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Enhanced Extraction of Phenolic Compounds from Moroccan *Silybum marianum* L. Gaertn Extract Utilizing Augmented Simplex Centroid Design

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Abstract: *Silybum marianum* (L.) Gaertn is a medicinal plant with significant amounts of bioactive compounds known for their pharmacological properties. A method for optimizing the total phenolic content (TPC) in the extracts of *S. marianum*'s flowers, leaves, and stems was developed using a mixture design methodology and the Augmented Simplex-Centroid plan. Three solvents (water, ethanol, and methanol) and their combinations were used for extraction. The TPC in the plant materials was estimated using a UV-spectrophotometer with standard gallic acid. Before the optimization process, a solvent screening was conducted using eight solvents to determine the most effective solvents for extracting TPC. The initial screening identified water, ethanol, and methanol as the optimal solvents for extraction. Optimization results showed that the optimal mixture for achieving a total phenolic yield of 14.66 ± 0.58 mg GAE/g from leaves was composed of 54% water, 24% ethanol, and 22% methanol. For the stems, a mixture of 100% water resulted in a total phenolic yield of 6.01 ± 0.51 mg GAE/g, and finally, for the flowers, a mixture of 44% water, 27% ethanol, and 30% methanol produced a total phenolic yield of 16.41 ± 0.72 mg GAE/g. The research demonstrated that employing mixture design as an optimization technique was highly effective in identifying the most suitable combination for extracting the highest levels of phenol compounds from different components of *Silybum marianum* L. This approach allowed for the precise determination of the optimal solvent mixtures for each plant part, enabling the extraction of a significant amount of bioactive compounds.

Keywords: *Silybum marianum* (L.) Gaertn; mixture design methodology; augmented simplex-centroid design; natural products; total phenolic content.

1. Introduction

Medicinal plants have been used for centuries in various cultures worldwide for their therapeutic properties [1]. In Morocco, where plant biodiversity is exceptional, these plants are vital in traditional medicine and local pharmacopeia [2]. Polyphenols present in these plants have numerous benefits for human health, including antioxidant, anti-inflammatory, and anti-cancer properties [3].

Silybum marianum (L.) Gaertn belongs to the Asteraceae family and is known for its uses in traditional European medicine [4], but unfortunately, it remains one of the most neglected plants in Morocco [5]. Numerous studies and scientific findings have verified *S. marianum's* capacity to exhibit several pharmacological properties, including antibacterial actions [6], antifungal abilities [7], and potentially antidiabetic effects [8]. These pharmacological properties are associated with phytochemical content, notably polyphenolic compounds [9]. As a result, *S. marianum* shows promising applications for addressing various illnesses and enhancing general public health.

In the field of medicinal plants, the choice of solvent used in the extraction process for medicinal plants is of critical importance. Solvents play a fundamental role in the extraction process, as they are responsible for isolating the active compounds from the plants [10].

Experimental designs are among the most common methods adopted to optimize phenolic compound extraction from different plant matrices [11-13].

They play an essential role in examining the possible interaction of different variables, providing the most appropriate combinations to minimize the experiment number and maximize the suitable responses [14].

This research investigates the extraction method utilizing ultrasound-assisted techniques from *S. marianum* plants. The study will specifically concentrate on optimizing solvent extraction to identify the best combination for achieving the highest polyphenol yields, employing an augmented simplex-centroid design.

2. Materials and Methods

2.1. Solvents and chemical products.

Water, ethanol, methanol, ethyl acetate, acetone, butanol, chloroform, methylene chloride, DPPH, Folin-Ciocalteu reagent, sodium carbonate, gallic acid.

2.2. Plant material.

Silybum marianum samples were collected at the flowering stage during March 2023 in the Kariat Ba Mohamed, Taounate region. Subsequently, we separated the aerial components of the plant, including stems, leaves, and flowers, to conduct individualized examinations on each part.

2.3. Ultrasonic assisted extraction.

To begin with, we carried out a solvent screening analysis using eight different solvents, which included both polar and non-polar solvents. These solvents were water, methanol, ethanol, ethyl acetate, acetone, butanol, chloroform, and methylene chloride. The extraction was performed using ultrasound at a frequency of 35 KHz, at dark for 30 min, and at a ratio of 0.1:10 (W/V). The resulting mixtures were filtered and stored at 4°C until experimental analysis. Quantification of total phenolic content

Total phenolic content (TPC) was quantified by spectrophotometry using the colorimetric method based on the Folin-Ciocalteu reagent described by [15]: 200 µl of extract and 1.5 ml of Folin-Ciocalteu reagent (10%) were mixed and incubated for 5 min in the dark.

