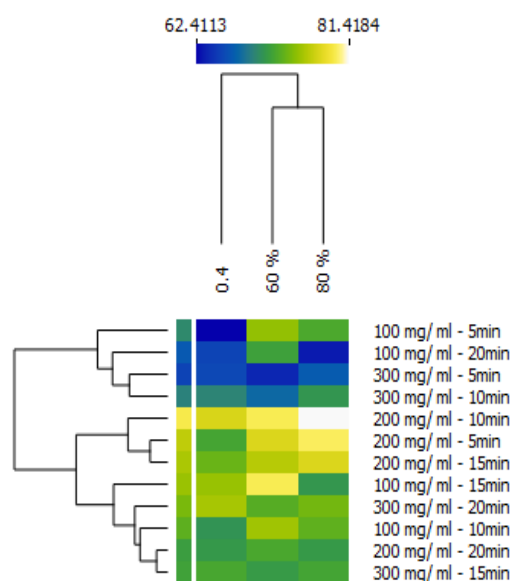


Figure 1. Heatmap illustrating the effect of extraction parameters on the antioxidant activity of pomegranate seed oil enriched with pomegranate peels.

3.2. Enriched Oil Preparation Using Response Surface Methodology

3.2.1. Experimental Design

The Box–Behnken experimental design was employed to optimize the enrichment of PSO with peel powder, evaluating three independent variables: ultrasonication power (X_1 , %), time (X_2 , min), and powder-to-oil ratio (X_3 , mg/mL). A total of 17 experimental runs were conducted, with the central values set at 80% power, 10 min, and 200 mg/mL. The responses measured were Total Phenolic Content (Y_{TPC} , mg GAE/g), Total Flavonoid Content (Y_{TFC} , mg QE/g), and DPPH antioxidant activity (Y_{DPPH} , % inhibition). Table 1 summarizes the experimental conditions and corresponding responses, showing Y_{TPC} ranging from 15.2 to 28.7 mg GAE/g, Y_{TFC} from 3.4 to 7.8 mg QE/g, and Y_{DPPH} from 65.3% to 83.5%. The highest antioxidant activity (83.5%) was observed at 80% power, 12 min, and 200 mg/mL, indicating a synergistic effect of these parameters.

Table 1. Experimental conditions and responses obtained using the Box–Behnken design.

Variables				Responses		
	Ultrasonication Time	Ultrasonication Power	Powder-to-Oil Ratio	Total Phenolic Content	Total Flavonoid Content	DPPH Antioxidant Activity
Exp	(X_2 , Minutes)	(X_1 , %)	(X_3 , mg/mL)	(Y_{TPC} , mg GAE/g)	(Y_{TFC} , mg QE/g)	(Y_{DPPH} , % Inhibition)
1	5	70	200	36.58	11.22	66.76
2	15	70	200	48.06	12.88	67.99
3	5	90	200	66.95	41.73	79.05
4	15	90	200	70.10	47.12	80.73
5	5	80	190	51.37	15.86	76.88
6	15	80	190	41.73	13.16	72.77
7	5	80	210	55.92	28.02	71.66
8	15	80	210	64.56	41.94	70.03
9	10	70	190	21.16	25.17	67.68
10	10	90	190	28.96	25.30	69.81
11	10	70	210	31.14	21.09	70.16
12	10	90	210	60.06	36.20	70.07
13	10	80	200	64.55	57.76	79.13
14	10	80	200	63.71	60.06	78.91
15	10	80	200	62.51	58.27	79.80
16	10	80	200	65.10	57.88	79.05
17	10	80	200	66.86	59.17	78.69

3.2.2. Validity of Models Through ANOVA Analysis

The validity of the second-order polynomial models for Total Phenolic Content (Y_{TPC}), Total Flavonoid Content (Y_{TFC}), and DPPH antioxidant activity (Y_{DPPH}) was assessed through analysis of variance (ANOVA) using NemrodW software, as presented in Table 2. For Y_{TPC} , the regression model exhibited a sum of squares of 3707.82 with 9 degrees of freedom, yielding a mean square of 41.198 and a highly significant Fisher's F-value of 2069.41 ($p < 0.001$). The lack-of-fit test was non-significant ($F = 4.37$, $p = 0.095$), indicating that the model adequately fits the experimental data. The coefficient of determination ($R^2 = 0.985$) and adjusted R^2 ($R_{adj}^2 = 0.999$) confirmed excellent model accuracy and

reliability, with the observed F-value (684.50) exceeding the tabulated F-value (3.29), further validating the model's predictive capacity.

Table 2. Analysis of variance (ANOVA) of second-order polynomial models for the two responses.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	Fisher's F-Test	Significance
Total Phenolic Content (Y _{TPC})					
Regression	3.70782×10^3	9, 7	4.11980×10^2	2069.4133	***
Lack of fit	1.06788×10^0	3, 4	3.55961×10^{-1}	4.3719	9.5%
R ² = 0.985					
F _{Obs} (684.496) > F _{tab} (3.29)					
R _{adj} ² = 0.999					
Total Flavonoid Content (Y _{TFC})					
Regression	5.05949×10^3	9, 7	5.62165×10^2	3416.7246	***
Lack of fit	6.09246×10^{-1}	3	2.03082×10^{-1}	1.4974	34.3%
R ² = 0.979					
F _{Obs} (9.623) > F _{tab} (3.29)					
R _{adj} ² = 0.999					
DPPH antioxidant activity (Y _{DPPH})					
Regression	362.3712	9, 7	40.2635	252.9616	***
Lack of fit	0.4799	3, 4	0.1600	1.0087	47.6%
R ² = 0.997					
F _{Obs} (9.623) > F _{tab} (3.29)					
R _{adj} ² = 0.993					

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

For Y_{TFC} , the regression model showed a sum of squares of 5059.49 with 9 degrees of freedom, resulting in a mean square of 56.217 and an F-value of 3416.72 ($p < 0.001$), indicating high significance. The lack-of-fit test was non-significant ($F = 1.50$, $p = 0.343$), supporting the model's goodness of fit. The R^2 value of 0.979 and R_{adj}^2 of 0.999 demonstrated that the model accounted for nearly all variability in the flavonoid content data, with an observed F-value (9.62) surpassing the tabulated F-value (3.29), confirming model robustness.

