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Fenugreek as a Potential Active Ingredient for the Development of Innovative Cosmetic Formulation

Ameni Ben Abdennebi [1], Emna Chaabani [1], Soumaya Bourgou [1], Majdi Hammami [1], Moufida Saidani Tounsi [1], Othmane Merah [2,3,*] and Iness Bettaieb Rebey [1,*]

[1] Laboratory of Aromatic and Medicinal Plants, Borj Cedria Biotechnology Center, BP. 901, Hammam-Lif 2050, Tunisia; benabdennebiameni9@gmail.com (A.B.A.); emna.chaabani@cbbc.rnrt.tn (E.C.); soumaya.bourgou@cbbc.rnrt.tn (S.B); majdi. hammami@cbbc.rnrt.tn (M.H.); moufida.saidani@cbbc.rnrt.tn (M.S.T.)

[2] Laboratoire de Chimie Agro-Industrielle (LCA), Université de Toulouse, INRAE, INPT, 31030 Toulouse, France

[3] Département Génie Biologique, IUT Paul Sabatier, Université Paul Sabatier, 32000 Auch, France

* Correspondence: ines.bettaieb@cbbc.rnrt.tn (I.B.R.); othmane.merah@ensiacet.fr (O.M)

Abstract: This study focuses on optimizing the extraction conditions for antioxidants from the fenugreek seeds (*Trigonella foenum-graecum* L.) through ultrasound-assisted extraction in the aim of creating a stable and effective cosmetic formulation. RSM was used to optimize the extraction parameters to ultrasonic power of 60%, with 50% ethanol concentration for 10 min. Under those conditions, the extract showed a phenolic-rich profile, with a total phenolic content equivalent to 18.56 mg GAE/g DM and a radical scavenging activity of 63.24%. Chromatographic analysis further confirmed the abundance of phenolic compounds, with epicatechin identified as the major compound at a concentration of 22.58 mg/g DM. The extract exhibited considerable antibacterial activity for a number of bacterial strains and it exhibited no cell toxicity on RAW 267.4 cells, supporting its safe use in cosmetic products. The cosmetic formulation maintained high stability, with pH values from 6.25 to 6.35, viscosity values from 7941.69 to 7956.70 cp, and less color change after 90 days preservation under varied temperature conditions. These findings validate fenugreek extract potential for producing a stable, eco-friendly, and effective cosmetic product, thus bringing skin health benefit and driving sustainable extraction methods in the cosmetic industry.

Keywords: *Trigonella foenum graecum* L; ultrasound-assisted extraction; epicatechin; radical scavenging activity; antibacterial activity; cytotoxicity; cosmetic formulation; skin health

1. Introduction

The skin, being the largest organ of the body, serves as a vital protective shield against harmful pathogens and substances, essentially acting as the body's first line of defense [1]. This protective role is shaped by both internal and external factors [2]. External influences include environmental conditions such as UV radiation, wind, humidity, and air conditioning, as well as physical damage and interactions with microorganisms like bacteria, fungi, viruses, and toxins. On the internal side, genetics, certain medications (like immunosuppressants and contraceptives), antibiotics, and diet all play a part. Furthermore, daily use of cosmetics, skincare products, detergents, soaps, and perfumes also has a notable impact on skin health [3]. which can accelerate skin aging through the production of reactive oxygen species (ROS). Oxidative stress, exacerbated by UV exposure, increases the activity of enzymes that degrade skin fibers, such as collagenase and elastase [4], thereby contributing to a loss of elasticity and tensile strength, leading to the appearance of wrinkles and increased dryness [4]. Oxidative stress also plays a key role in inducing inflammation [5], slowing down the cellular renewal of the epidermis and leading to a reduction in its thickness, which weakens the protective

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barrier [6]. UV radiation also triggers the production of free radicals (ROS), responsible for skin dehydration. To minimize skin damage, it is essential to reduce oxidative stress through the use of antioxidants. In recent years, interest in natural substances has significantly increased, particularly due to growing distrust of synthetic products. Many industries, including the cosmetics industry, are now turning towards the incorporation of natural molecules in their formulations, as these offer unique biological and chemical properties [7]. Studies have shown that many plants contain a wide variety of active compounds, such as terpenoids, alkaloids, and phenols [8]. These compounds, by neutralizing free radicals, slow down premature skin aging. Polyphenols, in particular, protect the skin from damage caused by these free radicals, thus reducing the development of wrinkles and visible aging indicators while supporting a radiant and balanced skin tone [9].

Trigonella foenum-graecum L., is an herbaceous plant belonging to the Leguminosae family, primarily cultivated in Western Asia, North Africa, Northern India, and the Mediterranean region [10]. Its seeds are widely used as food, spice, galactagogue, and in traditional medicine for the treatment of diabetes [11]. Furthermore, a toxicological study has confirmed the safety of using fenugreek seeds as a dietary supplement [12]. These seeds contain various phytocompounds such as alkaloids, saponins, and flavonoids (rutin, quercetin, vitexin), as well as galactomannans, which contribute to skin hydration through a humectant effect [13]. Numerous studies have demonstrated the anticancer, antimicrobial, antioxidant, and anti-inflammatory properties of fenugreek [14] Among its active compounds, epicatechin stands out for its ability to protect skin cells from damage caused by free radicals, thereby delaying premature aging and helping to prevent the appearance of wrinkles and fine lines [15]. The extraction of phenolic compounds from fenugreek seeds can be achieved through several techniques. In recent years, ultrasound-assisted extraction (UAE) has gained popularity due to its advantages, including more efficient extraction, minimal solvent use, reduced costs, and low environmental impact. UAE enhances extraction yields by facilitating the diffusion of active compounds through cavitation, a phenomenon where air bubbles rapidly form and collapse under the effect of ultrasound, generating local increases in pressure and temperature [16].

This study focuses on optimizing the ultrasound-assisted extraction (UAE) process for fenugreek seeds using response surface methodology (RSM) to maximize the recovery of phenolic compounds and their antioxidant activity. The application of RSM provides a systematic approach for evaluating and refining key extraction parameters to achieve high yields of bioactive substances with minimal environmental impact. The fenugreek extract obtained under optimal conditions was, formerly, evaluated for its antioxidant, antimicrobial, and anti-inflammatory activities. The extract was then incorporated into a cosmetic cream formulation, where its stability, bioactivity, and sensory properties were thoroughly assessed. By combining advanced extraction techniques with plant-derived bioactive compounds, this study contributes to the development of innovative skincare products that effectively target skin health while prioritizing environmental sustainability. The findings underscore the role of fenugreek extract as a safe and versatile component for next-generation cosmetic formulations.