The mixture was then treated with 1.5 ml sodium carbonate solution Na_2CO_3 (5%) and incubated in the dark for 2 hours. Absorbance was read at 725 nm, and the results were expressed as mg gallic acid equivalent per g of dried matter (mg GAE/g DM).

2.4. Mixture design methodology.

The current study aims to enhance the efficiency of polyphenol extraction methods by applying an Augmented simplex-centroid design. This design is based on a combination of different independent factors to formulate a set of equations that provide theoretical values [16]. In the Augmented simplex-centroid design, a triangular arrangement is employed to test different components of the solvent system, as outlined in Table 1. This triangular configuration, illustrated in Figure 1, features components at the apex, representing 100% of each solvent. The midpoint on either side corresponds to binary mixtures, while the central point signifies a ternary mixture. In addition, 12 experiments were carried out to examine the impact of solvents (Table 2). The components of the solvent system are shown in Table 1.

Table 1. Identification of solvent system factors.

Components	Coded variables	level -	level +
Solvent 1 (Water)	X_1	0	1
Solvent 2 (Ethanol)	X_2	0	1
Solvent3 Methanol)	X_3	0	1
Sum of proportions		**1**	

Table 2. Generated experience matrix.

N°of experiment	X_1	X_2	X_3
1	1,00	0,00	0,00
2	0,00	1,00	0,00
3	0,00	0,00	1,00
4	0,50	0,50	0,00
5	0,50	0,00	0,50
6	0,00	0,50	0,50
7	0,33	0,33	0,33
8	0,33	0,33	0,33
9	0,33	0,33	0,33
10	0,67	0,17	0,17
11	0,17	0,67	0,17
12	0,17	0,17	0,67

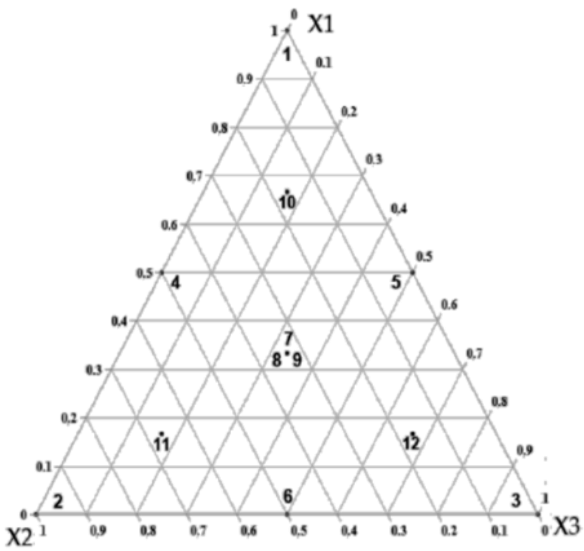

Figure 1. An augmented simplex centroid design was used to optimize the extraction. The points correspond to the mixtures in Table 2.

The model chosen in our study is a special cubic model; it is a linear model with interaction of order 2, having the following general form (1):

$$Y = \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{12} X_1 X_2 + \alpha_{13} X_1 X_3 + \alpha_{23} X_2 X_3 + \alpha_{123} X_2 X_3 + \varepsilon \quad (1)$$

With: Y is the response expressed in GAE/g; α_1, α_2, α_3, are the coefficients of the linear terms; α_{12}, α_{13}, α_{23}, are the coefficients of the binary terms; α_{123} is the ternary interaction term ε: The error term.

2.5. Statistical analysis.

An Augmented simplex Centroid design was used to assess the interaction between extraction solvents and total polyphenol TPC levels. The experiments involving selecting solvent fractions were conducted in three replicates, and the outcomes were presented as the mean ± standard error.

Design Expert 12 and JMP 16 software packages were used to determine the optimum solvent mixture for optimizing total polyphenol extraction. This model is a mathematical model that can be used to represent the relationship between two or more variables. The validity of the model was established by conducting an ANOVA test at a 95% significance level. To further evaluate the accuracy of the model with real data, the R^2 coefficient was used. This coefficient indicates the percentage of the total variance in the dependent variable explained by the model's independent variables. The model's goodness of fit was evaluated using the lack of fit test, which compares the model's well adjustment and the pure errors. Student t-test was used to determine the significance of the model coefficients. Additionally, the means were compared using the two-way ANOVA F-test and Tukey's multiple comparison test. For all statistical tests, p-values below 0.05 were considered statistically significant [11].