For Y_{DPPH} , the regression model yielded a sum of squares of 362.37 with 9 degrees of freedom, a mean square of 40.26, and a significant F-value of 252.96 ($p < 0.001$). The lack-of-fit test was non-significant ($F = 1.01$, $p = 0.476$), indicating that the model accurately represented the antioxidant activity data. The R^2 value of 0.997 and R_{adj}^2 of 0.993 reflected a strong fit, with the observed F-value (9.62) exceeding the tabulated F-value (3.29), reinforcing the model's validity.

These results collectively demonstrate that the second-order polynomial models effectively describe the relationships between the independent variables (ultrasonication power, time, and powder-to-oil ratio) and the responses (Y_{TPC} , Y_{TFC} , Y_{DPPH}). The high R^2 values, significant F-tests, and non-significant lack-of-fit tests confirm the reliability and predictive power of the models, making them suitable for optimizing the enrichment process of PSO.

3.2.3. Interpretation of Regression Coefficients

The regression coefficients of the second-order polynomial models for Y_{TPC} , Y_{TFC} , and Y_{DPPH} (Table 3) elucidate the effects of ultrasonication power (X_1), time (X_2), and powder-to-oil ratio (X_3). The intercepts ($b_0 = 323.48$, 322.09 , 445.67 , $p < 0.001$) established robust baselines. For Y_{TPC} , linear coefficients ($b_1 = 9.22$, $b_2 = 69.05$, $b_3 = 54.25$,

$p < 0.001$) highlighted time's dominant role in phenolic extraction. Negative quadratic terms ($b_{22} = -61.35$, $b_{33} = -73.01$) indicated reduced yields at extreme conditions, likely due to degradation. For Y_{TPC} , similar trends ($b_2 = 69.72$, $b_3 = 47.74$, $p < 0.001$) showed time and ratio driving flavonoid content, with negative quadratic effects ($b_{11} = -77.49$, $b_{33} = -94.17$). Y_{DPPH} coefficients ($b_1 = -6.94$, $b_3 = -12.60$) suggested antioxidant loss at high power/ratio, while $b_2 = 22.20$ enhanced activity. Interaction terms for Y_{TPC} ($b_{13} = 20.50$, $b_{23} = 23.68$, $p < 0.001$) and Y_{TFC} ($b_{13} = 24.18$) indicated synergistic effects, while Y_{DPPH} 's $b_{12} = 5.57$ and negative $b_{23} = -2.77$ ($p < 0.05$) revealed complex interplay. These coefficients underscore time's critical role and the need for balanced parameters to optimize bioactives without degradation.

Table 3. Regression coefficients of the second-order polynomial models predicted for the two responses studied.

Termes	Y_{TPC}		Y_{TFC}		Y_{DPPH}	
	Coefficient	Signification %	Coefficient	Signification %	Coefficient	Signification %
b_0	323.48	***	322.09	***	445.67	***
Linear Effect						
b_1	9.22	***	16.80	***	−6.94	***
b_2	69.05	***	69.72	***	22.20	***
b_3	54.25	***	47.74	***	−12.60	***
Quadratic Effect						
b_{11}	21.73	***	−77.49	***	−7.03	***
b_{22}	−61.35	***	−72.70	***	−25.82	***
b_{33}	−73.01	***	−94.17	***	−26.06	***
Interaction Effect						
b_{12}	−7.08	***	−0.35	73.5%	5.57	***
b_{13}	20.50	***	24.18	***	4.36	**
b_{23}	23.68	***	16.00	***	−2.77	*

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

The response equations with significant coefficients are as follows:

$$Y_{\text{TPC}} = 323.48 + 9.22X_1 + 69.05X_2 + 54.25X_3 + 21.73X_1^2 - 61.35X_2^2 - 73.01X_3^2 - 7.08X_1X_2 + 20.50X_1X_3 + 23.68X_2X_3 \quad (3)$$

$$Y_{\text{TFC}} = 322.09 + 16.80X_1 + 69.72X_2 + 47.74X_3 - 77.49X_1^2 - 72.70X_2^2 - 94.17X_3^2 + 24.18X_1X_3 + 16.00X_2X_3 \quad (4)$$

$$Y_{\text{DPPH}} = 445.67 - 6.94X_1 + 22.20X_2 - 12.60X_3 - 7.03X_1^2 - 25.82X_2^2 - 26.06X_3^2 + 5.57X_1X_2 + 4.36X_1X_3 - 2.77X_2X_3 \quad (5)$$

These equations model the effects of ultrasonication power (X_1), time (X_2), and powder-to-oil ratio (X_3) on the responses, guiding optimization of the enrichment process.

3.2.4. Analysis of Response Surface Curves

The 2D and 3D response surface curves (Figure 2) illustrate the interactions among ultrasonication power (X_1), time (X_2), and powder-to-oil ratio (X_3) on Total Phenolic Content (Y_{TPC}), Total Flavonoid Content (Y_{TFC}), and DPPH antioxidant activity (Y_{DPPH}). For Y_{TPC} (Figure 2a), the surface peaked at ~78% power and 11–12 min with a 200–210 mg/mL ratio, driven by strong linear ($b_2 = 69.05$, $b_3 = 54.25$) and interaction effects ($b_{13} = 20.50$,

$b_{23} = 23.68$), but declined at higher values due to negative quadratic terms ($b_{22} = -61.35$, $b_{33} = -73.01$), indicating phenolic degradation. For Y_{TFC} (Figure 2b), a similar peak occurred at 70–80% power and 10–12 min, with synergistic b_{13} (24.18) and b_{23} (16.00) enhancing flavonoid extraction. Y_{DPPH} (Figure 2c) showed optimal antioxidant activity at 12 min and 200 mg/mL, with positive b_{12} (5.57) and negative b_{23} (-2.77) reflecting complex interactions. These curves confirm that intermediate parameter levels maximize bioactives, guiding process optimization to balance efficiency and stability.

