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Optimization of Polyphenols and Antioxidants Extraction from Mentha Suaveolens Subspecies Timija

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ABSTRACT

This study delves into the influence of varying solvent compositions on the extraction of total phenolic compounds (TPC) and the antioxidant activities of extracts from *Mentha suaveolens* subsp. timija leaves and stems. The extraction process is conducted using ultrasound-assisted extraction via an ultrasonic bath. Employing a surface mixture design approach, we explored the singular use of water (W), methanol (M), and ethanol (E), as well as their combinations. The outcomes demonstrate that solvents' compositions significantly influence the extraction of TPC, with noteworthy synergistic effects observed in specific combinations, such as a ratio of W:M (1/2:1/2). The scavenging activity of DPPH and the total antioxidant capacity (TAC) were also evaluated, indicating that certain combinations of solvents, particularly those involving methanol and water, enhance the antioxidant activities. The results reveal that solvent compositions significantly influence TPC extraction, with notable synergistic effects observed in specific mixtures, such as W:M (1/2:1/2). The Pareto chart analysis highlights the substantial contributions of methanol, ethanol, and water to TPC recovery and antioxidant activities. The findings underscore the importance of solvent optimization for extracting bioactive compounds, providing valuable insights for researchers and industries seeking to harness the full potential of this species in various applications.

Keywords: *Mentha suaveolens*, phenolic compounds extraction, antioxidant activity, surface mixture design, solvent mixture design.

INTRODUCTION

Mentha suaveolens subsp. timija is a fragrant herbaceous perennial from the Lamiaceae family, indigenous to Southern and Western Europe, thriving in the humid regions of Morocco (El-Akhal et al., 2022). Also known as apple mint, this a botanical specimen renowned for its fragrant foliage and distinct medicinal properties including cytotoxic, antimicrobial, antioxidant, anti-inflammatory, hypotensive, and insecticidal properties, among others (Božović et al., 2015). Rich in phenolic compounds, *M. suaveolens* subsp. timija holds promising potential as a source of bioactive compounds with antioxidant benefits (Al-Mijalli et al., 2022). Harnessing the full therapeutic potential of this plant requires a deep understanding of the extraction process, emphasizing the importance of solvent composition optimization. Compounds displaying strong antioxidant capabilities are becoming increasingly attractive to both food producers and consumers. As concerns about the safety of synthetic chemicals in food grow, firms are increasingly looking into natural antioxidant reserves (Lourenço et al., 2019). Phenolic compounds derived from natural sources are pivotal antioxidants, offering diverse physiological benefits such

as antioxidant, antimutagenic, antiallergenic, anti-inflammatory, and antimicrobial effects (Liu et al., 2018). Researchers, particularly in recent years, have directed their focus towards discovering potent natural antioxidants, from herbal sources. The extraction process stands as a fundamental and pivotal phase in separating bioactive compounds from raw materials (Salomon et al., 2014), (Aznar-Ramos et al., 2022). however, traditional extraction methods are time-consuming, demand substantial solvent volumes, and may lead to the degradation of certain bioactive substances (Roseiro et al., 2013).

Ultrasonic-assisted extraction (UAE) distinguishes itself as a rapid and efficient technique, capitalizing on its robust cavitation effect and mechanical functionality (Rocha et al., 2017). The integration of response surface methodology (RSM), a powerful mathematical and statistical tool, is common for modeling and optimizing extraction parameters and exploring their interactions. This integrated approach is frequently employed for extracting bioactive substances from a variety of natural materials (Han et al., 2018).

Among the vast array of plant species, *Mentha suaveolens* subsp. timija, commonly known as apple mint, stands out for its rich phenolic content and antioxidant properties. Understanding the extraction dynamics of bioactive compounds from different plant parts is pivotal for unlocking their therapeutic potential. In this context, our study delves into the optimization of solvent compositions to enhance the extraction efficiency of total phenolic compounds (TPC) and antioxidant activities from *M. suaveolens* subsp. timija leaves and stems. Employing a surface mixture design approach, we systematically investigated the impact of individual solvents, including Water, Methanol, and Ethanol, as well as their combinations.

METHODOLOGY

Plant material

Samples of *Mentha suaveolens* subsp. timija were collected during May 2022 from Sefrou region in Morocco. The aerial parts of the plant of the plant were meticulously air-dried in the shade. Subsequently, the dried plant material was finely ground into a powder and securely stored in opaque containers for subsequent analyses.

Solvent extraction

The extracts were prepared in triplicate by combining 1 ml of the solvent (comprising both pure solvents and mixtures) with 50 mg of the plant powder. Subsequently, the mixture underwent sonication for 30 min in an ultrasonic bath at ambient temperature. Afterward, the extracts were recovered after centrifugation for 10 minutes at 10,000 rpm and stored in the dark at 4 °C. The initial extraction phase involved screening with solvents of varying polarities, including water, ethanol, methanol, acetone, ethyl acetate, dichloromethane, chloroform, hexane, petroleum ether, diethyl ether, and butanol. This screening aimed to identify the most suitable solvent for subsequent steps. The second step consists in extraction the pure selected solvents and their mixtures.

Total phenolic content

The total phenolic content (TPC) was determined spectrophotometrically, according to the colorimetric method using the Folin-Ciocalteu reagent (Ma et al., 2010). A volume of 50 µL of the sample was mixed with 450 µL of a solution of the Folin-Ciocalteu reagent diluted 10 times. After 5 minutes of incubation at room temperature, 450 µL of a solution of Na_2CO_3 (75 g L^{-1}) were added. All samples were subsequently incubated in the dark for 2 h at room temperature and their absorbance read at 760 nm in a spectrophotometer. The concentration of the calibration curve ($y = 2.8388x + 0.0556$, $R^2 = 0.9994$) ranged from 0.062 to 1 mg mL^{-1} in an ethanolic solution of gallic acid. The experiment was carried out in triplicate and the results are expressed in mg of gallic acid equivalent (GAE) g^{-1} of dry plant.

Total antioxidant capacity

The total antioxidant activity (TAC) of the samples was assessed by the formation of the phospho-molybdenum complex (Libbey and Walradt, 1968). A volume of 50 µl of the sample solution was combined with 1 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). After 90 min incubation in a water bath at 95 °C. The absorbance of the mixture was measured at 695 nm, against a blank, in a spectrophotometer. The aqueous solution of ascorbic acid, which served as the calibration curve ($y = 1.7355\ x +$

0.235 R^2 = 0.9999) had a concentration range of 1.0 to 0.0625 mg mL^{-1}. The experiment was carried out in triplicate and the results indicated (antioxidant activity in ascorbic acid equivalent) are average values expressed in g of ascorbic acid equivalents per g of dry plant.

Free radical scavenging activity: DPPH

DPPH (2,2-diphenyl-1-picrylhydrazyl) was produced as described by (DiCiaulaa et al., 2014) total polyphenol content, and antioxidant capacity of the crude extracts from the bark of Schinus terebinthifolius Raddi (anacardiaceae 25 µl) of different concentrations of samples or standards were added to a 60 µM ethanolic solution of DPPH (1 mL). The absorbance measurements were read at 517 nm, after 60 min of incubation time at room temperature. The absorption of a blank sample containing the same amount of methanol and DPPH solution served as a negative control. The experiment was performed in triplicate and the percentage inhibition of the free radical scavenging activity of each extract was calculated as follows:

$$Inhibition = [(Abs\ control - Abs\ sample) / Abs\ control] \times 100\% \quad (1)$$

Statistical protocol for solvent mixture design extraction study

The goal of this work was to optimize polyphenol extraction through a solvent mixture design, employing a simplex-centroid design with water (W), ethanol (E), and methanol (M) as solvents.