3. Results and Discussion

3.1. Screening of solvents' extractive power.

The Present study compared eight solvents to determine the most suitable solvents for extracting total polyphenol content (TPC) from *S. marianum* flowers, leaves, and stems. The results obtained are presented in Figure 2.

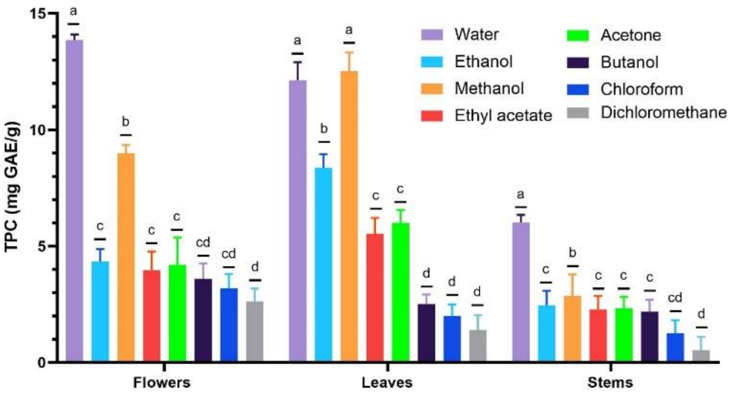

Figure 2. Total phenolic compounds in extracts from different parts of the *S. marianum* plant using different solvents.

For every plant part, solvents with different letters are statistically different. The result revealed that water was the most suitable solvent for extracting phenolic content in the two parts, flowers and stems, with 13.86 ± 0.14 and 6.01 ± 0.19 mg GAE/g, respectively. Methanol comes second for these two parts with 12.56 ± 0.08 mg GAE/g and 2.86 ±0.36 mg GAE/g for flowers and stems, respectively. For the part leaves, methanol comes first with 12.56 ± 0.08 mg GAE/g before water, which recorded 12.12 ± 0.45 mg GAE/g. Ethanol was in the third position for the three plant parts with 4.33 ± 0.35, 8.36 ± 0.32, and 2.45 ± 0.35 mg GAE/g for flowers, leaves and stems, respectively. The two-way ANOVA analysis shows a statistically significant effect of plant part and solvent factors on phenolic compound content (p-value<0.0001). Acetone and ethyl acetate gave moderate TPC values. In contrast, dichloromethane and chloroform showed the lowest amount of TPC.

The results of the complementary Tukey test indicate that for flowers, water and methanol are very distinct from other solvents, including ethanol, which gives the best third result, but this remains statistically equivalent to other solvents such as ethyl acetate and acetone. For the leaf part, the results of the Tukey test indicate that the total phenol content obtained by water and methanol can be considered statistically identical despite the slightly higher result for methanol compared to water.

The results obtained have also been reported by several researchers on the plant of *S. marianum*, indicating that high quantities of TPC were obtained in the aqueous extract compared with the ethanol and methanol extracts [17,18]. Similarly, SALEM *et al*. found chloroform to be the least extractable solvent for TPC, with 9.70 ±0.15 µg GAE/g DW for flowers, 2.02 ± 0.85 µg GAE/g DW for leaves and 3.57 ± 0.66 µg GAE/g DW for stems [19]. According to the results obtained, polyphenol content varies according to the part of the *S. marianum* plant: the highest TPC value was recorded for flowers, followed by leaves, while polyphenol content in stems remains the lowest for most of the solvents used in this study. The results are in line with those of GUEMARI, who conducted a comparative study of the polyphenol content of different parts of the *S. marianum* in Algeria. He obtained the following results in TPC: Flower extract 42.22 ± 2.07 mg EAG/ g MS, then leaf extract 22.25 ± 1.11 mg EAG/ g MS, and finally stem extract 9.05 ± 0.04 mg EAG/ g MS from the *S. marianum* plant [20]. On the other hand, Benchaaoucha obtained other results and a different ranking to our results: *Silybum marianum* leaves gave the highest polyphenol content (13.80 mg EAG/g DM on average), followed by flowers and finally stems, with values of around 8.13 and 7.68 mg EAG/g DM on average respectively [21].