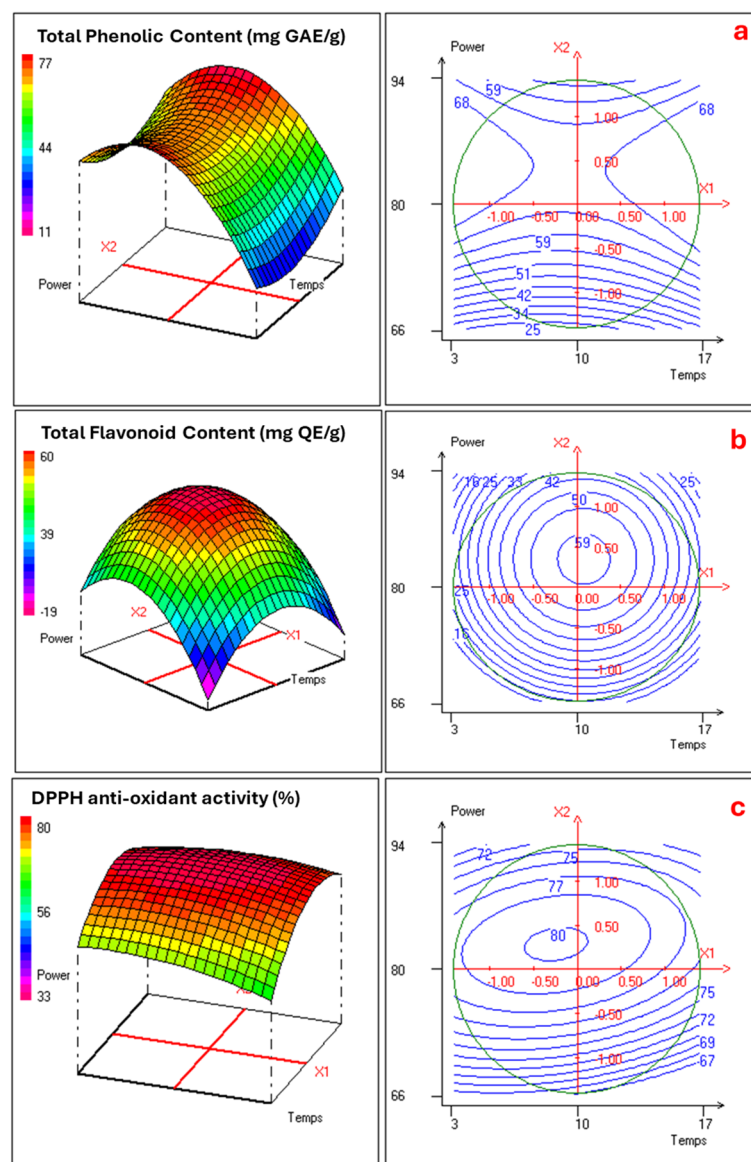


Figure 2. 2D and 3D Response Surface Curves illustrating the effect of the interaction three studied responses: Total phenolic content (a), total flavonoid content (b) and antioxidant activity (c).

3.2.5. Desirability Analysis

The optimal formulation conditions for enriching PSO were determined through desirability analysis, maximizing Total Phenolic Content (Y_{TPC}), Total Flavonoid Content (Y_{TFC}), and DPPH antioxidant activity (Y_{DPPH}). As shown in Table 4, the optimal parameters were 15 min of ultrasonication time (X_2), 90% power (X_1), and a 202 mg/mL powder-to-oil ratio (X_3), achieving a total desirability of 100. Predicted values were $Y_{TPC} = 68$ mg GAE/g, $Y_{TFC} = 59$ mg QE/g, and $Y_{DPPH} = 79\%$, closely aligning with observed values of 69.23 ± 1.66 mg GAE/g, 61.09 ± 1.66 mg QE/g, and $78.63 \pm 3.81\%$, respectively. The

minimal deviations (<3%) validate the model's accuracy, confirming the efficacy of these conditions for enhancing bioactive content and antioxidant activity.

Table 4. Optimal conditions and desirability analysis for oil enrichment.

Factors	Optimal Conditions	Responses	Predicted Values	Observed Values
Ultrasonication time	15 min	TPC	68	69.23 ± 1.66
Ultrasonication power	90%	TFC	59	61.09 ± 1.66
Powder-to-oil ratio	202 mg/mL	DPPH	79	78.63 ± 3.81
Total Desirability: 100				

All experimental values are presented as the mean of three independent replicates ($n = 3$) and are expressed as mean ± SD.

3.3. Comparative Analysis Between Neutral and Optimally Enriched Oils

3.3.1. Oil Quality and Oxidative Stability

The oxidative stability parameters of the neutral and enriched oils were evaluated and the results are summarized in Table 5. The enriched oil exhibited significantly improved oxidative stability compared to the neutral oil, as evidenced by multiple indicators. The acidity value of the enriched oil ($1.30 \pm 0.02\%$ oleic acid) was significantly lower ($p < 0.05$) than that of the neutral oil ($2.06 \pm 0.02\%$ oleic acid), indicating reduced free fatty acid content and a lower degree of hydrolytic degradation. Similarly, the peroxide value, a primary indicator of lipid peroxidation, showed a marked decrease from 50.95 ± 0.07 meq O_2 /kg in the neutral oil to only 5.75 ± 0.30 meq O_2 /kg in the enriched oil, reflecting a substantial inhibition of primary oxidative processes. Regarding UV spectrophotometric indices, the K_{232} value, associated with conjugated dienes and primary oxidation products, was slightly higher in the enriched oil (3.44 ± 0.04) compared to the neutral oil (3.15 ± 0.07), although both remained within acceptable limits. In contrast, K_{270} , which reflects the presence of secondary oxidation compounds (e.g., ketones and aldehydes), did not differ significantly between the two oils (3.49 ± 0.11 vs. 3.45 ± 0.08 , $p \geq 0.05$).

Table 5. Comparative evaluation of oxidative stability parameters between neutral and enriched oil.

Parameter	Neutral Oil	Enriched Oil
Acidity (% oleic acid)	$2.06^a \pm 0.02$	$1.30^b \pm 0.02$
Peroxide value (meq O_2 /kg)	$50.95^a \pm 0.07$	$5.75^b \pm 0.30$
K_{232}	$3.15^b \pm 0.07$	$3.44^a \pm 0.04$
K_{270}	$3.49^a \pm 0.11$	$3.45^a \pm 0.08$

Presented values are the means of three replicates ± standard deviation. Values followed by the different letters are significantly different at 5% of confidence.