2. Materials and Methods

2.1. Plant Material

Fenugreek, or *Trigonella foenum-graecum* L. is a plant renowned for its seeds with numerous uses. These seeds were purchased in dried form from spice merchants, particularly from the Beja province.

2.2. Ultrasound-Assisted Recovery of Bioactive Antioxidants

The ultrasound-assisted extraction was conducted using an ultrasonic bath (Sonorex Digital 10 P, Bandelin, GmbH, Germany) with hydro-ethanolic solvent mixture, prepared in varying ratios. Following each extraction, the solution was subjected to centrifugation at 5000 rpm for 10 minutes.

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The supernatant, which contained the extracted antioxidants, was then collected for further analysis [17].

2.3. Design of Experiments and Statistical Analysis

We designed an experimental plan using the NemrodW program to optimize the experimental conditions for extracting antioxidants from fenugreek and increase this extraction using ultrasound. With three independent variables and two responses, this design consists of 19 trials arranged using the Box-Behnken model (Table 1).

This plan consists of 19 experiments with 3 independent variables and 2 responses, arranged using a Box-Behnken model (Table 1). The factors investigated include ultrasonic power (X_1), ethanol % (X_2), and extraction time (X_3). 60%, 50%, and 10 minutes are their respective central values. The variation steps selected are 10% for X_1, 25% for X_2, and 5 minutes for X_3. A second-degree polynomial model was used to analyze the change of DPPH free radical scavenging activity (Y_{PI}) and total phenolic content (Y_{TPC}) with respect to the three variables that were chosen, X_1, X_2, and X_3, as indicated in the equation above:

$$Y_{i=} \beta 0 + \beta 1x1 + \beta 2x2 + \beta 3x3 + \beta 11(x1x1) + \beta 22(x2x2) + \beta 33(x3x3) + \beta 12(x1x2) + \beta 13(x1x3) + \beta 23(x2x3)$$

- Yi denotes the observed response variables
- β0 is a constant
- β1, β2, β3 Linear coefficients for X_1, X_2 ,X_3, respectively.
- $\beta_{1,1}$, $\beta_{2,2}$,$\beta_{3,3}$: Coefficients for the quadratic terms $x1x1$, $x2x2$ and $x3x3$ respectively.
- $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$ Coefficients for the interaction terms $x1x2$, $x1x3$ and $x2x3$ respectively.

ANOVA was conducted with IBM SPSS Statistics (Version 20.0, IBM SPSS Inc., Armonk, NY, USA), followed by Duncan's multiple range test to determine significant differences among means at $p < 0.05$.

2.4. Total Phenolic and Flavonoid Contents

The total polyphenol content was determined by spectrophotometric analysis using the Folin-Ciocalteu method The aluminum chloride colorimetric method was used to determine the total flavonoid content of the sample, in accordance with the procedure described by Condensed tannins were also measured by subjecting them to depolymerization in the presence of sulfuric acid, followed by a reaction with vanillin [17].

2.5. Chromatographic Phenolic Composition Assessment

Phenolic compounds were identified and quantified using an Agilent 1260 system (Agilent Technologies, Waldronn, Germany), equipped with a photodiode array detector. Separation was achieved on a Zorbax Eclipse XDB C18 reverse-phase column (100 mm x 4.6 mm, 5 µm), maintained at 25°C. The mobile phase consisted of HPLC-grade water with 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.7 ml/min. The gradient was programmed as follows: 90% A/10% B (0-40 min), 50% A/50% B (40-41 min), 100% B (41-50 min), and 90% A/10% B (50-59 min). Quantification was performed using standards at 280 nm, with calibration curves constructed for concentrations from 10 to 1000 µg/ml. Analyses were conducted in triplicate, and results were reported as mg/g of extract.

2.6. Biological Activities

2.6.1. Antioxidant Activities

The evaluation of antioxidant activity was carried out using four distinct methods. First, the total antioxidant capacity was determined by the reduction of molybdenum (Mo6+) to molybdenum (Mo5+) [18]. The antiradical activity was then assessed using the DPPH and ABTS assays, which

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measure the extracts' ability to neutralize DPPH and ABTS free radicals, with results expressed as a percentage of inhibition [19]. The reducing power of the extracts was evaluated using the ferricyanide method, where the reduction of ferric iron (Fe3+) to ferrous iron (Fe2+) served as an indicator of antioxidant potential. The effective concentration (EC_{50} µg/mL) required to achieve an absorbance of 0.5 at 700 nm was calculated [19]. All tests were conducted in triplicate.

2.6.2. Antibacterial Activity

Assessment of Eco-Extract via Disc Diffusion Technique

The antibacterial efficacy of the fenugreek eco-extract was assessed using the agar diffusion method against several pathogenic bacteria, including *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 8739), and *Salmonella typhimurium* (ATCC 14028). A single colony from each pure culture was transferred to physiological saline. The pathogenic strains were inoculated at 100 µL (10^8 CFU/mL) into 10 mL of soft agar, which was then overlaid onto a Petri dish containing 50 ml of MH agar [20]. Discs of 6 mm in diameter were placed on the agar surface and loaded with 10 µL of the extract. The plates were first incubated at 4°C for 2 hours, then at 37°C for 24 hours. The zones of inhibition of bacterial growth were measured to assess the antibacterial effectiveness of the extract.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

A response surface model with an equation containing 10 coefficients was used to analyze the results. For determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), serial dilutions of the green fenugreek extract in a 10% aqueous DMSO solution were added to wells inoculated with 10 µl of a bacterial suspension (10^6 CFU/ml) and Muller Hinton broth. After overnight incubation at 37°C, the MIC was determined by the highest dilution showing no growth. For the MBC, 200 µL from wells with no growth were plated on agar and incubated overnight at 37°C, with the highest dilution showing no bacterial colonies recorded as the MBC [21].

2.6.3. Anti-Inflammatory Activity

The RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) under standard conditions. After a 24-hour incubation period to allow for cell adhesion, the medium was replaced with fresh DMEM containing different concentrations of the extracts. The cells were then incubated for an additional 24 hours.

Cell viability was assessed using the resazurin assay [22], and the fluorescence intensity (F) was measured. Cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{F\ sample}{F\ control} \times 100$$

where F sample represents the fluorescence of treated cells, and F control represents the fluorescence of untreated control cells.