Modelingapproach

The linear model (main effects) assesses the linear impact of each individual solvent (WEM) through coefficients (β_1, β_2, β_3). Simultaneously, the quadratic models (interaction effects) investigate the interaction effects among solvents (WEM) using coefficients (β_{12}, β_{13}, β_{23}), while the special cubic model explores WEM to comprehend the joint impact of all three solvents. Collectively, these models offer a nuanced understanding of the relationship between solvent composition and the response variable in your solvent mixture design experiments.

Evaluation of solvent effects by simplex axial design

In the simplex-centroid design, various conditions were tested to form a triangle, with pure components at the top, representing 100% of each solvent. The central points on each side represented the permutations of binary mixtures (1/2: 1/2: 0; 1/2: 0: 1/2; 0: 1/2: 1/2), and the focal point as a ternary mixture (1: 1: 1). The predicted response was calculated by a second-order polynomial equation. The linear Eq. 2, quadratic Eq. 3, and special cubic Eq. 4 mathematical models were evaluated.

$$\text{Linear: } Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \quad (2)$$

$$\text{Quadratic: } Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 \quad (3)$$

$$\text{Special cubic: } Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4)$$

where: Y is the response variable, X_1, X_2, X_3 are the predictor variables (solvent proportions or concentrations); β_1, β_2, β_3 are the coefficients for the linear terms, representing the imp act of each individual solvent; β_{12}, β_{13}, β_{23} are the coefficients for the interaction terms. For example, β_{12} represents the impact of the interaction between X_1 and X_2; Y is the response variable; X_1, X_2, X_3 are the predictor variables representing the proportions or concentrations of water (W), ethanol (E), and methanol (M), respectively; β_1, β_2, β_3 are the coefficients for the linear terms; β_{12}, β_{13}, β_{23} are the coefficients for the interaction terms, representing the impact of the interactions between the corresponding solvents; β_{123} is the coefficient for the special cubic term, indicating the joint impact of all three solvents.

Statistical analysis

All analytical measurements were conducted at least in triplicate, and the mean values with standard deviations (±SD) were reported. Mixture response surfaces, ANOVA calculations, and regression coefficients were obtained using Statistica 8.0 software (Tulsa, USA; Free Version). The polynomial equation's goodness of fit was assessed through regression analysis (ANOVA), considering the coefficient of determination.

RESULTS AND DISCUSSION

Extraction solvents screening

The extraction of total phenolic compounds from plant leaves and stems using a variety of solvents with various polarity has yielded insightful findings. According to the Figure 1, water extraction emerged as the most effective method, demonstrating its capability to extract a substantial quantity of phenolic compounds from both plant parts. This is consistent with the predicted behavior because phenolic chemicals are often polar, and especially most phenolic glycosides are water soluble (Lattanzio, 2013). As polar solvents, methanol and ethanol showed good extraction performance, with methanol consistently outperforming ethanol in the leaves and stems. These observations are supported by previous research, indicating that phenolic compounds, particularly those in highly hydroxylated aglycone forms, generally exhibit solubility in water, alcohols such as ethanol and methanol, as well as their combinations (Dorta et al., 2012). The present study evaluates the effect of solvent (methanol, ethanol, acetone, water, methanol:water [1:1], ethanol:water [1:1], and acetone:water [1:1]. The solvents with intermediate polarity, including acetone and ethyl acetate, showed a moderate amount of efficacy, with acetone often producing greater TPC levels. The non-polar solvent hexane showed only moderate efficacy, especially when it came to extracting phenolic chemicals. The observed trend suggests that non-polar solvents may not be suitable for extracting polar phenolic compounds. Furthermore, butanol, a polar solvent, showed reduced effectiveness, suggesting its limits in terms of extracting certain phenolic chemicals found in the plant. According to (Nawaz et al., 2020) extraction in highly polar solvents resulted in a higher extract yield but a lower phenolic and flavonoid content than extraction in non-polar solvents. The polarity-related increase in total antioxidant activity and reducing properties suggests that potent antioxidant compounds can be extracted in polar solvents. The differences in solvent extraction efficiency between them emphasize how crucial it is to take solvent polarity and specificity into account when creating extraction methods. The differences observed between leaves and stems may be attributed to variations in the composition of phenolic compounds in different plant parts.

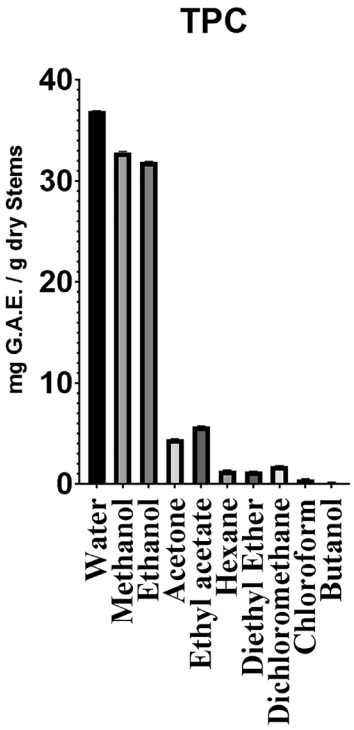

Figure 1. Total phenolic compounds of stems (a), and leaves (b) based on different solvents used

Extraction using solvent mixtures

Various solvent combinations have been employed in the literature for extracting antioxidants from plant materials, including fruits, vegetables, and other food items. The recovery of phenolic compounds is contingent upon the choice of solvent for extraction and its polarity (Boeing et al., 2014a). The levels of total phenolic compounds and antioxidant activities of the extracts obtained using the three selected pure solvents (water, ethanol, and methanol) and their mixtures are illustrated in Table 1. The results for TPC extracted from leaves and stem using various solvents and their mixtures ranged from 26.60 ± 1.12 to 46.29 ± 0.97 mg GAE/gdw (Gallic acid equivalent per gram of dry weight) in stems, and from 29.93 ± 0.04 to 42.08 ± 1.03 GAE/gdw in leaves. The results demonstrate that the equi-proportional binary mixture of methanol with water was able to extract the highest quantity of total polyphenols from the leaves and stems. These extracts exhibited the highest total antioxidant activity. They were followed by those extracted using the equi-proportional mixture of the three solvents. Indeed, multiple studies have affirmed that incorporating water in conjunction with organic solvents generates a moderately polar environment conducive to

Table 1. Simplex axial design and results for mixture tested of TPC, DPPH, and TAC

Crude extract	Extract (solvent proportions)	TPC mg Gallic acid/g dry plant		DPPH (%)		TAC mg Ascorbic acid/g dry plant	
		Leaves	Stems	Leaves	Stems	Leaves	Stems
1	Water (W) (1)	31.44 ± 0.06	26.60 ± 1.12	68.64 ± 0.17	56.18 ± 0.13	48.79 ± 0.02	46.81 ± 0.02
2	Methanol (M) (1)	33.32 ± 0.04	33.03 ± 0.02	74.14 ± 0.59	61.23 ± 0.11	47.32 ± 0.01	44.86 ± 0.03
3	Ethanol (E) (1)	29.93 ± 0.04	32.05 ± 0.02	63.99 ± 0.17	59.40 ± 0.26	44.98 ± 0.04	42.43 ± 0.02
4	W:M (1/2:1/2)	42.08 ± 1.03	46.29 ± 0.97	90.28 ± 0.22	87.86 ± 0.17	65.91 ± 0.03	63.96 ± 0.04
5	W:E (1/2:1/2)	36.57 ± 0.04	39.11 ± 0.04	85.31 ± 0.74	76.89 ± 0.23	56.80 ± 0.55	50.71 ± 0.04
6	E:M (1/2:1/2)	30.65 ± 0.06	36.68 ± 0.06	74.80 ± 0.40	62.98 ± 0.28	43.76 ± 0.03	40.81 ± 1.04
7	W:E:M (1/3:1/3:1/3)	39.94 ± 0.04	40.42 ± 0.04	87.45 ± 0.23	88.19 ± 0.34	54.38 ± 0.04	53.53 ± 0.04
All runs		34.85 ± 4.55	36.31 ± 6.11	77.80 ± 9.51	70.39 ±13.00	51.71 ± 7.46	49.02 ± 7.55