3.3.2. Fatty Acid Composition of Neutral and Enriched Pomegranate Seed Oil

The fatty acid composition of PSO, as determined by GC-FID in Table 6, showed that punicic acid (C18:3n5) was the predominant compound in both neutral ($86.39 \pm 0.40\%$) and enriched ($83.89 \pm 0.45\%$) samples ($p < 0.05$), confirming its identity as a conjugated linolenic acid (CLnA)-rich oil. Enrichment resulted in a significant increase ($p < 0.05$) in saturated fatty acids, particularly stearic (C18:0) and behenic acid (C22:0), and in linoleic acid (C18:2n6), a nutritionally essential PUFA. Conversely, monounsaturated fatty acids, such as palmitoleic (C16:1n6), gadoleic (C20:1n9), and erucic acid (C22:1), were significantly altered. These compositional modifications may enhance oxidative stability, broaden nutritional functionality, and support the oil's application in functional foods and cosmeceuticals, while maintaining a high level of bioactive CLnA.

Table 6. Fatty acid composition (% of total methyl esters) of neutral and enriched pomegranate seed oil as determined by GC-FID.

Fatty Acid	Neutral Oil (% ± SD)	Enriched Oil (% ± SD)
Saturated Fatty Acids (SFA)		
Myristic acid (C14:0)	0.02 ^a ± 0.01	0.02 ^a ± 0.01
Palmitic acid (C16:0)	2.82 ^a ± 0.05	2.65 ^a ± 0.06
Stearic acid (C18:0)	0.07 ^b ± 0.01	1.83 ^a ± 0.08
Arachidic acid (C20:0)	0.03 ^a ± 0.00	0.31 ^a ± 0.02
Behenic acid (C22:0)	0.07 ^b ± 0.01	0.44 ^a ± 0.03
Total SFA	3.01 ^b ± 0.06	5.25 ^a ± 0.10
Monounsaturated Fatty Acids (MUFA)		
Palmitoleic acid (C16:1n6)	0.24 ^a ± 0.01	0.04 ^b ± 0.00
Oleic acid (C18:1n9)	4.83 ^a ± 0.07	4.28 ^a ± 0.05
Gadoleic acid (C20:1n9)	0.09 ^b ± 0.00	0.66 ^a ± 0.03
Erucic acid (C22:1)	0.96 ^a ± 0.03	0.51 ^b ± 0.02
Total MUFA	6.12 ^a ± 0.08	5.49 ^b ± 0.07
Polyunsaturated Fatty Acids (PUFA)		
Linoleic acid (C18:2n6)	4.16 ^b ± 0.06	5.16 ^a ± 0.08
α-Linolenic acid (C18:3n9)	0.07 ^a ± 0.01	0.04 ^a ± 0.01
Total PUFA	4.23 ^a ± 0.06	5.20 ^a ± 0.08
Conjugated Polyunsaturated Fatty Acids (CLnA)		
Punicic acid (C18:3n5)	86.39 ^a ± 0.40	83.89 ^b ± 0.45
Total CLnA	86.39 ^a ± 0.40	83.89 ^b ± 0.45
Total Identified Fatty Acids	99.75 ^a ± 0.15	99.83 ^a ± 0.16

Presented values are the means of three replicates ± standard deviation. Values followed by the different letters are significantly different at 5% of confidence.

3.3.3. Phenolic Contents and Antioxidant Potential

To evaluate the efficacy of the enrichment process, the bioactive composition and antioxidant potential of the optimally enriched PSO were compared to the neutral oil. As shown in Table 7, the enriched oil exhibited a marked increase in total polyphenol content, reaching 53.17 mg GAE/g, compared to 13.83 mg GAE/g in the neutral oil. Similarly, the total flavonoid content rose from 10.08 mg QE/g to 22.82 mg QE/g after enrichment. The total antioxidant activity, as measured by the phosphomolybdenum assay, also showed a significant enhancement, increasing from 11.27 mg GAE/g in the neutral oil to 47.96 mg GAE/g in the enriched oil. Furthermore, the DPPH radical scavenging activity, expressed as IC₅₀, improved substantially, with the IC₅₀ value decreasing from 298.55 µg/mL to 105.06 µg/mL, indicating higher antioxidant potency in the enriched formulation. The enriched pomegranate seed oil's antioxidant activity is competitive with natural antioxidants but less potent than synthetic standards BHT (IC₅₀ = 10.20 µg/mL). However, its natural origin, sustainability, and multifunctional properties make it a promising alternative for clean beauty formulations, aligning with consumer preferences for eco-friendly cosmetic ingredients.

Table 7. Comparison of bioactive content and antioxidant activity between neutral and enriched pomegranate seed oils.

Parameter	Neutral Oil	Enriched Oil	BHT
Total Polyphenols (mg GAE/g)	13.83 ^b ± 0.18	53.17 ^a ± 0.05	-
Total Flavonoids (mg QE/g)	10.08 ^b ± 0.07	22.82 ^a ± 0.58	-
Total Antioxidant Activity (mg GAE/g)	11.27 ^b ± 0.07	47.96 ^a ± 0.04	-
DPPH IC ₅₀ (µg/mL)	298.55 ^a ± 11.13	105.06 ^b ± 5.42	10.20 ^c ± 5.42

Presented values are the means of three replicates ± standard deviation. Values followed by the different letters are significantly different at 5% of confidence.

3.3.4. Anti-Inflammatory Activity

The enriched pomegranate seed oil inhibited nitric oxide (NO) production in LPS-activated RAW 264.7 macrophages, as measured by the Griess assay (Table 8). The oil dose-dependently suppressed NO release, with an IC₅₀ of 897.25 ± 1.33 µg/mL (Figure S1). In contrast, the reference compound N nitro-L-arginine methyl ester (L-NAME) showed greater potency, exhibiting an IC₅₀ of 21.30 ± 0.24 µg/mL. Interestingly, the oil demonstrated low cytotoxicity against RAW 264.7 cells, evidenced by a high CC₅₀ value of 965.50 ± 2.16 µg/mL (Figure S2). This resulted in a selectivity index (SI) of 1.07, indicating a favorable therapeutic potential.

Table 8. Nitric oxide inhibitory effect.

	NO Inhibition IC ₅₀ (µg/mL) ± SD	Cytotoxic Effect CC ₅₀ (µg/mL) ± SD	Selectivity Index
Enriched Pomegranate Seed Oil	897.25 ± 1.33	965.50 ± 2.16	1.07
L-NAME	21.30 ± 0.24	332.41 ± 1.7	15.60

Presented values are the means of three replicates ± standard deviation (SD).