The anti-inflammatory activity of the extracts was assessed using RAW 264.7 cells stimulated with lipopolysaccharide (LPS). Cells were seeded in 24-well plates at a density of 2×10^4 cells/mL and incubated for 24 hours to allow for adhesion. The cells were then pretreated with varying concentrations of the extracts (25-200 µg/mL) for 1 hour, followed by stimulation with 1 µg/mL LPS for an additional 24 hours. After incubation, the culture supernatants were collected and mixed with an equal volume of Griess reagent. The mixture was incubated at room temperature for 10 minutes to allow for color development. Absorbance was measured at 540 nm using a microplate reader. Nitrite levels, indicating nitric oxide (NO) production, were quantified using a sodium nitrite ($NaNO_2$) standard curve. The half-maximal inhibitory concentration (IC_{50}) for each extract was determined [17,22]. All experiments were conducted in triplicate.

2.7. Cosmetic Cream Formulation

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The formulated cream is an oil-in-water (O/W) emulsion, where the oil phase is dispersed within the aqueous phase. The preparation of this cream involves two distinct steps. First, the aqueous phase, which constitutes 70.6% of the formula, is prepared by dissolving xanthan gum, a co-emulsifier, in distilled water. Simultaneously, the oil phase is created by combining 17% refined sweet almond oil with 8.5% stearic glycerides, which act as an emulsifier. Both phases are prepared in a water bath at approximately 70°C with continuous stirring. After the preparation of the two phases, they are mixed using a helical disperser at 1300 rpm for 10 minutes, until the mixture cools down to 40°C. Finally, the active ingredient and preservative are added, and the mixture is stirred for an additional 10 minutes [17,22].

2.8. Stability Testing Evaluation

A stability assessment was conducted by storing the formulation at 4°C, 25°C, and 40°C for 90 days. Centrifugation was conducted at 3000 rpm for 30 minutes at room temperature, followed by macroscopic analysis to assess the appearance and uniformity of the sample. pH was measured with a pH meter at a controlled temperature of 25 ± 2°C. Color analysis was performed using a portable colorimeter (PCE-XXM 30, PCE Instruments, Germany), and viscosity was measured with a rotary viscometer (VISCOSIMETER PCE-RVI 2, PCE Instruments, France).

2.9. Sensory Analysis

Sensory evaluation was performed with a group of 60 participants to *evaluate* assess the cream *formulation*. The analysis focused on several factors, including color, fragrance, texture, spreadability, stickiness, and absorption. Participants rated each aspect on a scale from 1 to 10. Following the evaluation, an aggregate score was calculated for the cream based on the ratings of all parameters. This sensory analysis provided insights into user perceptions and an overall evaluation of the cosmetic cream [23].

3. Results and Discussion

3.1. Design of Experiment

Response Surface Methodology (RSM) was utilized to optimize the extraction process by evaluating the effects of extraction time (X_1, min), ultrasonic power (X_2, %), and the EtOH/H_2O ratio (X_3, % v/v). These factors were examined for their impact on two key response variables: total phenolic content (Y_{TPC}) and DPPH free radical scavenging activity (Y_{PI}). Based on initial experimental data (not shown), the study focused on specific ranges for each factor: extraction time (5 to 15 min), ultrasound power (50 to 70%), and ethanol concentration (25 to 75%). The results for total phenolic content (Y_{TPC}) and DPPH scavenging activity (Y_{PI}) from the 19 experiments are presented in Table 1.

Table 1. Box-Behnken Design.

Exp	Independent variables			Responses	
	Time (min)	Ultrasonic Power (%)	EtOH/H_2O ratio (% v/v)	Y_{TPC} (mg GAE /g DM)	Y_{PI} (%)
1	5	50	25	18.53	63.70
2	5	70	25	13.85	51.63
3	5	50	75	5.00	35.05
4	5	70	75	6.85	52.61
5	15	50	25	15.07	63.05
6	15	70	25	11.77	51.38
7	15	50	75	9.33	39.34
8	15	70	75	18.68	63.85

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9	10	50	50	19.20	62.12
10	10	70	50	19.20	66.21
11	10	60	25	14.57	56.57
12	10	60	75	9.12	50.82
13	5	60	50	15.62	55.84
14	15	60	50	19.75	54.58
15	10	60	50	18.28	58.84
16	10	60	50	19.62	60.42
17	10	60	50	19.40	61.70
18	10	60	50	19.02	60.71
19	10	60	50	19.05	59.17

3.2. Interpretation of Coefficients

The significance of the models coefficients was evaluated using analysis of variance (ANOVA). Table 2 indicate the considerable effect of studied coefficients on respective response variables. The coefficients of the second-degree polynomial model for both responses (Y_{PI}, Y_{TPC}) were evaluated using Student's t-test with a 95% confidence interval (α = 0.05). A coefficient is deemed significant when the p-value is below 0.05. Table 2 presents the coefficient values for both models. The results show that ultrasonic power (X_1), ethanol percentage (X_2), and extraction time (X_3) all significantly affect YPI ($p < 0.05$), while only X_2 and X_3 impact Y_{TPC}. Additionally, all three factors influence the DPPH test (YPI) quadratically, whereas only X_2 affects Y_{TPC} quadratically. Significant interactions were found between ultrasonic power and extraction time (X_1-X_3), and ethanol percentage and extraction time (X_2-X_3). Higher coefficient values indicate more significant factors, with the X_1-X_3 interaction notably affecting Y_{TPC}.

Table 2. Regression coefficients of the second-order polynomial models for the analyzed responses: total phenolic content (TPC) and DPPH radical scavenging activity (PI).