the extraction of phenolic compounds (Liyana-Pathirana and Shahidi, 2006). Furthermore, consistent with the results reported by (Nawaz et al., 2020), the use of a combination of polar and nonpolar solvents to increase the extraction efficiency of phytochemicals with good antioxidant quality. Additionally, it is noteworthy that the binary mixture of the two organic solvents, namely ethanol and methanol, ranked the lowest in terms of extraction efficiency. Consistent findings were reported by (Aazza, 2021) for binary mixtures of organic solvents, specifically ethanol and methanol. Regarding DPPH antioxidant activity, the mixture of water and ethanol proved to be the most effective in the leaves, followed by the mixtures of the three solvents. Conversely, in the extraction from stems, the mixture of the three solvents was the most efficient, followed by the mixture of water and methanol. Furthermore, the equi-proportional mixture of ethanol and water ranked third as an efficient solvent for yielding extracts rich in phenolic compounds and endowed with high antioxidant activity. It is essential to highlight that extract yielded with pure water exhibited the lowest DPPH antioxidant activity.

In summary, the equi-proportional ternary mixture of water, methanol, and ethanol demonstrated superior extraction efficiency, especially for phenolic compounds and antioxidant activity. Notably, the binary mixture of water and ethanol was also effective, while pure water extracts exhibited the lowest DPPH antioxidant activity. The results emphasize the importance of solvent composition in optimizing the extraction of bioactive compounds from plant materials.

Validation of experimental models by statistical analysis

The simplex-centroid design enables the assessment of interaction effects when solvents are concurrently mixed, achieved through the adjustment of solvent proportions. Regression analysis is employed to evaluate these interactions, providing a comprehensive understanding of how the combined effects of solvents influence the desired responses (Marcheafave et al., 2019). The statistical analysis offered here strengthens the reliability of the study's findings by providing useful insights into the efficacy of different solvents in extracting bioactive chemicals from various plant components. The validation of experimental models through rigorous statistical analysis is a crucial step in ensuring the reliability and generalizability of scientific findings obtained from the extraction of total phenolic compounds (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and TAC from leaves and stems of a plant using various solvents. The statistical studies enable a thorough evaluation of the relevance of linear, quadratic, and special cubic terms related to the polarity of each solvent. These analyses include analysis of variance (ANOVA) for TPC, DPPH, and TAC. The generated F-values, R-squared values, lack of fit and p-values are critical indicators of the model's trustworthiness and capacity to capture the subtle connections within the experimental data shown in the Tables 2 and 3. A significant Lack of Fit indicates that the model is inadequate to explain the data, and the model should be modified (do Carmo et al., 2018).The provided ANOVA results (Table 2), offer insights into the relationships between solvent polarity and the TPC extraction efficiency

Table 2. Variance analysis (ANOVA) results for mixing models

Specification		SS	df	MS	F	p	R-Sqr	R-Sqr
TPC - Leaves	Linear	340.39	18	18.91	1.93	0.174641	0.176	0.085
	Quadratic	8.67	15	0.58	191.33	0.000000	0.979	0.972
	Special cubic	2.16	14	0.15	42.14	0.000014	0.995	0.993
	Total adjusted	413.23	20	20.66				
DPPH-Leaves	Linear	1558.88	18	86.60	1.45	0.261352	0.139	0.043
	Quadratic	3.81	15	0.25	2040.45	0.000000	0.998	0.997
	Special cubic	2.44	14	0.17	7.88	0.013980	0.999	0.998
	Total adjusted							
TAC - Leaves	Linear	847.15	18	47.06	2.83	0.085342	0.239	0.155
	Quadratic	29.09	15	1.94	140.61	0.000000	0.974	0.965
	Special cubic	0.61	14	0.04	654.77	0.000000	0.999	0.999
	Total adjusted	1809.53	20	90.48				
TPC - Stem	Linear	708.93	18	39.39	0.49	0.618888	0.052	0.000
	Quadratic	28.96	15	1.93	117.40	0.000000	0.961	0.948
	Special cubic	4.42	14	0.32	77.67	0.000000	0.994	0.992
	Total adjusted	747.75	20	37.39				
DPPH - Stem	Linear	3264.37	18	181.35	0.31	0.734952	0.034	0.000
	Quadratic	81.61	15	5.44	195.01	0.000000	0.976	0.968
	Special cubic	0.75	14	0.05	1511.25	0.000000	1.000	1.000
	Total adjusted	3378.00	20	168.90				
TAC - Stem	Linear	833.29	18	46.29	3.32	0.059265	0.269	0.188
	Quadratic	2.98	15	0.20	1395.39	0.000000	0.997	0.997
	Special cubic	2.16	14	0.15	5.31	0.037032	0.998	0.997
	Total adjusted	1140.65	20	57.03				

Table 3. Statistical parameters using response surface methods

Specification		SS	df	MS	F	p
TPC - Leaves	Model	411.06	6	68.51	443.65	0.000000
	Total error	2.16	14	0.15		
	Lack of fit	0.00	0	0.00		
	Pure error	2.16	14	0.15		
	Total adjusted	413.23	20	20.66		

and the antioxidant activities from both leaves and stems of a plant. The substantial F-values and low p-values ($p < 0.0001$) for all models confirm the statistical models' suitability for defining the associations between solvent polarity and the extraction of TPC, DPPH, and TAC from both leaves and stems. These findings verify the models' ability to accurately represent the inherent patterns within the experimental data. This establishes a solid foundation for drawing meaningful conclusions regarding the impact of solvent polarity on the desired chemical attributes.

The highly significant F-values across all models, supported by exceedingly low p-values (Table 3), underscore the appropriateness of the chosen models in capturing the intricate relationships between solvent polarity and the targeted chemical properties of the yielded extracts. These findings affirm the models' efficacy in explaining the variance within the experimental data, lending a high degree of statistical confidence to the observed trends. Additionally, the lack of fit and pure error values, consistently minimal across all models, further attests to the models' ability to accurately represent the underlying patterns

in the extraction processes. This robust model fit enhances the credibility of the study's outcomes, reinforcing the validity of the reported relationships and providing a solid basis for scientific inference regarding the influence of solvent polarity on the extraction of bioactive compounds from different plant parts.The findings of our investigation demonstrate excellent model performance with exceptionally high R-squared (R^2) values. The models explain 99.5% (leaves) and 99.4% (stems) variability (R^2), as evidenced by adjusted R-squared (adjusted R^2) values of 0.993 and 0.992 for TPC. With R^2 values of 0.999 (leaves) and 1.000 (stems), and corresponding Adjusted R^2 values of 0.998 and 1.000, the DPPH Radical Scavenging Activity models demonstrate near-perfect fits. TAC models perform exceedingly well, explaining 99.9% (leaves) and 99.8% (stems) variability (R^2), with Adjusted R^2 values of 0.999 and 0.997, respectively. These findings highlight the accuracy of our models in forecasting bioactive component levels, which improves our understanding of plant extraction processes.

In conclusion, the comprehensive statistical analyses presented in the two tables collectively emphasize the robust model fit for the extraction of TPC, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and TAC from both leaves and stems of the studied plant.