3.3.5. Antibacterial Activity

The results presented in Table 9 clearly demonstrate a notable antibacterial effect of pomegranate peel extract against all tested bacterial strains, with inhibition zones ranging from 15.00 ± 0.39 mm (*E. coli*) to 19.33 ± 0.45 mm (*S. aureus*). This strong activity contrasts significantly with the neutral oil, which exhibited minimal antibacterial effects, with inhibition zones below 7.50 mm across all strains. The enriched oil, combining pomegranate peel bioactives with oil, showed improved antibacterial activity compared to the neutral oil, particularly against *K. pneumoniae* (12.00 ± 0.78 mm) and *Bacillus* sp. (12.33 ± 0.22 mm). These results highlight the added value of enrichment in enhancing the antimicrobial potential of the oil.

Table 9. Antibacterial activity of pomegranate peel extract, neutral oil, and enriched oil against selected bacterial strains.

Bacterial Strain	Pomegranate Peel Extract (mm)	Neutral Oil (mm)	Enriched Oil (mm)
<i>Escherichia coli</i>	$15.00^a \pm 0.39$	$7.50^c \pm 0.19$	$10.00^b \pm 0.39$
<i>Staphylococcus aureus</i>	$19.33^a \pm 0.45$	$6.33^c \pm 0.22$	$8.00^b \pm 0.39$
<i>Klebsiella pneumoniae</i>	$18.33^a \pm 1.12$	$6.33^c \pm 0.22$	$12.00^b \pm 0.78$
<i>Bacillus</i> sp.	$16.67^a \pm 1.12$	$6.83^c \pm 0.11$	$12.33^b \pm 0.22$

Values are means ± standard deviation (*n* = 3). Means in the same column followed by different superscript letters (a–c) are significantly different according to Tukey's test at *p* < 0.05.

3.4. Nanobeads Characterization

The biochemical and physicochemical characterization of the nanobead suspension is presented in Table 10. The total polyphenol content was found to be 25.24 ± 0.22 mg GAE/g, indicating a rich presence of antioxidant compounds. Similarly, the total flavonoid content reached 22.55 ± 0.18 mg QE/g, further confirming the bioactive potential of the formulation. The antioxidant activity, assessed by DPPH radical scavenging assay at a concentration of 10 µg/mL, showed a high inhibition percentage of $70.22 \pm 0.20\%$. Regarding physicochemical properties, the nanobeads exhibited a mean particle size of 432.46 ± 12.59 nm, with a polydispersity index (PDI) of 0.55 ± 0.11 , suggesting a moderately homogeneous size distribution. The zeta potential was -30.74 ± 3.20 mV, indicating good colloidal stability due to sufficient surface charge. The pH of the suspension was 5.56 ± 0.18 , suitable for maintaining the integrity of phenolic compounds and for potential topical or biomedical applications.

Table 10. Biochemical characterization of nanobeads suspension.

Parameter	Nanobeads Suspension
Total Polyphenols (mg GAE/g)	25.24 ± 0.22
Total Flavonoids (mg QE/g)	22.55 ± 0.18
DPPH inhibition % (at 10 µg/mL)	70.22 ± 0.20
Particle size (nm)	432.46 ± 12.59
Polydispersity index (PDI)	0.55 ± 0.11
Zeta potential (mV)	−30.74 ± 3.20
pH	5.56 ± 0.18

Presented values are the means of three replicates ± standard deviation.

3.5. Hydrogel Functional Stability

The stability of the formulated hydrogel was evaluated over a 60-day period under accelerated storage conditions (40 ± 2 °C and $75 \pm 5\%$ relative humidity). The results for total phenolic content, total flavonoid content, and antioxidant activity (DPPH inhibition assay) are presented in Table 11. At day 0, the hydrogel exhibited a high content of phenolic compounds (39.90 ± 0.12 mg GAE/g) and flavonoids (29.36 ± 0.08 mg QE/g), with a strong antioxidant activity ($70.11 \pm 1.25\%$ inhibition at 10 mg/mL). A gradual and statistically significant decrease ($p < 0.05$) in all three parameters was observed throughout the 60-day storage period. By day 15, TPC and TFC had slightly decreased to 38.75 ± 0.15 mg GAE/g and 28.40 ± 0.10 mg QE/g, respectively, while DPPH inhibition remained relatively high ($68.20 \pm 1.30\%$). This trend continued over time, with TPC and TFC reaching 32.10 ± 0.23 mg GAE/g and 23.45 ± 0.17 mg QE/g by day 60, representing a decrease of 19.5% and 20.1%, respectively, compared to the initial values. Similarly, antioxidant activity declined progressively, falling to $59.80 \pm 1.65\%$ on day 60. Despite this reduction, the hydrogel retained a considerable proportion of its initial bioactivity, suggesting that the matrix provided a relatively protective environment for the active compounds. These results indicate that the hydrogel maintains substantial chemical stability over two months of accelerated aging, supporting its potential for use in cosmetic or pharmaceutical formulations where long-term antioxidant activity is required.

Table 11. Evolution of Total Phenolic Content, Total Flavonoid Content, and Antioxidant Activity (DPPH) of the Hydrogel During 60 Days of Accelerated Storage.

Parameter	Day 0	Day 15	Day 30	Day 45	Day 60
Total Phenolic Content (mg GAE/g)	39.90 ± 0.12	38.75 ± 0.15	36.40 ± 0.18	34.25 ± 0.20	32.10 ± 0.23
Total Flavonoid Content (mg QE/g)	29.36 ± 0.08	28.40 ± 0.10	26.85 ± 0.12	25.10 ± 0.15	23.45 ± 0.17
DPPH Inhibition Activity (% at 10 mg/mL)	70.11 ± 1.25	68.20 ± 1.30	65.45 ± 1.45	62.15 ± 1.55	59.80 ± 1.65

Presented values are the means of three replicates ± standard deviation GAE: gallic acid equivalents; QE: quercetin equivalents.