Termes	DPPH		TPC	
	Coefficient	**Signification %**	**Coefficient**	**Signification %**
*b*0	60.195	***	18.776	***
		Linear Effect		
b 1	2.242	**	0.322	31.9%
b 2	-4.466	***	-2.481	***
b 3	1.337	*	1.474	***
		Quadratic Effect		
b 11	3 .936	**	0.787	20.3%
b 22	-6.533	***	-6.561	***
b 33	-5.013	**	-0.720	24.7%
		Interaction Effect		
b 12	8.228	**	2.399	***
*b*13	0 .920	16.5%	1.111	**
b 23	2.055	**	2.714	***

*** : Very significant. ** : Significant. * : Slightly significant.

3.3. Model Validation via ANOVA Analysis

The statistical significance of regression equation was checked by Fisher's F-test (Table 3). According to Fisher's F-test, the observed F-ratios, which are the ratios of the mean square of the

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regression to the mean square of the residuals, are higher than the tabulated values ([$F_{observed}$ = 43.5432] PI > $F_{tabulated}$ = 3.18; [$F_{observed}$ = 48.2308] TPC > $F_{tabulated}$ = 3.18). Furthermore, the ratio between the mean square of validity and the estimated experimental variance is lower than the tabulated value, confirming the results of Fisher's test and the validity of the proposed model. The R^2 coefficients for the responses Y_{PI} and Y_{TPC} are 0.978 and 0.980, respectively, indicating a strong correlation between the experimental values and those predicted 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_{PI}					
Regression	1.17486	9	1.30540	43.5432	***
Validity	2.15036	5	4.30071	3.1404	14.5 %
R^2 = 0.978	$F_{Obs}(43.54) > F_{tab}(3.18)$				
Y_{TPC}					
Regression	402.59	9	44.7329	48.2308	***
Validity	7.3166	5	1.4633	5.6794	6.0 %
R^2=0.980	$F_{Obs}(48.23) > F_{tab}(3.18)$				

***: Very significant. **: Significant. *: Slightly significant.

3.4. Analysis of Response Surface Curves

The isoresponse curves, displayed in both 2D and 3D, were utilized to visually represent the mathematical models that describe the impact of significant factors on the radical scavenging activity (PI) and total phenolic content (TPC) of the extract. Figure 1 illustrates the effects of interactions between key independent variables, such as EtOH/H_2O ratio (X_2) and extraction time (X_3), on the total phenolic content of the fenugreek extract. With ultrasonic power set at 60%, the total polyphenol content exceeds 19.75 mg GAE/g DW when the EtOH/H_2O ratio ranges from 25% to 50%, and the extraction time spans 10 to 15 minutes. Regarding the DPPH test, Figure 1 also shows how the interactions between ultrasonic power (X_1) and EtOH/H_2O ratio (X_2) influence the ability of the fenugreek extract to neutralize the DPPH radical. Specifically, when the extraction time is held at 10 minutes, DPPH radical inhibition can reach up to 60.54% with ultrasonic power levels between 50% and 60%, and EtOH/H_2O ratios from 25% to 50%.

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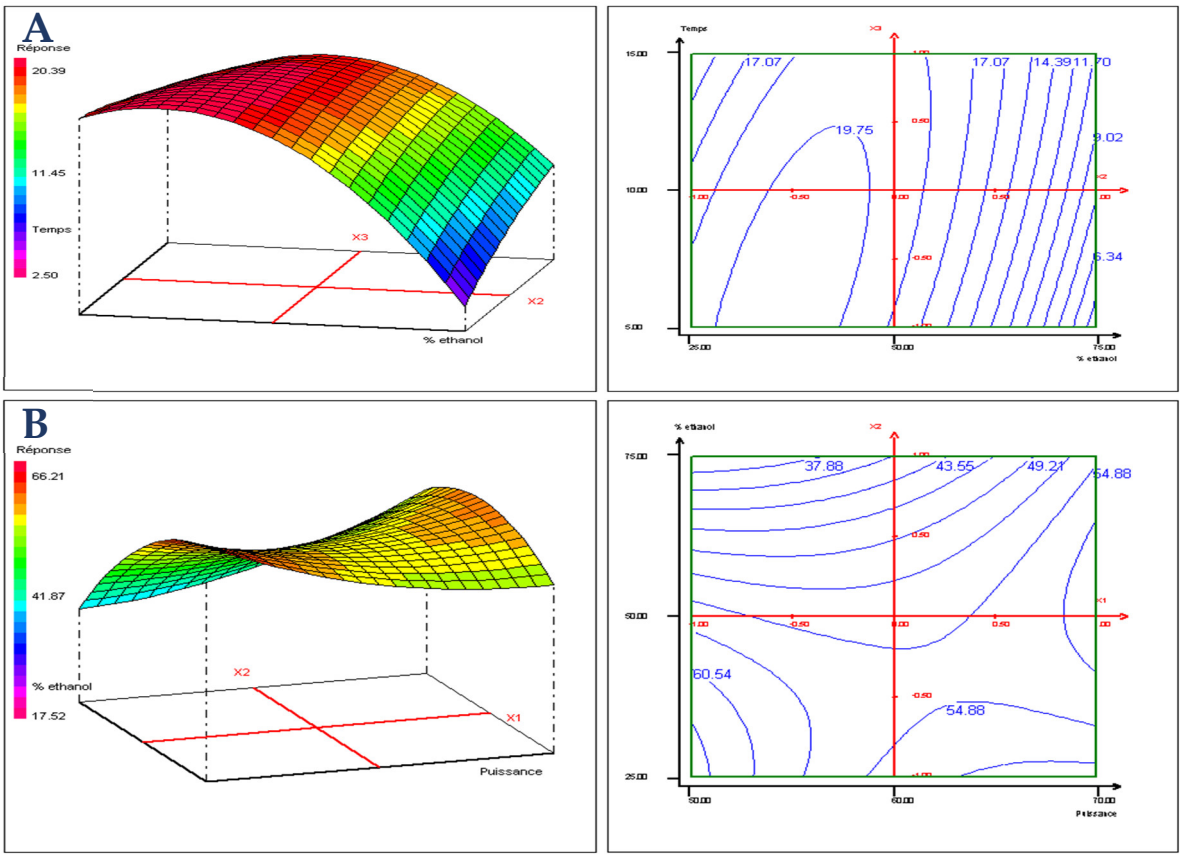

Figure 1. (A) 2D and 3D Response surface curves showing the effect of interaction between extraction time (X_1) and EtOH/H_2O ratio (X_2) on TPC. (B) 2D and 3D Response surface curves showing the effect of the interaction between time (X_1) and EtOH/H_2O ratio (X_2) on PI.

3.5. Optimization of Antioxidant Extraction Conditions from Fenugreek

The optimization of the ultrasound-assisted extraction (UAE) process for fenugreek seeds was achieved using response surface methodology (RSM). The optimal extraction conditions included an EtOH/H_2O ratio of 50%, an extraction time of 10 minutes, and an ultrasonic power of 60% (Table 4). These parameters yielded experimental values for DPPH radical scavenging activity of 63.24% and total phenolic content (TPC) of 18.56 mg GAE/g DM. The close alignment of these experimental values with the RSM-predicted values (60.20% for DPPH and 18.87 mg GAE/g DM for TPC) underscores the accuracy of the optimization process (Table 4). These results align with those reported by **Dastan et al.** [24], who optimized fenugreek seed extraction using ultrasound and a solvent/water mixture (50/50), obtaining a similar total phenolic content of **16.96 mg GAE/g DW**. Furthermore, **Yang et al.** [25], using an ultrasound-assisted extraction method with slightly different parameters (72% ethanol, an extraction time of 41 minutes, and a solvent-to-material ratio of 35 mL/g), reported a high radical scavenging activity of **80.33%**. These observations corroborate our findings, reinforcing the reliability of the applied method. The results confirm that UAE effectively enhances the extraction of phenolic compounds and antioxidant activity. The identified conditions maximize extraction efficiency while aligning with sustainable practices, such as reducing solvent use and energy consumption. Compared to conventional methods, UAE offers superior performance and promotes environmental sustainability, making it a valuable technique for recovering bioactive compounds [26].