Total phenolic compound extraction

The investigation into the impact of varying solvent compositions on the recovery of TPC is illustrated in Figure 2 a-b through the utilization of a surface mixture design approach. The utilization of different solvents and their combinations in the extraction process significantly influenced the extraction of TPC from both leaves and stems of the studied plant, as evident in the presented Figure 2 a-b. Water (W), methanol (M), and ethanol (E) as individual solvents yielded varying TPC concentrations in both leaves and stems, reflecting their distinct polarities and extraction efficiencies. Notably, the addition of water to organic solvents increased their ability to extract phenolic compounds up to a certain level; however, exceeding 70 percent water led to a decline in extractive efficiency. This corroborates (Aazza, 2021; Ksibi et al., 2015) findings on solvent extractive capacity for polyphenols, indicating that adding water improves extraction rates. However, excessive water content simultaneously extracts other compounds, leading to reduced phenolic concentrations in the extracts. This underscores the need for careful solvent composition optimization to maximize phenolic compound extraction while minimizing co-extraction of undesired compounds. Intriguingly, solvent binary mixtures, such as W:M (1/2:1/2), W:E (1/2:1/2), and the ternary mixture W:E:M (1/3:1/3:1/3), demonstrated synergistic effects, resulting in elevated TPC levels compared to individual solvents. Particularly, the W:M (1/2:1/2) mixture showcased a remarkable increase in TPC extraction, indicating a potential positive interaction between water and methanol.

The water-ethanol binary combination was highly effective in extracting phenolic compounds from leaves and stems, aligning with findings by (Liu et al., 2018). They observed an increase in extraction yield with ethanol concentration up to 50%, beyond which a decline occurred due to reduced solution polarity. Maintaining around 50% ethanol concentration is essential for optimal extraction. Similar findings have shown that too low or too high ethanol concentration in the extraction solvent is unfavorable for the extraction of total phenolic and TPC, or total monomeric anthocyanins from eggplant peels (Liao et al., 2022).

The polynomial model equation, derived from the solvent mixture design method, provides a mathematical representation of the relationship between solvent composition and total phenolic compound (TPC) extraction. The equation below (Eq. 5) and (Eq. 6), captures the combined linear, quadratic, and cubic effects of the solvent proportions on the response variable in the special cubic model.

$$TPC_{Leaves} = 31.44 \times (\%W) + 33.32 \times (\%M) + 29.93 \times (\%E) + 38.82 \times (\%W \times \%M) + 23.56 \times (\%W \times \%E) - 3.91 \times (\%M \times \%E) + 50.76 \times (\%W \times \%M \times \%E) \quad (5)$$

$$TPC_{Stem} = 26.60 \times (\%W) + 33.03 \times (\%M) + 32.05 \times (\%E) + 65.92 \times (\%W \times \%M) + 39.15 \times (\%W \times \%E) + 16.56 \times (\%M \times \%E) - 98.57 \times (\%W \times \%M \times \%E) \quad (6)$$

where: $\%W$ – percentage of water, $\%M$ – percentage of methanol, $\%E$ – percentage of ethanol; positive coefficients show a positive contribution, whereas interaction terms show synergistic or antagonistic effects amongst solvents.

The equations highlight the nuanced impact of solvent interactions on TPC. Positive coefficients and synergistic interaction terms emphasize the

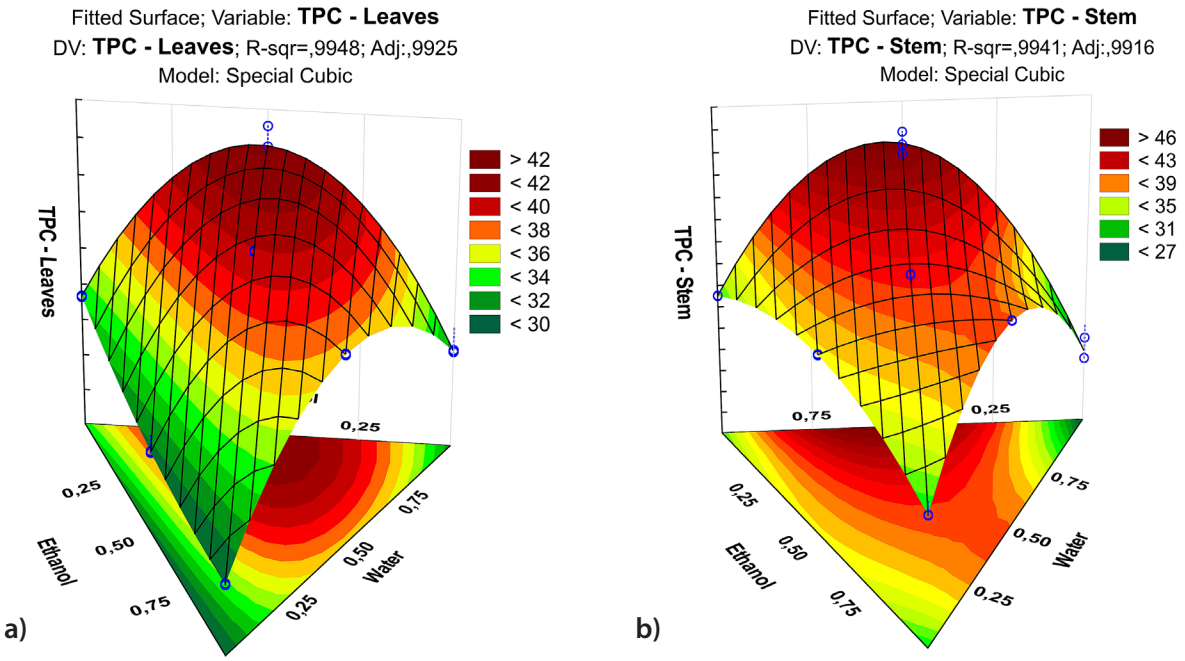

Figure 2. Response surface of the Special Cubic Model predicts TPC based on the proportions of Water, Ethanol, and Methanol of leaves (a) and stems (b)

importance of solvent choice for maximizing phenolic compound extraction, while negative interaction terms suggest potential reduction. Positive coefficients (31.44, 33.32, 29.93) imply that increasing water, methanol, and ethanol favorably contributes to TPC in leaves (TPC-Leaves). TPC is enhanced by synergistic effects from interaction factors (% water × % methanol and % water × % ethanol), whilst a negative interaction term signals possible decrease. The occurrence of a significant positive interaction term (50.76% water × % methanol × % ethanol) suggests a strong synergistic impact. A similar pattern appears for stems (TPC-Stem), stressing the favorable impact of individual solvents (26.60, 33.03, 32.05) and synergistic effects. Positive interaction factors (% water × % methanol and % water × % ethanol) improve TPC meaning that the combination of water and methanol improves TPC extraction, but negative interaction words indicate probable decline. A significant negative interaction term (-98.57% water × % methanol × % ethanol) suggests a strong antagonistic impact.

DPPH scavenging activity

The DPPH assay method is one of the two most widely employed spectrophotometric methods for assessing the radical scavenging activity of herbs, food, and vegetables. This method utilizes stable radicals that are easy to use and exhibit a high level of sensitivity. It is particularly effective in determining the antioxidant activity of various pure compounds, vegetables, fruits, and tea extracts (Arnao, 2000). The impact of solvent selection and their combinations extended beyond TPC extraction, influencing the DPPH radical scavenging activity and TAC. This suggests a nuanced relationship between solvent properties and their impact on the extraction of bioactive compounds. Figure 3a-b**,** illustrates a surface contour plot depicting the impact of solvent mixture composition on the DPPH scavenging activity of the yielded extracts. The general trend reveals that pure solvent extracts demonstrated lower DPPH free radical scavenging activities compared to solvent mixtures. Among the pure solvents, water extracts displayed the highest activity, followed by methanol. This observation suggests that the combination of solvents, particularly in mixtures, enhances the antioxidant activity of the extracts, showcasing a potential synergistic effect in scavenging DPPH free radicals. The graph shows a synergistic impact between water and the two organic solvents, which is most noticeable when the volume of water in the combination equals the volume of the organic solvent. Beyond these ratios, the mixture's capacity to extract antioxidants begins to deteriorate,