3.2. Optimizing extractive solvents: solvent mixture.

Numerous studies suggest that the use of aqueous mixtures and organic solvents is the appropriate method for extracting phenol compounds from plants. In this context, an Augmented simplex-centroid design with different combinations of three solvents (water, ethanol, and methanol) was used. The total polyphenol TPC responses recorded for each experiment are presented in Table 3.

Table 3. TPC results for each experiment and each part of the *S. marianum* plant.

Experiment number	Proportion des solvents			TPC (mg GAE/g)		
	Water	Ethanol	Methanol	Flowers	Leaves	Stems
1	1.0000	0	0	10.99±0.45	13.86±0.14	6.01± 0.19
2	0	1.0000	0	8.36± 0 .35	4.33±0.32	2.46± 0.36
3	0	0	1.0000	12.56±0.08	9±0.21	2.86± 0.35
4	0.5000	0.5000	0	16.09±0.49	11.74±0.13	4.74± 0.39
5	0.5000	0	0.5000	15.80±0.22	11.44±0.22	4.99± 0.78
6	0	0.5000	0.5000	13.15±0.06	7.68±0.04	3.1± 0.27
7	0.3333	0.3333	0.3333	15.44±0.59	13.88±0.04	4.9± 0.47
8	0.3333	0.3333	0.3333	16.87±0.15	13.53±0.32	4.44± 0.64
9	0.3333	0.3333	0.3333	16.21±0.02	13.79±0.27	4.89± 0.38

Experiment number	Proportion des solvents			TPC (mg GAE/g)		
	Water	Ethanol	Methanol	Flowers	Leaves	Stems
10	0.6667	0.1667	0.1667	15.16±0.13	14.99±0.1	5.51± 0.1
11	0.1667	0.6667	0.1667	14.16±0.35	10.68±0.15	3.78± 0.07
12	0.1667	0.1667	0.6667	15.72±0.22	11.82±0.87	3.68± 0 .12

Results revealed that the mixture of equal proportions of the three solvents was the most effective at extracting TPCs from *S. marianum* flowers. In addition, a ternary mixture of 67% water, 16% ethanol, and 16% methanol showed significant TPC values for leaves, while pure water proved most effective for stems.

3.2.1. Statistical validation of models.

The results presented in Table 4 showed that the main effect of the regression was significant for all the responses studied, with low p-values (0.0005 for flowers, <0.0001 for leaves, and <0.0002 for stems). In addition, the three postulated models did not show a lack of fit since the p-values were greater than 0.05.

Table 4. The analysis of variance (ANOVA) of studied fitted models.

Model	Flowers					Leaves					Stems				
	DF	SS	MS	F	p-value	DF	SS	MS	F	p-value	DF	SS	MS	F	p-value
R	6	68,82	11,47	37,19	0,0005*	6	104,60	17,43	76,60	<0,0001*	6	13,13	2,19	53,31	0,0002*
r	5	1,54	0,31			5	1,14	0,23			5	0,21	0,04		
Lof	3	0,50	0,17	0,32	0,81	3	1,07	0,36	10,82	0,09	3	0,07	0,02	0,32	0,81
Pe	2	1,04	0,52			2	0,07	0,03			2	0,14	0,07		
Total	11	70,36				11	105,73				11	13,33			
R^2	0.97					0.98					0.98				

DF: degrees of freedom; SS: sum of squares; MS: mean square; R: regression; r: residual; Lof: Lack of fit; Pe: pure error, *: statistically significant.

The quality of the model studied was confirmed by the coefficient of determination (R^2). The latter is equal to 0.97, 0.98, and 0.98 for flowers, leaves, and stems, respectively. Both adjusted and predicted R-squared values demonstrate the strong predictive quality of the models chosen. This is further supported by the excellent correlation between experimental and predicted results (Figure 3).