Table 4. Experimental and predicted values of the responses analyzed under optimal conditions.

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Factor			Experimental Value		Predicted Value	
Ultrasound Power (%)	EtOH/H_2O ratio (%v/v)	Time (min)	PI (%)	TPC (mgGAE/g DM)	PI (%)	TPC (mgGAE/g DM)
60	50	10	63.24±0.83	18.56±0.03	60.20	18.78

3.5. Phenolic Content and Antioxidant Activities in Fenugreek Extract

In this study, after optimizing the experimental parameters to maximize phenolic compound content and antioxidant activity, the following conditions were established: 60% ultrasonic power, 50% hydro-ethanolic solution, and a 10-minute extraction time. These optimized parameters were consistently applied in all subsequent analyses. The spectrometric evaluation of T. foenum-graecum (fenugreek) eco-extract revealed its substantial phenolic content and antioxidant activity (Table 5), emphasizing its potential as a rich source of bioactive compounds for diverse applications. Specifically, the total phenolic content (TPC) of the eco-extract, measured at 18.56 mg GAE/g DM, highlights the presence of bioactive molecules known for their capacity to mitigate oxidative damage, a key factor in skin aging and deterioration, through well-documented mechanisms such as free radical scavenging and metal chelation [27]. Phenolic compounds are particularly valued for their hydrogen-donating properties, which make them highly effective against free radicals [28]. Moreover, the **total flavonoid content (TFC)** was determined to be **19.26 mg CE/g DM**, further emphasizing the extract's rich flavonoid profile. Flavonoids are particularly valued in cosmetic formulations for their photoprotective, anti-inflammatory, and antioxidant properties [29]. Their presence in the extract enhances its ability to protect the skin from environmental stressors, such as UV radiation, and combat visible signs of aging, including wrinkles and fine lines [30]. Additionally, the presence of **condensed tannins** (10.35 mg EC/g DM) strengthens the overall antioxidant capacity of the extract. Tannins have been shown to provide supplementary antioxidant effects by effectively scavenging free radicals and inhibiting oxidative damage [31].

Similarly, the antioxidant activity of the fenugreek seed eco-extract was evaluated using four complementary methods: total antioxidant capacity (TAC), DPPH radical scavenging assay, ABTS radical scavenging assay, and ferric reducing antioxidant power (FRAP) test (Table 5).

The total antioxidant capacity (TAC) of the extract was determined, yielding a value of 218.75 mg GAE/mL. The DPPH and ABTS assays assessed the extract's ability to donate hydrogen atoms or electrons to stabilize free radicals. The results revealed IC_{50} values of 77.17 µg/mL for DPPH and 93.69 µg/mL for ABTS, indicating strong radical scavenging activity. These values are comparable to those reported for other phenolic-rich plant extracts, such as green tea and rosemary, which are known for their antioxidant properties [32]. In addition, the FRAP assay demonstrated the extract's reducing power, with an EC_{50} value of 131.77 µg/mL, highlighting its ability to reduce Fe3+ to Fe2+. This activity is attributed to flavonoids and condensed tannins, compounds known for their potent antioxidant properties. These compounds help chelate metal ions and prevent oxidative chain reactions, further enhancing the extract's ability to combat oxidative stress [33].

Table 5. Phenolic contents and antioxidant activities of fenugreek eco-extract.

Assay	Values
TPC (mg EAG/g DM)	18.56±0.54
TFC (mg EC/g DM)	19.26±1.63
TCT (mg EC/g DM)	10.35±0.72
TAC (mg/mL)	218.75 ± 1.87
DPPH (µg/mL)	CI_{50} =77.17±0.75

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ABTS (µg/mL)	CI_{50} = 93.69±0.86
RP (µg/mL)	CE_{50} = 131.77±5.88

The combination of high phenolic content and antioxidant activity positions fenugreek extract as a promising candidate for cosmetic applications, particularly in anti-aging and skin-rejuvenating products. The antioxidant capacity, validated by experimental data and supported by the predictive models, highlights the extract's ability to protect the skin from oxidative damage caused by environmental stressors, UV exposure, and pollution. This also suggests that fenugreek extract may help in reducing signs of premature aging, such as wrinkles and loss of skin elasticity [34]. Additionally, the validation experiments confirm the robustness of the optimized extraction process, supporting the use of ultrasound-assisted extraction as a sustainable and effective method for producing bioactive compounds. The consistency of the results obtained from the validation experiments strengthens the potential of fenugreek extract as an eco-friendly and effective ingredient for next-generation cosmetic formulations. Incorporating fenugreek extract into skincare products offer enhanced antioxidant protection, promote skin health, and reduce visible signs of aging, aligning with current consumer trends toward natural, sustainable, and high-performance cosmetic ingredients [34].

3.6. Phytochemicals Identification by RP-HPLC

To further understand the bioactive potential of the fenugreek extract, the specific phenolic compounds responsible for its antioxidant activity were identified and quantified using reverse-phase high-performance liquid chromatography (RP-HPLC). The analysis of the 50% hydroethanolic extract of *T. foenum-graecum* L. by RP-HPLC, highlighting the extract's rich phenolic profile and reinforcing its antioxidant potential (Figure 2).