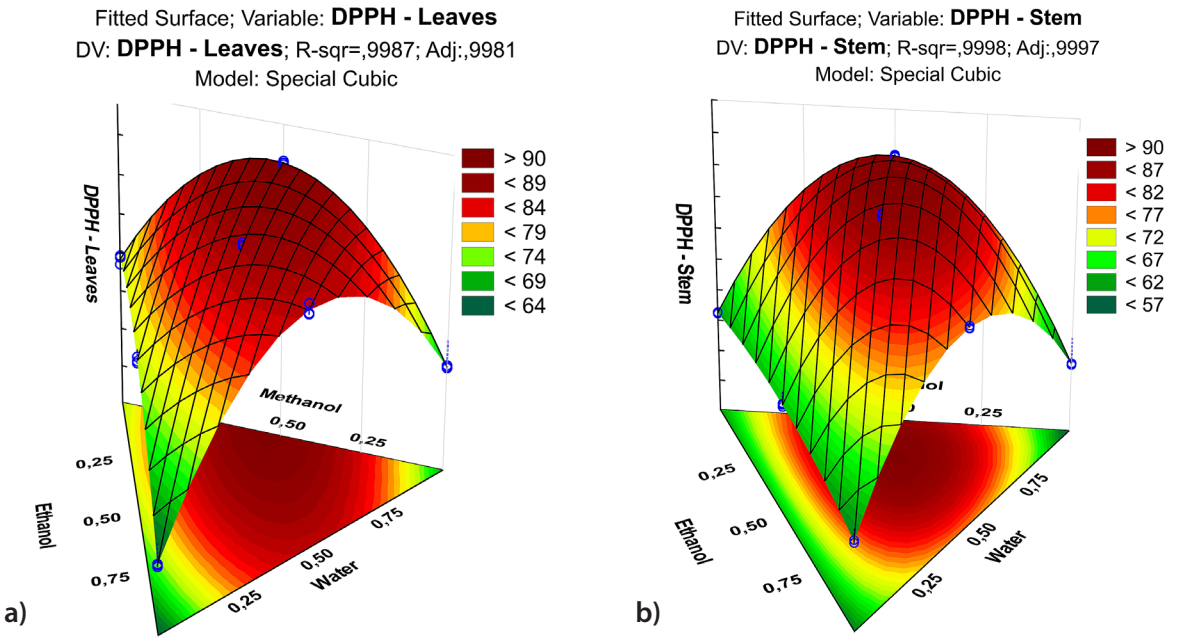

Figure 3. The response surface of the special cubic model predicts DPPH based on the proportions of water, ethanol, and methanol of leaves (a) and stems (b)

reaching a bare minimum as compared to pure solvents. This synergy might be attributed to the ability of water to improve the solubility of certain antioxidant compounds. Whereas, excessive water content could lead to saturation or competition for solubilizing capacity, resulting in a decrease in the extraction efficiency of antioxidants. Beyond a certain threshold, the solvent mixture may become less effective in extracting antioxidants. Equivalent findings indicated that extracts obtained using combinations of ethanol and water (50/50, v/v) exhibited the most elevated antioxidant capacities (Boeing et al., 2014b).

The optimal range for solvent mixtures that yield extracts with high DPPH antioxidant activity from both leaves and stems is defined by three key points: the equiportional mixture of the three solvents, the binary mixture of 75% water and 25% methanol, and the binary mixture of 25% water and 75% methanol. Extracts with increased DPPH antioxidant activity are likely to fall within this range for both leaves and stems, highlighting the relevance of solvent composition in attaining optimum antioxidant results across diverse plant parts. The polynomial model equations generated from the solvent mixture design technique for DPPH scavenging activity in leaves (Eq. 7) and stems (Eq. 8) indicate complicated correlations between solvent composition and antioxidant potential.

$$DPPH_{Leaves} = 68.64 \times (\%W) + 74.14 \times (\%M) + 63.98 \times (\%E) + 75.56 \times (\%W \times \%M) + 75.98 \times (\%W \times \%E) + 22.98 \times (\%M \times \%E) - 23.31 \times (\%W \times \%M \times \%E) \quad (7)$$

$$DPPH_{Stems} = 56.18 \times (\%W) + 61.23 \times (\%M) + 59.40 \times (\%E) + 116.61 \times (\%W \times \%M) + 76.39 \times (\%W \times \%E) + 10.65 (\%M \times \%E) + 178.94 \times (\%W \times \%M \times \%E) \quad (8)$$

where: $\%W$ – percentage of water, $\%M$ – percentage of methanol, $\%E$ – percentage of ethanol; positive coefficients show a positive contribution, whereas interaction terms show synergistic or antagonistic effects amongst solvents.

Positive coefficients (68.64, 74.14, 63.98) for leaves (DPPH-Leaves) imply that increasing water, methanol, and ethanol positively correlates to DPPH scavenging activity. Synergistic effects of interaction words (% water × % methanol and % water × % ethanol) increase activity, whilst a negative interaction term indicates possible decline. Similar patterns appear for stems (DPPH-Stem), emphasizing the favorable impact of specific solvents (56.18, 61.23, 59.40) and synergistic effects. A significant positive interaction term (178.94% water × % methanol × % ethanol) suggests a strong synergistic.

Total antioxidant capacity

The influence of solvent mixtures on TAC of the resulting extracts is a central focus in this analysis. Examining how different solvent combinations impact the total antioxidant capacity is critical for improving extraction methods and using the bioactive characteristics of plant extracts. The contour plots in Figure 4a-b graphically represent the relationship between solvent mixture compositions and TAC, offering a thorough comprehension of the complicated dynamics at work. This contour plot provides a visual representation of the intricate relationship between solvent composition and total antioxidant activity, offering insights into the optimal conditions for extracting antioxidants from both leaves and stems. The objective is to discover the ideal conditions that boost antioxidant activity in extracted chemicals, revealing understanding on the complex interplay between solvent composition and plant TAC. Figure 4a-b, shows that extracts from pure solvents have lower antioxidant activity than combinations. Methanol in particular proves to be the most efficient solvent, producing extracts with the highest values for TAC. Subsequently, ethanol produces extracts with intermediate TAC values, while water produces extracts with the lowest antioxidant activity. This solvent hierarchy emphasizes the significant influence of solvent selection on the antioxidant capacity of the yielded extracts. As evident in Figure 4, combining water with organic solvents such as methanol and ethanol significantly increases TAC, indicating a synergistic effect. These findings are consistent with our main goal of understanding how various solvent combinations contribute to total antioxidant capacity. Notably, the equiportional mixture of the three solvents and specific binary mixtures, such as 75% water – 25% methanol and 25% water – 75% methanol, emerge as prominent regions associated with elevated total antioxidant activity. The polynomial model equations for TAC in leaves and stems, obtained through the solvent mixture design method, unveil the complex influence of solvent composition on the antioxidant capacity of the extracts.

$$TAC_{Leaves} = 48.79 \times (\%W) + 47.32 \times (\%M) + 44.98 \times (\%E) + 71.42 \times (\%W \times \%M) + 39.65 \times (\%W \times \%E) - 9.58 \times (\%M \times \%E) - 106.20 \times (\%W \times \%M \times \%E) \quad (9)$$

$$TAC_{stems} = 46.81 \times (\%W) + 44.86 \times (\%M) + 42.43 \times (\%E) + 72.50 \times (\%W \times \%M) + 24.34 \times (\%W \times \%E) - 11.36 \times (\%M \times \%E) - 18.00 \times (\%W \times \%M \times \%E) \quad (10)$$

where: $\%W$ – percentage of water, $\%M$ – percentage of methanol, $\%E$ – percentage