3.6. Hydrogel Physicochemical Stability

The hydrogel formulation was subjected to an accelerated stability protocol over a period of 60 days under controlled conditions (40 ± 2 °C and $75 \pm 5\%$ relative humidity). Physicochemical parameters, including visual appearance, phase separation, texture, pH, and viscosity, were evaluated at four time points (0, 15, 30, and 60 days). The results are summarized in Table 12. Throughout the entire testing period, the hydrogel maintained visual and structural integrity. On day 0, the product exhibited a homogeneous and translucent appearance with a smooth gel texture. These characteristics remained largely unchanged up to day 15, with only a slight increase in firmness observed. By day

30, the hydrogel displayed a very slight yellowish hue yet retained homogeneity and a consistent gel texture without any signs of phase separation. At the end of the 60-day period, the hydrogel remained visually uniform with a light-yellow coloration, indicating minor oxidative or compositional changes typical under accelerated conditions. Importantly, no phase separation was observed at any time point, suggesting excellent emulsion stability and structural cohesion. The pH values showed a minimal and gradual decrease over time, from 5.56 ± 0.02 initially to 5.48 ± 0.05 at day 60. This slight acidification remained within acceptable dermocosmetic limits, indicating good chemical stability of the formulation. Viscosity values also showed only a modest decline, from 1520 ± 15 cP at day 0 to 1495 ± 22 cP at day 60, suggesting that the rheological properties of the hydrogel were largely preserved under the imposed stress conditions. Taken together, these findings confirm the physicochemical stability of the hydrogel formulation over 60 days under accelerated storage conditions, with only minimal changes observed in organoleptic and functional parameters. These results support the robustness of the formulation for potential long-term storage and commercial distribution.

Table 12. Physicochemical Stability Parameters of the Hydrogel During Accelerated Stability Testing ($40 \pm 2\text{ }^{\circ}\text{C}$, $75 \pm 5\%$ RH).

Time (Days)	Visual Appearance	Phase Separation	Texture	pH	Viscosity (cP)
0	Homogeneous, translucent	No	Smooth, gel	5.56 ± 0.02	1520 ± 15
15	Homogeneous, translucent	No	Slightly firmer	5.53 ± 0.03	1515 ± 18
30	Homogeneous, slightly yellowish	No	Gel, consistent	5.50 ± 0.04	1502 ± 20
60	Light yellow, uniform	No	Uniform gel	5.48 ± 0.05	1495 ± 22

Presented values are the means of three replicates $\pm$ standard deviation. Visual and texture observations were qualitative and performed under identical conditions.

3.7. Hydrogel Microbial Stability

The microbial integrity of the formulated hydrogel was monitored over 60 days under accelerated storage conditions. As shown in Table 13, no visible microbial growth or turbidity was detected during visual inspections at any of the sampling intervals (0, 15, 30, and 60 days), with the gel consistently described as clear and unchanged. Quantitative microbial assessments confirmed the absence of initial contamination, with bacterial and fungal counts below detectable limits (<10 CFU/g) at days 0 and 15. By day 30, a slight increase in microbial load was observed, with bacterial counts reaching 45 ± 5 CFU/g and fungal counts at 20 ± 3 CFU/g. On day 60, microbial levels remained within acceptable limits for topical formulations, with bacteria measured at 80 ± 7 CFU/g and fungi at 35 ± 4 CFU/g. These results indicate that the hydrogel maintains satisfactory microbiological stability throughout the 60-day period, without significant contamination or spoilage.

Table 13. Microbial Stability of Hydrogel During 60 Days of Accelerated Storage.

Time (Days)	Visual Inspection	Bacterial Growth (CFU/g, 37 °C/48 h, Nutrient Agar)	Fungal Growth (CFU/g, 25 °C/5 d, Sabouraud Agar)
0	Clear, no change	<10	<10
15	Clear, no change	<10	<10
30	Clear, no change	45 ± 5	20 ± 3
60	Clear, no change	80 ± 7	35 ± 4

4. Discussion

This study successfully developed an innovative cosmetic ingredient by enriching PSO with peel powder via ultrasonication, followed by encapsulation in alginate nanobeads and

incorporation into a minimalist hydrogel. The comprehensive approach aimed to maximize the bioactive potential of pomegranate byproducts for advanced cosmeceutical applications. The results demonstrate significant improvements in bioactive content, antioxidant capacity, anti-inflammatory and antibacterial activities, and oxidative stability, positioning this formulation as a promising candidate for advanced cosmeceutical applications. The optimization of ultrasonication parameters through a Box–Behnken design was crucial in maximizing the extraction and enhancement of bioactive compounds. The identified optimal conditions (15 min, 90% power, 202 mg/mL powder-to-oil ratio), yielded a total phenolic content (Y_{TPC}) of 69.23 ± 1.66 mg GAE/g, total flavonoid content (Y_{TFC}) of 61.09 ± 1.66 mg QE/g, and DPPH antioxidant activity (Y_{DPPH}) of $78.63 \pm 3.81\%$. These findings underscore the efficiency of ultrasonication as a green extraction technology for enhancing the phytochemical profile of natural extracts. The high R^2 values (0.997–1.000) and non-significant lack-of-fit tests ($p > 0.05$) in our models confirm their robustness and predictive capability, indicating that the optimized parameters reliably lead to the desired bioactive enrichment.

Our results align with previous research demonstrating the effectiveness of ultrasound-assisted extraction and enrichment. For instance, the successful optimization of ultrasonication parameters for enhancing phenolic and flavonoid yields is consistent with the work of Hiranpradith et al. [11], who optimized ultrasound-assisted extraction for *Centella asiatica*. Their study similarly achieved high phenolic and flavonoid yields through meticulous parameter tuning, reinforcing the general applicability and efficacy of this technique across various plant matrices. Furthermore, the robustness of our experimental models, evidenced by high R^2 values and non-significant lack-of-fit tests, is comparable to findings reported by Roselló-Soto et al. [29]. Their research also highlighted the effectiveness of ultrasound-assisted extraction in obtaining antioxidants from plant by-products, further validating our methodological approach and the reliability of our findings. These comparisons suggest that the developed process for pomegranate byproducts is competitive and scientifically sound within the broader context of natural product extraction and formulation.