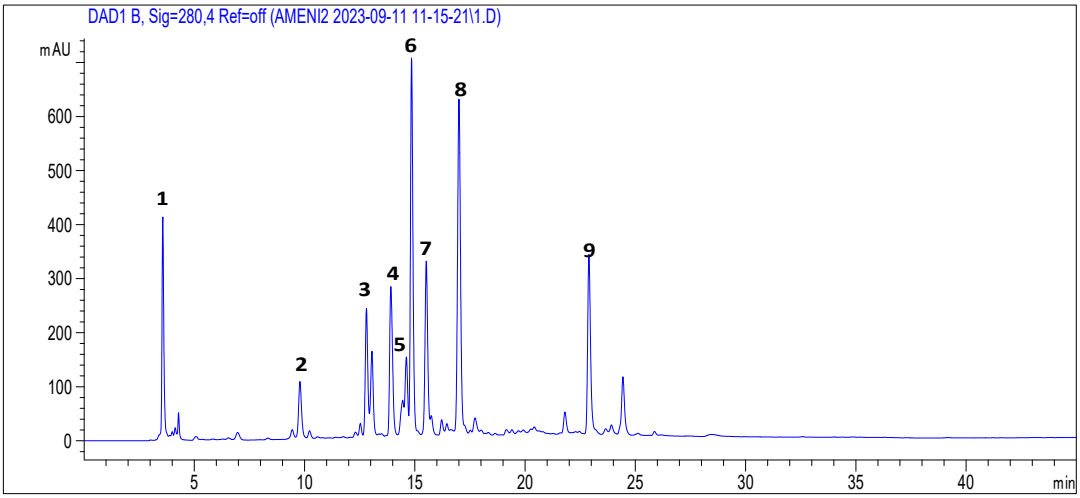

Figure 2. HPLC-DAD profile of fenugreek seed eco extract. 1. Ascorbic acid, 2. Ferulic acid, 3. Catechin, 4. Catechol, 5. Caffeic acid, 6. Epicatechin, 7. Myrectin, 8. Luteolin, 9. Apigenin.

Among the nine identified compounds, epicatechin emerged as the predominant phenolic compound (Table 6), with a concentration of 22.58 mg/g DM, followed by catechin (8.21 mg/g DM), vanillic acid (9.85 mg/g DM), myricetin (6.73 mg/g DM), and luteolin (5.6 mg/g DM). Additional compounds such as ferulic acid, apigenin and caffeic acid further enriched the extract's bioactivity.

Table 6. HPLC analysis of optimal *T. foenum-graecum* L**.** extract.

Compounds	mg/g DW	Calibration curve	R^2
Ferulic acid	0.81±0.05	Y=20.505x - 8.728	1
Vanillic acid	9.85±0.08	Y=9.02x - 1.55	0.995

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Catechol	5.74±0.01	Y=3.632x + 1.8	0.998
Caffeic acid	1.74±0.02	Y=23.496x + 5.57	0.999
Myrecitin	6.73±0.05	Y=6.7915x - 35.35	0.994
Epicatechin	22.58±0.01	Y=3.632x + 1.8	0.998
Catechin	8.21±0.04	Y=3.632x + 1.8	0.998
Luteolin	5.6±0.02	Y=7.4296x + 13.16	0.996
Apigenin	1.35±0.05	Y=12.418x + 59.908	0.997

Epicatechin, known for its broad therapeutic properties including antioxidant, anti-inflammatory, neuroprotective, and anti-aging effects plays a pivotal role in mitigating oxidative stress, preventing collagen degradation, and enhancing skin elasticity [35]. These attributes make epicatechin, alongside other phenolic compounds like catechin and luteolin, invaluable for natural skincare formulations [36]. The phenolic composition underscores the extract's potential not only as a natural antioxidant but also as a bioactive agent for anti-aging and skin-rejuvenating applications. This aligns with growing consumer and industry trends favoring plant-derived, eco-friendly ingredients for cosmetics.

3.9. Antibacterial Activity

The antibacterial efficacy of the fenugreek seed eco-extract was demonstrated against four pathogenic bacterial strains, including *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*. The extract exhibited a concentration-dependent inhibitory effect, as shown by increasing inhibition zone diameters with higher extract concentrations (Table 7). The Minimum Inhibitory Concentration (MIC) values were consistent at 50 mg/mL for all strains except *E. coli*, which required a slightly higher bactericidal concentration (MBC) of 70 mg/mL.

These findings highlight the broad-spectrum antibacterial activity of the extract, effective against both Gram-positive (*E. faecalis*, *S. aureus*) and Gram-negative (*E. coli*, *S. typhimurium*) bacteria. This dual activity may be attributed to the presence of bioactive phenolic compounds, such as epicatechin, catechin, and vanillic acid, which have been previously reported to disrupt bacterial cell membranes and interfere with essential metabolic pathways [36]. Specifically, epicatechin is known to cause oxidative damage to bacterial cells by generating reactive oxygen species (ROS), leading to membrane integrity loss [37].

Table 7. Results of the antibacterial activity of T. foenum graecum L. eco-extract.

Gram	ATCC	Strains	MIC (mg/ml)	MBC (mg/ml)
Gram +	**29212**	*Entrococcus feacalis*	50	50
Gram +	**25923**	*Staphylococcus aureus*	50	50
Gram -	**3739**	*Escherichia coli*	50	70
Gram -	**14028**	*Salmonella thyphimirium*	50	50

3.7. Evaluation of Anti-Inflammatory Activity

3.7.1. Evaluation of Cytotoxicity of Fenugreek Eco-Extract

The cytotoxicity of the fenugreek eco-extract was assessed using the resazurin assay on RAW 264.7 macrophage cells at varying concentrations ranging from 50 to 200 µg/mL (Figure 3). The resazurin assay is a widely used method to evaluate cell viability, as it measures the metabolic activity of cells [22]. The extract did not induce any significant decrease in cell viability, even at the highest concentration tested (200 µg/mL). In fact, at a low concentration of 50 µg/mL, cell viability increased by 32% compared to the control, suggesting a potential stimulatory effect on cell growth. These results indicate that the fenugreek eco-extract is non-toxic at the tested concentrations, and it does

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not exhibit cytotoxic effects on RAW 264.7 cells. This finding is consistent with previous studies on fenugreek extracts, which have shown no cytotoxicity up to concentrations of 250 µg/mL towards J744.A1 rat macrophage cell line [38]. Consequently, the extract's safety profile supports its potential for use in cosmetic formulations, where it could promote skin health without adverse effects on cellular viability.