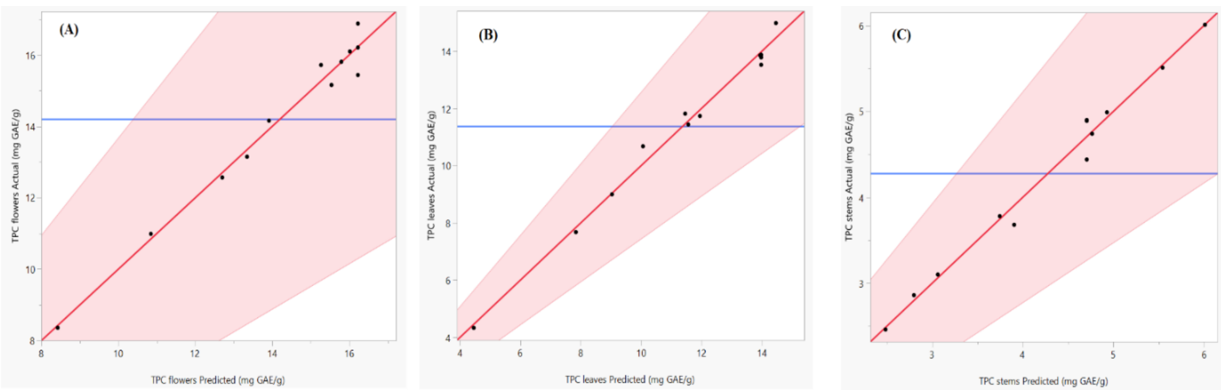

Figure 3. Curves of actual values in terms of predicted ones for **(A)** TPC flowers; **(B)** TPC leaves; **(C)** TPC stems.

3.2.2. Factor and model effects.

The estimated regression coefficients of the special cubic model are presented in Table 5. Regression equations with significant coefficients were used to determine the relationship between all the parameters studied and the polyphenol-level responses obtained.

Table 5. Estimated regression coefficients of the special cubic model

Terme	Coefficient	Flowers		Leaves		Stems	
		Estimation	P-value	Estimation	P-value	Estimation	P-value
Water	α_1	10,84	<,0001*	13,94	<,0001*	6,01	<,0001*
Ethanol	α_2	8,42	<,0001*	4,45	0,0002*	2,48	<,0001*
Methanol	α_3	12,70	<,0001*	9,03	<,0001*	2,79	<,0001*
Water*Ethanol	α_{12}	25,53	0,0002*	11,00	0,0051*	2,07	0,0896
Water*Methanol	α_{13}	16,09	0,0019*	0,29	0,9047	2,09	0,0865
Ethanol*Methanol	α_{23}	11,13	0,0092*	4,37	0,1178	1,68	0,1483
Water*Ethanol*Methanol	α_{123}	-7,87	0,6149	83,54	0,0012*	7,87	0,2016

*significantif p < 0.05

For flower extracts, the response TPC is notably influenced by significant factors, including the linear effects of water X_1, Ethanol X_2, and methanol X_3, the interaction terms X_1X_2, X_1X_3, and X2X3. The relationship between the extraction yield and the variables was elucidated by formulating a second-order polynomial equation (2)

$$Y_{\text{Tpc flowers}} = 10.84\, X_1 + 8.42\, X_2 + 12.70\, X_3 + 25.53\, X_1X_2 + 16.09\, X_1X_3 + 11.13\, X_2X_3 + \varepsilon \quad (2)$$

The response of total phenolic content (TPC) in leaf extracts is notably impacted by significant factors, which include the linear effects of water X_1, ethanol X_2, and methanol X_3, as well as the interaction terms X_1X_2 and $X_1X_2X_3$. A third-order polynomial equation (3) was formulated to clarify the relationship between extraction yield and the variables.

$$Y_{\text{Tpc flowers}} = 13.95\, X_1 + 4.45\, X_2 + 9.03\, X_3 + 11\, X_1X_2 + 83.54\, X_1X_2X_3 + \varepsilon \quad (3)$$

Finally, for the TPC for stems, only the coefficients of the pure solvents that are significant are X_1, X_2, and X_3. The TPC response for stems was determined using the following mathematical model (4):

$$Y_{\text{Tpc flowers}} = 6.01\, X_1 + 2.48\, X_2 + 2.79\, X_3 + \varepsilon \quad (4)$$

3.2.3. Strategic optimization of solvent systems.

3.2.3.1. Quantification of total polyphenols in flower extracts.

The Influence of solvent mixtures (water, ethanol, methanol) on total polyphenol content (TPC) through surface curves was presented in Figure 4. The TPC of 12 extracts ranged from 8.36 to 16.87 mg GAE/g (table 4). Analyzing the surface curves (Figure 4A), it is evident that a combination of water, ethanol, and methanol is necessary to achieve TPC around 16 mg GAE/g. Furthermore, the desirability plot (Figure 5A) indicates a maximum achievable TPC of 17.13 mg GAE/g for flowers. Notably, this value can be obtained with 99% desirability using a 44:27:29 (v/v/v) water:ethanol: methanol mixture.