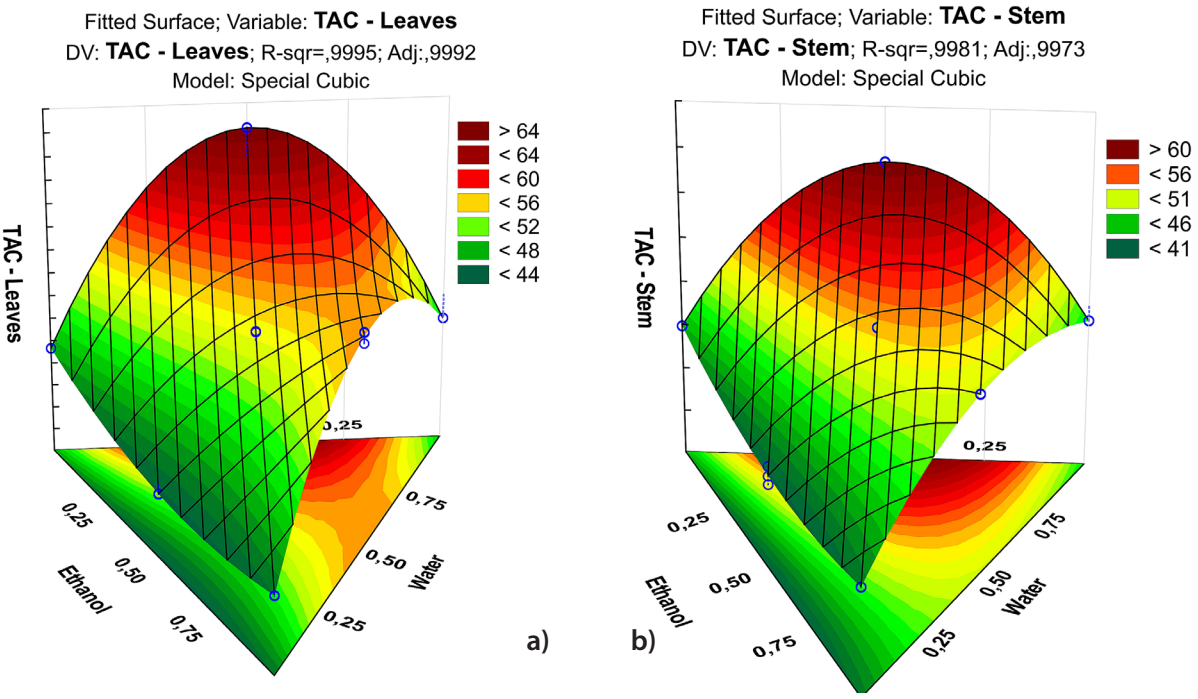

Figure 4. The response surface of the special cubic model predicts TAC based on the proportions of water, ethanol, and methanol of leaves (a) and stems (b)

of ethanol; positive coefficients show a positive contribution, whereas interaction terms show synergistic or antagonistic effects amongst solvents.

Positive coefficients (48.79, 47.32, 44.98) for leaves (TAC-Leaves) imply that increasing water, methanol, and ethanol positively correlates to TAC. TAC is increased by synergistic effects from interaction variables (% water × % methanol and % water × % ethanol), whilst negative interaction terms signal possible decrease. A significant negative interaction term (-106.20% water %methanol %ethanol) implies a strong antagonistic impact. A similar pattern appears for stems (TAC-Stem), emphasizing the favorable impact of individual solvents (46.81, 44.86, 42.43) and synergistic effects. Positive interaction variables (% water × % methanol and % water × % ethanol) increase TAC, but negative interaction terms indicate a possible drop. The presence of a negative interaction term (-18.00% water × % methanol × % ethanol) shows that an antagonistic impact is probable.

Pareto analysis

The Pareto chart was performed to visually represent and prioritize the factors influencing each outcome. The absolute values of the coefficients in the regression models were used to determine the relative importance of each factor. This concept is based on the Pareto principle, commonly known as the 80/20 rule, which states that the majority of problems or outcomes in a given scenario are due to a limited number of factors. The diagram helps to identify the few critical factors that are responsible for the majority of quality problems (Carpenter, 2011). In the context of our work, the Pareto analysis for TPC, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and TAC could be conducted based on the coefficients in the respective regression models. These coefficients represent the contribution of each solvent and their interactions to the extraction of TPC, DPPH, and TAC.

The results of the Pareto chart analysis illustrating the impact of pure solvents and their mixtures on the recovery of total phenolic compounds from *M. suaveolens* subsp. timija leaves and stems are presented in Figure 5 a-b, respectively. In the case of leaves, methanol (B) showed the greatest effect with a Pareto coefficient of 146.8598, closely followed by ethanol (A) and water (C) with coefficients of 138.555 and 131.9245, respectively. Combinations such as AB (methanol-ethanol) and AC (methanol-water) also showed positive effects on TPC recovery, contributing 34.92 and 21.19, respectively. However, the combination ABC (methanol-ethanol-water) had a lower coefficient of 6.49, indicating a comparatively lower effect. On the other hand, BC (ethanol-water) exhibited a negative impact (–3.51), suggesting a potential antagonistic effect. Relating to stems, methanol (B) remained an important factor with a Pareto coefficient of 101, closely followed by ethanol (A) and water (C) with coefficients of 98 and 131.9245, respectively. Combinations such as AB, AC and BC also showed positive effects, contributing 41, 24 and 10, respectively. The combination ABC showed a negative coefficient of -8, indicating a possible antagonistic effect. **(**The length of the bars in the chart indicates the magnitude of each factor's impact, helping to identify the most influential contributors. This visual representation enhances our understanding of the significant variables affecting TPC extraction from both leaves and stems of *M. suaveolens* subsp. timija)

The Pareto chart analysis, focusing on the effects of pure solvents and their combinations on the antioxidant activities using DPPH scavenging activity (Figure 6a-b) and the total antioxidant activity (Figure 7a-b) of the extracts from leaves and stems gives useful insights into the key components. Methanol (B), ethanol (A), and water (C) all had substantial beneficial effects on DPPH scavenging activity in leaves, with coefficients of 307, 265, and 284, respectively. Combinations AB and AC increased activity by 64 points apiece, but ABC had a lesser influence (19). BC, on the other hand, exhibited a negative coefficient (-2), indicating a potential antagonistic action. The coefficients for stems were higher, with methanol (B) leading at 458, followed by ethanol (A) and water (C) with coefficients of 444 and 420, respectively. Combinations AB, AC, and ABC all had positive effects, contributing 178, 116 and 38 points, respectively, whereas BC just had a positive coefficient (16). The results of the Pareto chart analysis illustrating the impact of pure solvents and their mixtures on the recovery of total antioxidant capacity from *M. suaveolens* subsp. timija leaves and stems are