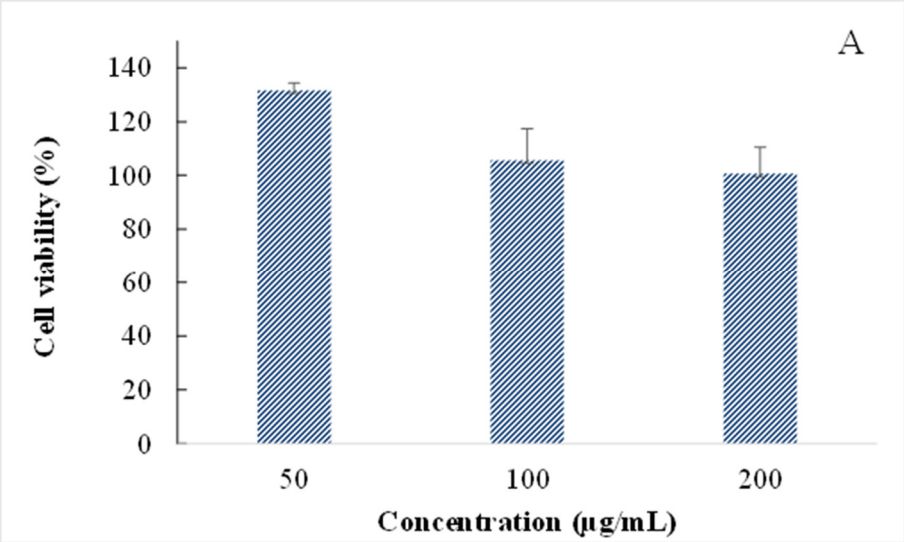

Figure 3. The viability of Raw 264.7 murine macrophage cells treated with *T. foenum graecum* L. eco-extract.

3.6.2. Measurement of Nitrite Production (NO)

To evaluate the anti-inflammatory potential of the fenugreek eco-extract, nitrite production was measured in RAW 264.7 macrophage cells stimulated with lipopolysaccharide (LPS). Nitrite, a stable product of nitric oxide (NO), serves as an indicator of inflammatory responses, particularly the activation of inducible nitric oxide synthase (iNOS) by LPS [39]. After treating the cells with increasing concentrations of the extract (ranging from 25 to 200 µg/mL) for 1 hour, followed by stimulation with 1 µg/mL LPS for 24 hours, the nitrite levels in the culture supernatants were quantified using the Griess reagent assay. The results showed a dose-dependent reduction in nitrite production, with the extract demonstrating significant inhibition of NO production. At concentrations of 100 µg/mL and 200 µg/mL, nitrite production was reduced by 34% and 48%, respectively, compared to the control group. These findings suggest that the fenugreek eco-extract has anti-inflammatory activity, likely through the inhibition of iNOS expression and NO production. This inhibition is important for modulating the inflammatory response and protecting against inflammation-related skin damage. Recent advances, on Fenugreek seeds, have shed light on their antioxidative and anti-inflammatory activities, making them attractive candidates for applications in cosmeceuticals. Notably, the study by Eaknai et al. [34] explored the anti-aging potential of ethanolic fenugreek extracts, identifying a significant collagenase inhibitory activity (IC50 = 0.57 mg/mL), which was 2.6 times more effective than vitamin C (IC50 = 1.46 mg/mL).

3.8. Incorporation of Fenugreek Eco-Extract into Cosmetic Cream Formulation

Through ultrasonic-assisted extraction and design of experiments, we obtained a fenugreek extract with high levels of antioxidants compounds with potential antimicrobial and antiinflammatory properties, making it a promising ingredient for cosmetic formulations. The extract's high content of phenolic compounds such as epicatechin, catechin, and luteolin provides powerful antioxidant effects, which help combat oxidative stress a major contributor to skin aging [36]. These findings align with previous study highlighting the benefits of fenugreek in promoting skin elasticity, reducing wrinkles, and enhancing skin radiance [34].

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In addition to its potent antioxidant activity, the extract exhibits significant anti-inflammatory properties, as demonstrated by its ability to suppress nitric oxide (NO) production in macrophage cells. These findings suggest that the extract holds promise for managing inflammatory skin diseases. Furthermore, its lack of cytotoxic effects reinforces its safety and suitability for use in skincare formulations. Previous studies have demonstrated the antiinflammatory properties of fenugreek *[38,40]*. In addition, the use of ultrasound-assisted extraction (UAE) also ensures that the bioactive compounds are efficiently recovered in an eco-friendly manner, aligning with sustainable production practices and green chemistry principles [41].

Based on these promising results, we have formulated an anti-aging and anti-wrinkle cream featuring the active eco-extract of T. foenum-graecum L. as the key ingredient.

3.9. Analysis and Stability of the Cosmetic Cream

The stability of the cosmetic cream containing fenugreek eco-extract was assessed over a 90-day period under different storage conditions (4°C, 25°C, and 40°C) (Table 8). This comprehensive evaluation included physical property measurements such as pH, viscosity, and color, as well as centrifuge stability to ensure the cream's consistency and quality during storage. Throughout the storage period, the pH of the cream remained stable, ranging from 6.25 to 6.35, which is within the optimal range for skin care formulations. Maintaining a stable pH is critical, as it ensures the safety and effectiveness of the product on the skin [42]. These findings are in agreement with other studies, such as that by Tan et al. [43], which reported similar pH stability in creams containing natural extracts, demonstrating that fenugreek extract does not significantly alter the pH of the formulation, maintaining skin compatibility. The viscosity of the cream was monitored with values ranging from 7941.69 to 7956.70 cp over the 90 days (Table 8). This stability in viscosity indicates that the cream preserved its texture and spreadability, key characteristics for consumer satisfaction and effective application. Okafo et al. [44] also reported similar viscosity stability in *Psidium guajava* ethanol extract creams formulated with plant extracts, further supporting the reliability of our results in maintaining product consistency. Color analysis revealed minor changes, with a slight shift towards yellow (b* values increasing from 4.3 to 4.8) (Table 8). Centrifuge tests were conducted at 4°C, 25°C, and 40°C to evaluate the cream's homogeneity. The cream remained stable under all conditions, with no separation or phase inversion observed. These results are consistent with findings from Chaabani et al. [17], where creams containing *Cymodocea nodosa* extract demonstrated similar stability, with no significant phase separation under various temperature conditions. This suggests that the fenugreek eco-extract does not compromise the physical stability of the cream.

The zeta potential (ZP) of the cream was measured to evaluate its colloidal stability, with values ranging from -36.18 to 37.25 mV. A negative ZP indicates good stability by preventing aggregation of particles in the formulation.

In conclusion, the cosmetic cream containing fenugreek eco-extract demonstrated excellent stability over 90 days, maintaining its physical properties, color, and consistency under various storage conditions. These findings align with previous studies on the stability of plant-based cosmetic formulations and highlight the potential of fenugreek extract as a safe, effective, and sustainable ingredient for next-generation skincare products.

Table 8. Stability parameters of the cream.