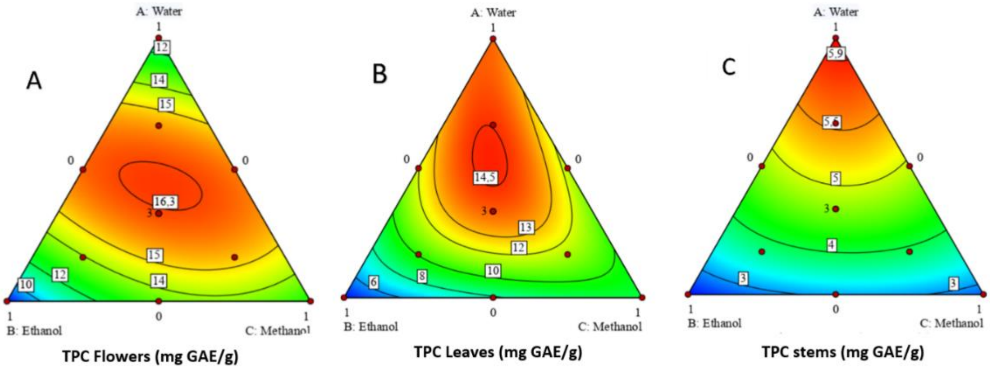

Figure 4. 2D-contour plots showing the optimum zone leading to the best TPC for **(A)** Flowers; **(B)** leaves; **(C)** stems.

The results obtained far surpass those of Anvari *et al*., who found a concentration of 2.29±0.09 mg GAE/100g in extracts from *S. marianum* flowers collected in the vicinity of Mahabad, in the West Azerbaijan province, Iran, and treated with absolute ethanol using the ultrasound-assisted extraction method [22].

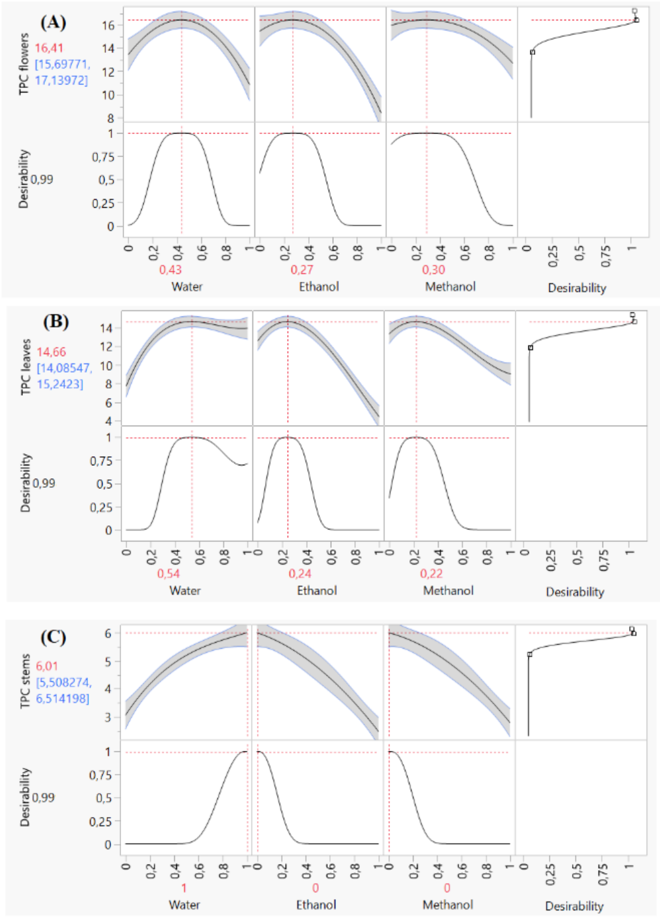

Figure 5. Desirability plots of TPC **(A)** Flowers; **(B)** TPC leaves; **(C)** TPC stems.

3.2.3.2. Quantification of total polyphenols in leaf extracts.

For leaves, the TPC of 12 extracts showed a variation in TPC between 4.33 and 14.99 mg GAE/g (Table 4). Analysis of the surface curves (Figure 4B) reveals that a mixture of water, ethanol, and methanol is required to obtain a TPC of around 14.5 mg GAE/g. Furthermore, according to the desirability plot (Figure 5B), the maximum achievable TPC is 15.24 mg GAE/g for leaves. Notably, this value can be achieved with 99% desirability using water: ethanol: methanol mixture of 54:24:22 (v/v/v).