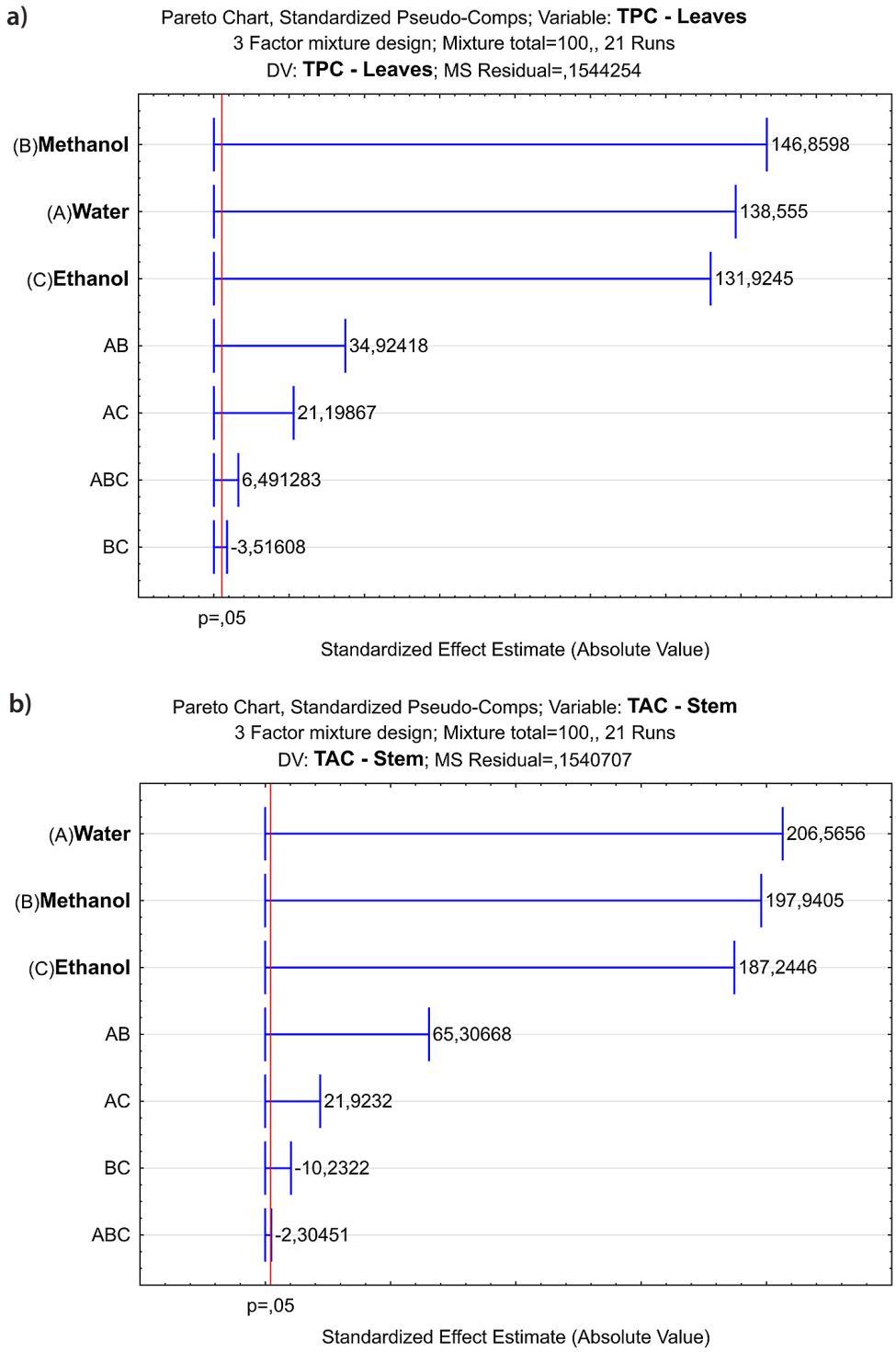

Figure 5. Pareto's graph analysis of standardized effects for TPC of leaves (a) and stems (b)

presented in Figure 7a-b, respectively. Methanol (B), ethanol (A), and water (C) all favorably contributed to TAC in stems, with coefficients of 197, 187, and 206, respectively. Combinations AB and AC contributed 65 and 21, respectively, to the TAC. ABC and BC, on the other hand, had negative coefficients (-2 and -10), indicating potential antagonistic effects.

Total antioxidant capacity

The desirability function is a widely employed tool for optimizing a large number of responses based on specific criteria. The present investigation employed this approach to ascertain optimal conditions by means of a predetermined desirability value (Vera Candioti et

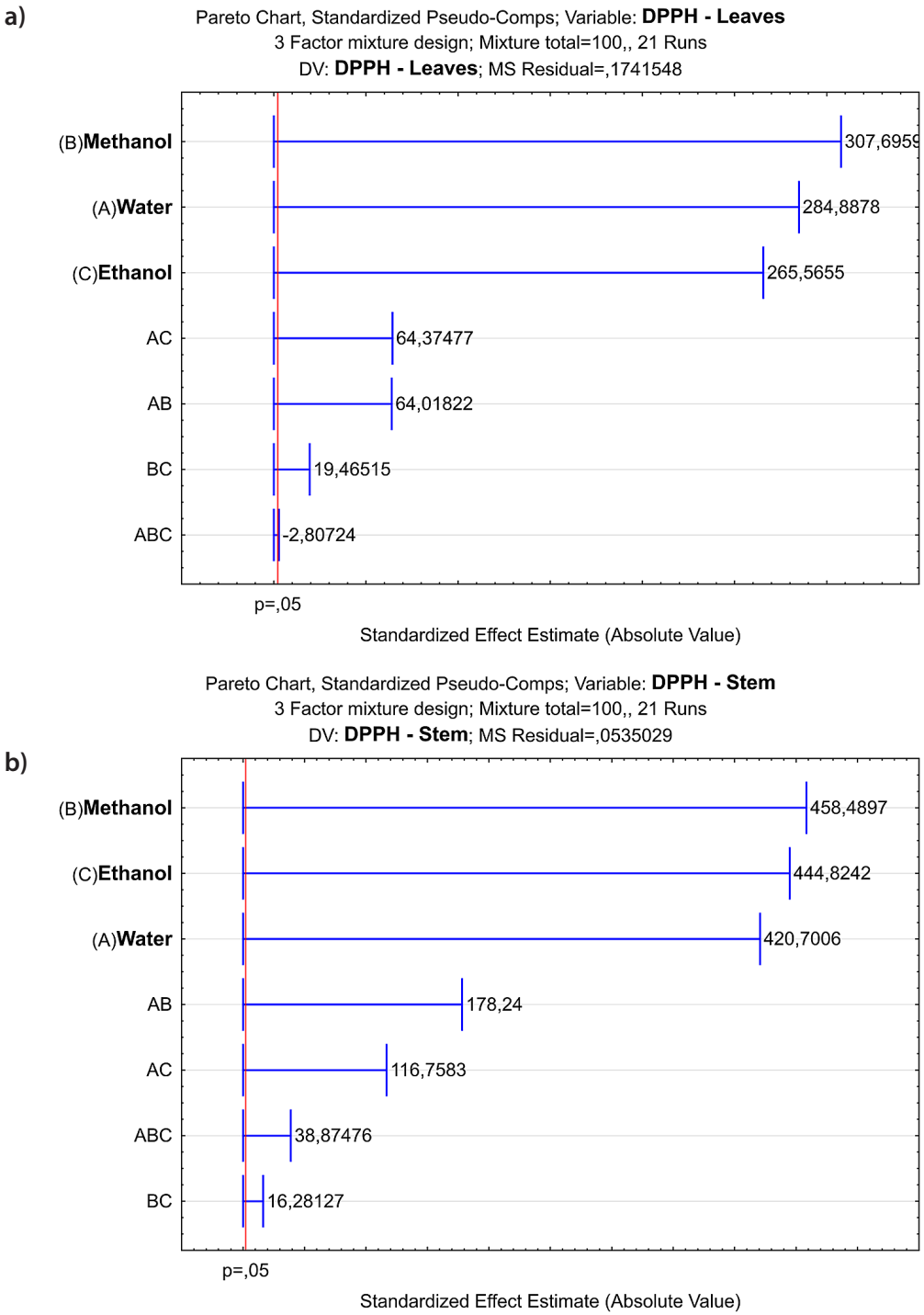

Figure 6. Pareto's Graph analysis of standardized effects for DPPH of leaves (a) and stems (b)

al., 2014). The desirability analysis was conducted to determine the optimal solvent mixture for the extraction of bioactive compounds from *Mentha suaveolens* subsp. timija leaves and stems Figure 8. The findings show that solvent composition has a substantial impact on the extraction of bioactive components from *Mentha suaveolens* subsp. timija leaves and stems. The optimum solvent mixture for TPC was 50% methanol and 50% water, which consistently provided the greatest concentrations, with a maximum TPC value of 43.1 in leaves and 47.3 in stems. In terms of DPPH free radical scavenging activity, the suggested solvent combination demonstrated exceptional antioxidant capacity, with the greatest DPPH activity reported in leaves at 90.5 and 88.5 in stems. Similarly, the ideal solvent mixture maximized