	Day 0	Day 30	Day 60	Day 90
pH	6.34	6.25	6.32	6.35
Viscosity (cp)	7941.69	7956.70	7967.68	7973.62
Z-Average (d.nm)	321.20[a] ±5.32	332.79[a] ±9.05	339.11[a] ±18.07	320.60[a] ±2.18
Zeta Potential (mV)	-37.25[a] ±1.45	-36.49[a] ±2.16	-36.18[a] ±2.67	-36.70[a] ±3.86

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L*	82.5	82.5	82.5	82.5
a*	-2.4	-2.4	-2.5	-2.6
b*	4.3	4.7	4.4	4.8
Color differnece 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

3.10. Evaluation of Sensory Characteristics of the Cosmetic Cream

The sensory properties of the cosmetic cream formulated with fenugreek eco-extract were evaluated by a panel of 60 participants to assess key attributes. Each parameter was rated on a 10-point scale, and an overall score was calculated based on participant feedback. The results obtained are highly encouraging, as illustrated by the radar graph (Figure 4). The cream received consistently high scores across all attributes, demonstrating strong consumer appeal. The color was rated at an average of 9.38, indicating that the cream's natural appearance was well-received. The fragrance scored 9.09, reflecting the pleasant and subtle aroma of the formulation, aligning with preferences for mild fragrances in skincare products. Texture-related attributes also performed exceptionally well, with consistency scoring 9.41, spreadability at 9.6, and stickiness at 9.8, highlighting the cream's smooth application and non-greasy finish. As well, absorption was rated the highest, with an average score of 9.81, showing the cream's ability to penetrate the skin effectively without leaving a residue.

These results are in agreement with previous studies on natural cosmetic formulations. For instance, Chaabani et al. [17] reported similar consumer satisfaction scores for creams enriched with plant-based extracts, emphasizing the importance of ease of application and lightweight textures.

Eventually, the overall score of 9.51 highlights the cream's ability to meet user expectations, demonstrating strong sensory appeal and functional benefits. Combined with its proven stability, antioxidant, anti-inflammatory, and antimicrobial properties, the fenugreek eco-extract-based cosmetic cream shows great promise as a versatile ingredient for innovative and commercially viable skincare products.

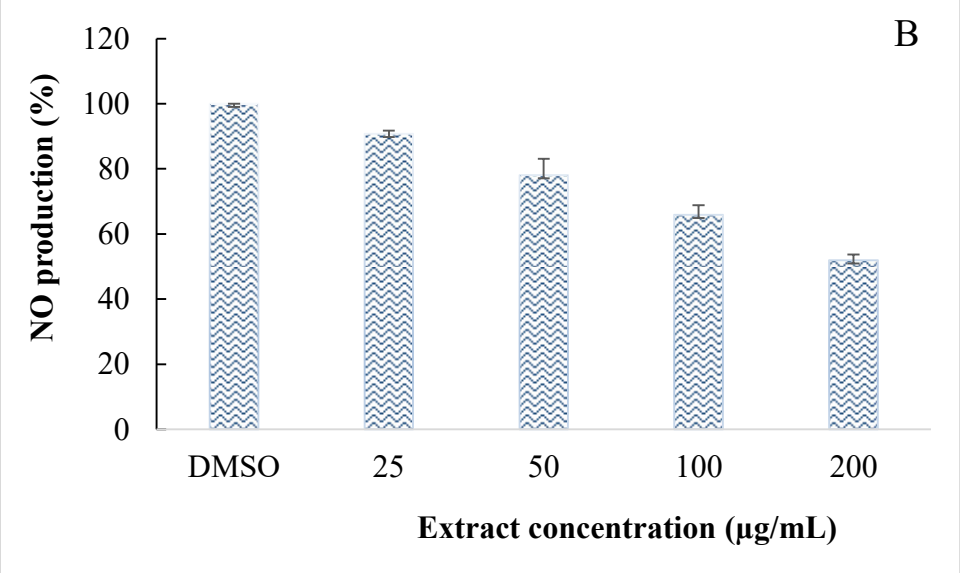

Figure 4. Antiinflammatory activity of *T. foenum graecum* L. eco-extract.

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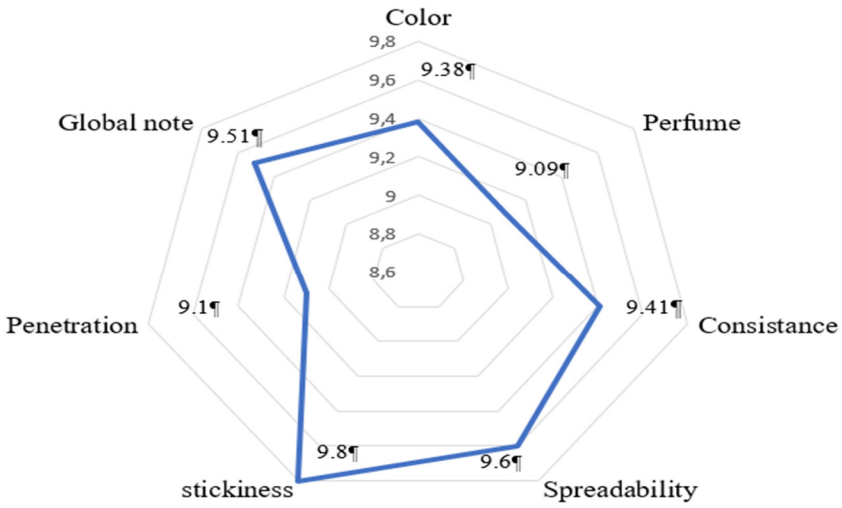

Figure 4. Sensory analysis of *T. foenum-graecum* L. cosmetic cream.

4. Conclusions

In summary, this study successfully optimized the ultrasound-assisted extraction process for fenugreek seeds, enabling the efficient recovery of bioactive compounds with proven antioxidant, antimicrobial, and anti-inflammatory properties. Chromatographic analysis revealed the presence of phenolic compounds linked to anti-aging and protection against oxidative damage. The formulated cream demonstrated exceptional stability under various environmental conditions, retaining its physical and sensory characteristics. These findings highlight the potential of fenugreek seeds as a sustainable and versatile ingredient for eco-conscious cosmetic formulations, delivering diverse benefits for skin health. By integrating advanced green extraction methods with plant-derived actives, this study paves the way for next-generation skincare products that combine efficacy with environmental sustainability, meeting the growing demand for innovative and responsible solutions in the cosmetics industry.

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