For leaves, the TPC obtained is higher than in previous studies using only simple solvents, as in the case of Jing Sun *et al*., who obtained only 11.84 ± 0.21 mg GAE/g using an ethanolic extract [23].

For the leaves and flowers of *S. marianum*, the acceptable reason for the higher quantities of polyphenols in this study is that the combined effects (synergy) of the solvents used, namely water, ethanol, and methanol, are superior to simple methods of dissolving polyphenols.

Similar results were revealed for other plants, such as *Pimpinella anisum* and *Anacyclus pyrethrum*, in which mixture design recorded superior results for polyphenols compared with simple methods [24, 25]. In another study, Ines Sifaoui *et al*. found that the highest recovery of TPC from olive leaves was obtained using 12.7% water, 14.8% ethanol, and 72.5% methanol by ultrasound-assisted extraction [26]. With the same objective of maximizing the recovery of total phenolic compounds, Aazza found that the most suitable combination for maximizing the recovery of phenolic compounds from *Cannabis sativa* waste required mixing 12.5% water, 12.5% methanol, and 75% ethanol [27]. In addition, a solvent system consisting of 50% methanol proved the most suitable for recovering large quantities of PTCs from *Capsicum chinense* [28].

3.2.3.3. Quantification of total polyphenols in stem extracts.

For stems, the 12 extracts examined showed a variation in TPC between 2.46 and 6.01 mg GAE/g (Table 4). Analysis of the surface curves (Figure 4C) reveals that to obtain a TPC of around 6.01 mg GAE/g, the use of pure water is necessary. Furthermore, according to the desirability plot (Figure 5C), the maximum achievable TPC is 6.51 mg WAS/g for stems. In particular, this value can be achieved with 99% desirability using a 100:0:0 (v/v/v) pure water mixture.

The results obtained are in line with other studies, including Benchaachoua's study in the community of Sidi Bel Abbes Algerie, which obtained the highest TPC (10.88 mg GAE/g MS) with pure water extraction compared with other solvents, ethanol, methanol, and acetone by the decoction extraction method [23].

3.2.4. Experimental validation of optimal conditions.

An experiment was carried out to assess the adequacy of the prediction model by comparing predicted and experimental values. The coordinates of the selected test point were in agreement with the proportions of the optimal solvent system obtained.

According to Table 6, the observed and predicted values are in agreement, demonstrating that the choice of postulated models was judicious.

Table 6. Predicted and experimental values for the test points were achieved using the optimal mixtures found for each plant part.

Plant part	Parameters	Solvent proportions	Predicted value	Experimental value
Flowers	Water	44%	16.41±0.72	17.05±0.22
	Ethanol	27%		
	Methanol	29%		
Leaves	Water	54%	14.66±0.58	14.67±0.3
	Ethanol	24%		
	Methanol	22%		
Stems	Water	100%	6.01±0.51	6.40±0.22
	Ethanol	0%		
	Methanol	0%		

4. Conclusions

In the present study, the extraction of polyphenolic compounds from Silybum marianum was optimized using an augmented simplex-centroid design. A solvent system containing water, ethanol, and methanol was optimized to enhance the TPC response from different parts. Validated mathematical models were used to optimize the polyphenol response for each part.

The results indicate that the ternary mixture of water: ethanol: methanol (44:27:29 v/v/v) is the most suitable for maximizing TPC from *S. marianum* flowers, the mixture

(54:24:22 v/v/v) being the most suitable for extracting polyphenols from leaves. In the end, extraction with pure water was the best choice.

Optimized responses were 17.05±0.22 mg GAE/g, 14.67±0.3 mg GAE/g, and 6.40±0.22 GAE/g for TPC from flowers, leaves, and stems, respectively.

The statistical mixing method proved highly effective in improving the extraction of polyphenols from different parts of *S. marianum*. The results of this study could represent a significant advance in how we manage the extraction of phenolic compounds, particularly in the food and pharmaceutical sectors, to improve product quality. In addition, our research has confirmed that the use of solvent mixing is a valuable and efficient means of organizing and optimizing experimental parameters, enabling the best results to be obtained with the minimum number of trials.

In terms of future prospects, it is necessary to explore the mixing design by testing other experimental parameters such as temperature and time.

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Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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