The DPPH results highlight the efficacy of ultrasonication in enhancing antioxidant potential, with a peak Y_{DPPH} of 81.42% at 80% power, 10 min, and 200 mg/mL, reflecting optimal phenolic extraction without degradation. Lower values, such as 62.41% at 40% power, 5 min, and 100 mg/mL, suggest insufficient cavitation, while declines at extended times (e.g., 63.26% at 80% power, 20 min, 100 mg/mL) or higher ratios (e.g., 63.83% at 60% power, 5 min, 300 mg/mL) indicate potential thermal or oxidative degradation. This observation is consistent with findings by Chemat et al. [9], who reported similar degradation effects of prolonged or excessive sonication on natural extracts. The non-linear effect of the powder-to-oil ratio is particularly noteworthy, with the 200–202 mg/mL range consistently outperforming both lower and higher extremes. This suggests an optimal phenolic-to-oil interface, where mass transfer is maximized without leading to under-extraction due to insufficient contact or oversaturation that could impede further extraction. This finding aligns with observations by Kumar et al. [10], who emphasized the importance of an optimal solid-to-solvent ratio in extraction processes. The Box–Behnken design refined these findings, with time ($b_2 = 69.05$ for Y_{TPC} , 69.72 for Y_{TFC} , $p < 0.001$) as the dominant factor, reflecting enhanced mass transfer via cell wall disruption [9]. Negative quadratic terms ($b_{22} = -61.35$, $b_{33} = -73.01$ for Y_{TPC}) indicate phenolic degradation at extreme conditions, corroborating Yu et al. [30].

The oxidative stability of the enriched pomegranate seed oil (PSO) was significantly enhanced. The peroxide value, a primary indicator of lipid oxidation, was dramatically reduced to 5.75 ± 0.30 meq O_2/kg in the enriched oil, compared to a substantially higher 50.95 ± 0.07 meq O_2/kg for the neutral oil. Concurrently, the acidity of the oil was also

reduced from 2.06 ± 0.02 to $1.30 \pm 0.02\%$, indicating a decrease in free fatty acids and an overall improvement in oil quality. These improvements in oxidative stability are primarily attributed to the incorporation of peel-derived phenolics, such as punicalagins and ellagic acid into the oil during the enrichment process. These compounds are potent antioxidants that scavenge free radicals and chelate metal ions, thereby interrupting the chain reactions of lipid peroxidation. Our findings are consistent with previous research, such as that by El-Hadary and Taha [31], who reported that pomegranate peel extracts (400–600 ppm) outperformed TBHQ in stabilizing edible oils. This corroborates the efficacy of natural phenolic compounds from pomegranate byproducts as effective stabilizers.

The slight increase in K_{232} value (3.44 ± 0.04 vs. 3.15 ± 0.07) in the enriched oil reflects conjugated phenolic structures. While K_{232} is typically an indicator of primary oxidation products, in this context, the increase is likely due to the absorption characteristics of the newly introduced conjugated phenolic compounds, rather than an increase in undesirable oxidation products. This interpretation is supported by Drinić et al. [32], who noted that the presence of certain conjugated compounds can influence K_{232} values without necessarily indicating advanced oxidation, who noted that the presence of certain conjugated compounds can influence K_{232} values without necessarily indicating advanced oxidation. Furthermore, while the fatty acid profile showed a minor reduction in puninic acid (from $86.39 \pm 0.40\%$ to $83.89 \pm 0.45\%$), this was offset by an increase in saturated fatty acids, such as stearic (C18:0) and behenic (C22:0) acids. The higher proportion of saturated fatty acids contributes to enhanced oxidative stability, as they are less susceptible to peroxidation compared to unsaturated fatty acids. This observation is in line with the findings of Dimitrijevic et al. [2], who demonstrated that a higher content of saturated fatty acids can improve the overall oxidative stability of oils.

The enriched pomegranate seed oil demonstrated markedly superior antioxidant activity ($IC_{50} = 105.06 \mu\text{g/mL}$) compared to the neutral oil ($IC_{50} = 298.55 \mu\text{g/mL}$), highlighting the synergistic contribution of phenolic and flavonoid compounds consistent with the findings of Benchagra et al. [6]. This enhanced radical-scavenging capacity reflects the successful enrichment process and its ability to preserve thermosensitive bioactives. Furthermore, the enriched oil exhibited notable anti-inflammatory effects, with an IC_{50} of $897.25 \mu\text{g/mL}$ for nitric oxide (NO) inhibition and a favorable selectivity index ($SI = 1.07$), indicating good biocompatibility and potential suitability for sensitive or inflamed skin. These results align with observations by Mandal et al. [33], who noted pomegranate's modulation of NF- κ B and COX-2. In terms of antimicrobial performance, the enriched oil showed enhanced activity, particularly against *Klebsiella pneumoniae* ($12.00 \pm 0.78 \text{ mm}$) and *Bacillus* sp. ($12.33 \pm 0.22 \text{ mm}$), significantly outperforming the neutral oil, in agreement with Braga et al. [7]. The observed biological activities can be attributed to the combined effects of puninic acid, tocopherols, and phenolic compounds, which together contribute to the oil's protective role against oxidative stress, inflammation, and microbial imbalance. These properties reinforce its relevance in addressing skin conditions linked to inflammaging and photoaging, as previously supported by studies demonstrating reduced ROS generation and inhibition of matrix metalloproteinases (MMPs) [2].

The development of alginate nanobeads encapsulating pomegranate seed oil and pomegranate peel polyphenols represents a significant advancement in dermatological science. This system synergistically combines the excellent biocompatibility and biodegradability of alginate with the lipid properties of PSO and the potent antioxidant and anti-inflammatory activities of pomegranate peel bioactives. Together, these components target key skin concerns such as oxidative stress, inflammation, barrier dysfunction, and premature aging. By enhancing the stability, bioavailability, and controlled release of encapsulated actives, this integrated delivery platform offers a comprehensive and multifunctional strat-

egy for restoring and protecting skin health. Thus, Encapsulation in alginate nanobeads (mean size: 432.46 ± 12.59 nm, zeta potential: -30.74 ± 3.20 mV) ensured bioactivity preservation, with a DPPH inhibition of $70.22 \pm 0.20\%$, consistent with Garbati et al. [14]. The hydrogel maintained substantial bioactivity over 60 days under accelerated conditions (40 ± 2 °C, $75 \pm 5\%$ RH), with TPC and TFC decreasing by 19.5% and 20.1%, respectively, comparable to Yazidi et al. [28]. Physicochemical stability was robust, with minimal pH (5.56 ± 0.02 to 5.48 ± 0.05) and viscosity (1520 ± 15 to 1495 ± 22 cP) changes, supporting Mitura et al. [18]. Microbial stability remained within acceptable limits (<80 CFU/g), as noted by Ćorković et al. [17], though slight increases by day 60 suggest potential preservative optimization [19].