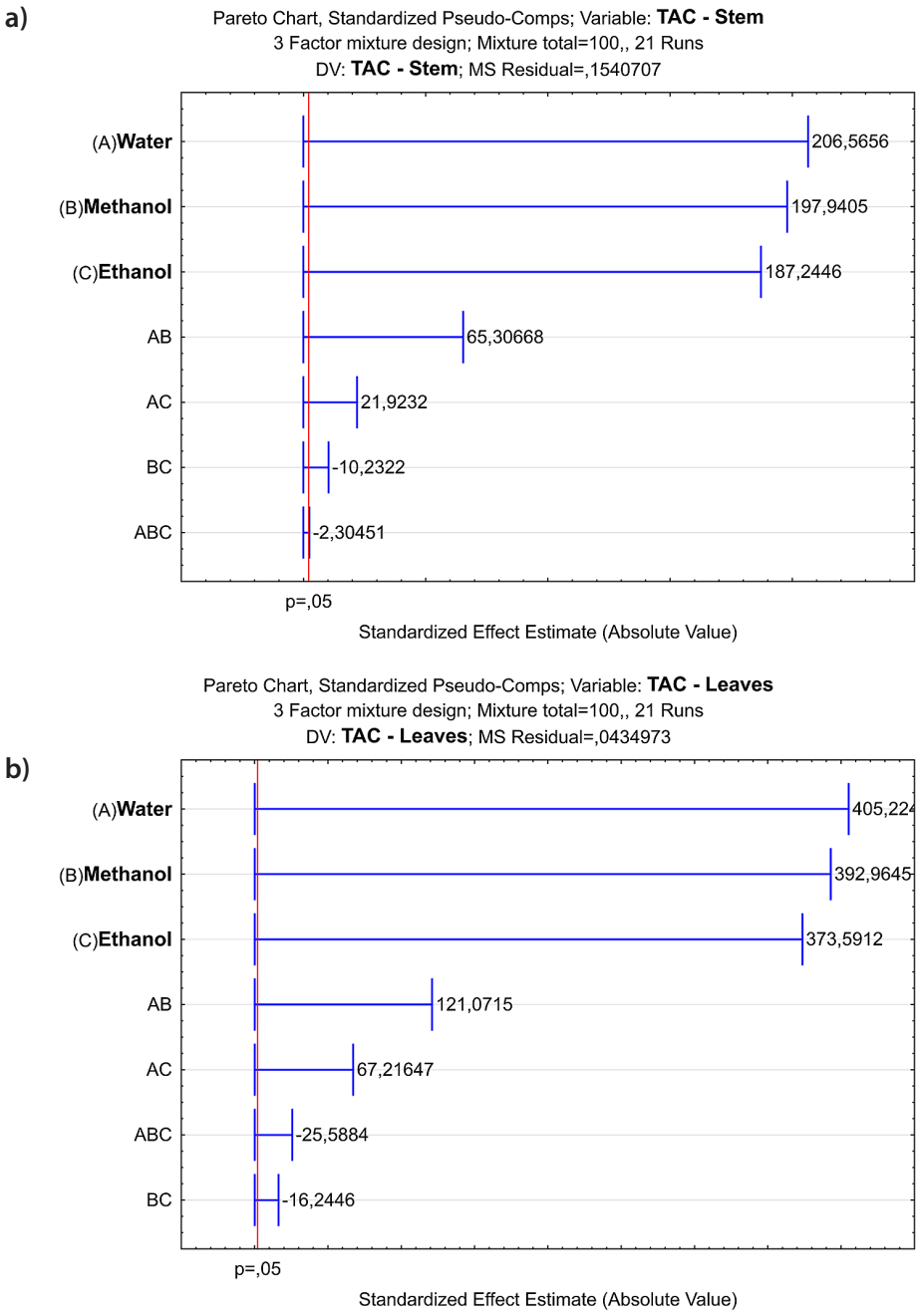

Figure 7. Pareto's Graph analysis of standardized effects for TAC assay of leaves (a) and stems (b)

TAC, with values of 65.9 in leaves and 64.1 in stems. These findings highlight the importance of solvent selection in improving the extraction efficiency of bioactive chemicals, with implications for future research.

CONCLUSIONS

In conclusion, this comprehensive investigation on *Mentha suaveolens* subsp. timija underscores the crucial role of the design of solvent mixtures in optimizing the extraction of compounds with biological activity from the leaves and stems. The outcomes this research highlight the profound influence of solvent composition on the extraction of TPC, and the generation of extracts exhibiting high DPPH free radical scavenging activity and TAC. The analysis of desirability recognized an optimal mixture of solvents consisting of 50% methanol and 50% water, consistently yielding the highest concentrations of TPC, substantial DPPH activity, and elevated TAC levels in both

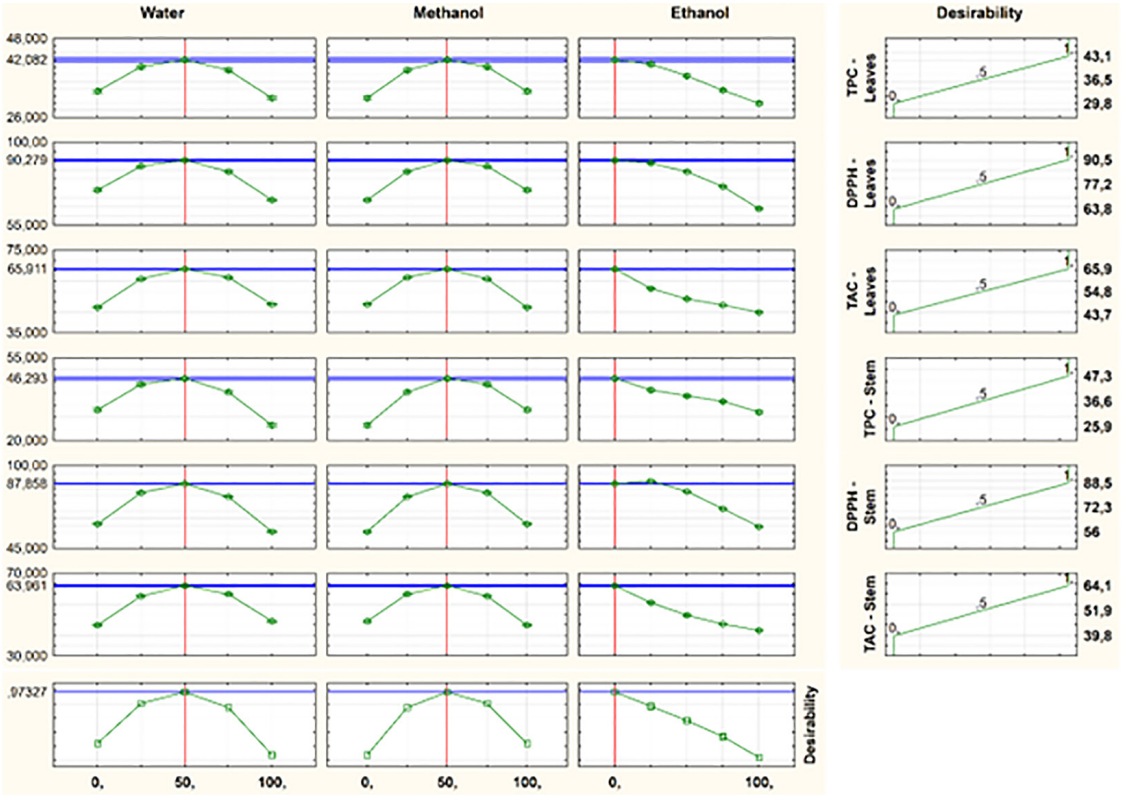

Figure 8. Optimization of desirability function values of the phenolic compounds and the antioxidant activities obtained as a function solvent mixture

the leaves and stems. The findings highlight the significance of customized solvent mixtures in fully harnessing the potential of natural resources for applications in the pharmaceutical and nutraceutical sectors.

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