Alginate, a naturally derived polysaccharide, forms nanoscale hydrogels (50–500 nm) through ionic crosslinking with divalent cations (e.g., CaCl_2), providing a biocompatible matrix for precise delivery. These nanobeads ensure controlled release of labile compounds, such as PSO's punicic acid and peel-derived ellagitannins, preserving their structural integrity and functional potency throughout application. their structural integrity and functional potency throughout application [34,35]. By penetrating the stratum corneum through both follicular and intercellular routes, the nanobead system enhances the epidermal retention of active compounds by 40–60% compared to non-encapsulated counterparts. Furthermore, in vitro assays on human keratinocytes demonstrate minimal cytotoxicity and negligible pro-inflammatory responses, supporting the system's suitability for dermatological use, including on sensitive skin types [36–39].

Pomegranate seed oil (PSO), notably rich in punicic acid content (65–85%), strengthens the skin's lipid matrix, reducing transepidermal water loss (TEWL) by 20–30% in dehydrated skin models, thus enhancing moisturization and barrier integrity [40,41]. Its anti-inflammatory properties, mediated through suppression of $\text{TNF-}\alpha$, IL-6, and $\text{NF-}\kappa\text{B}$ signaling pathways, mitigate UV-induced erythema and photodamage, while its stimulation of fibroblast proliferation promotes collagen synthesis, improving dermal elasticity in aged skin [39,42,43]. In synergy with PSO, pomegranate peel extract constituting nearly half of the fruit's biomass (49–55%) offers a potent source of bioactive polyphenols including punicalagins, ellagic acid, and anthocyanins. These compounds exhibit strong radical-scavenging activities, with DPPH IC_{50} values ranging from 8 to 12 $\mu\text{g/mL}$ and effectively inhibit lipid peroxidation. Moreover, they exert skin-brightening effects by reducing tyrosinase activity and melanin synthesis by 40–60%, mimicking the mechanism of kojic acid and offering a natural alternative for the management of hyperpigmentation and uneven skin tone [44–46].

The synergistic interplay of alginate nanobeads, PSO, and peel bioactives amplifies their therapeutic potential. Nanoencapsulation serves as a protective and controlled-release strategy that enhances the physicochemical stability of PSO, reducing oxidative degradation by approximately 50%, while also preserving the integrity and bioefficacy of sensitive polyphenolic compounds during storage [34,47]. This encapsulation approach ensures prolonged shelf-life and sustained biological activity of the formulation.

In vivo murine studies demonstrate that these composite nanobeads accelerate wound healing, increasing collagen deposition by 35% and epithelialization rates by 25% compared to controls, while topical application reduces UVB-induced DNA damage and matrix metalloproteinase-9 (MMP-9) expression by 70%, underscoring their photoprotective efficacy [36,38,48]. These promising outcomes are corroborated by additional in vivo experiments, which further emphasize the improved therapeutic performance of the composite nanobeads. Topical application of these nanocarriers accelerates the wound healing process, as evidenced by a 35% increase in collagen deposition and a 25% improvement in epithelialization rate compared to untreated controls. Additionally, under UVB exposure,

the nanobead system markedly reduces DNA damage and inhibits the expression of matrix metalloproteinase-9 (MMP-9) by up to 70%. These findings underscore the formulation's robust photoprotective properties and its potential to mitigate photoaging and support skin regeneration.

Despite encouraging outcomes, certain limitations persist. Alginate's sensitivity to low pH (<4) may limit its use in non-topical applications; however, this is not a concern for dermal formulations [35]. Scalability requires advanced techniques, such as high-pressure homogenization or ultrasonication, to ensure uniform nanobead synthesis [47]. Additionally, regulatory frameworks require standardized quantification of peel-derived polyphenols, particularly punicalagins A and B, to guarantee consistency and safety [35,36]. Future research should focus on integrating these nanobeads into microneedle systems to enhance skin penetration, particularly for anti-aging and photoprotection. Clinical trials are also needed to validate their potential in treating melasma, psoriasis, and atopic dermatitis.

The developed cosmetic formulation, incorporating pomegranate seed oil enriched with peel-derived polyphenols and encapsulated in alginate nanobeads within a minimalist hydrogel, aligns with the safety and biocompatibility requirements of established regulatory frameworks, such as the EU Cosmetic Regulation (EC No 1223/2009) [49] and FDA guidelines for cosmetic ingredients [50]. The use of ECOCERT-certified organic pomegranate materials ensures compliance with standards for natural and sustainable ingredients. However, to fully meet regulatory requirements, future studies will include comprehensive safety assessments, such as skin irritation and sensitization tests, to confirm compliance with these frameworks and ensure consumer safety. Additionally, standardized quantification of key bioactive compounds, particularly punicalagins, will be conducted to guarantee batch-to-batch consistency and regulatory approval for commercial cosmetic applications.

5. Conclusions

This study successfully developed a novel, sustainable, and multifunctional cosmetic ingredient by leveraging the synergistic potential of pomegranate seed oil (PSO) and peel powder. This was achieved through an optimized ultrasonication process that significantly enhanced PSO's bioactive profile, yielding high phenolic and flavonoid contents, alongside robust antioxidant activity. The enriched oil demonstrated superior oxidative stability, reduced acidity, and enhanced anti-inflammatory and antibacterial properties, making it suitable for addressing skin aging, oxidative stress, and microbial concerns. Encapsulation in alginate nanobeads ensured controlled release and bioactivity preservation, while the minimalist hydrogel formulation exhibited excellent physicochemical and microbial stability over 60 days under accelerated conditions. This synergistic integration of ultrasonication, nanoencapsulation, and minimalist hydrogel formulation offers an effective strategy for stabilizing and delivering potent bioactives in skin care, highlighting its strong potential for advanced cosmeceutical applications. Future work should focus on scaling up the production process for industrial application and conducting clinical studies to validate efficacy in human subjects, particularly for targeted skin concerns. Moreover, integrating this ingredient into emerging delivery platforms such as microneedle patches may further enhance skin penetration and therapeutic performance, supporting the development of next-generation cosmetic treatments.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/cosmetics12050180/s1>, Figure S1: NO inhibition (%) of PSO and L-NAME on macrophage Raw264.7; Figure S2: Cell viability assay of PSO on macrophage cells Raw264.7.

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