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Optimisation of Total Phenolic Compound Extraction and Antioxidant Activity from Dried Inflorescence of *Ammi Visnaga* Using Mixture Design and Triangular Surfaces

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Abstract

Ammi visnaga L. (Apiaceae) is a medicinal plant widely distributed in the Mediterranean area. In Morocco, the umbel is traditionally prescribed for mouth care, toothache, and diabetes. This study was designed to examine the affinity of different solvents towards total phenolic compounds (TPC) from the dried umbels of *Ammi visnaga.* Mixture Design Response Surface Methodology was performed to maximize phenolic compounds and antioxidant activity using the selected solvents from the first step. Plant extracts were prepared using the optimized solvent mixtures. Ternary mixture with 66.67% acetone, 16.67% water, and 16.67% methanol was the most proper mixture to obtain high TPC (29.62 ± 0.60 mg EGA/g dw). Pure methanol was appropriate for TAC followed by binary mixture of 50% water and 50% acetone and ternary mixture with equal proportion of three studied solvents. Special cubic model explained the variance of the total phenolic content, and the antioxidant activity of our extracts at levels of R^2 are greater than 95%. In general, the total phenolic content and the antioxidant activity of our extracts increased with the increasing amount of water in the methanol or acetone solvent mixture. The extraction ability of phenolic compounds was also influenced positively and significantly by the water content.

Keywords: *Ammi visnaga L.*, Phenolic content, Antioxidant activity, Optimization, Extraction.

1. Introduction

Ammi visnaga L. is a widespread herb in the basin of the Mediterranean Sea, Nile Valley, West of Africa, Europe, and Asia (Khalil et al., 2020). This species belongs to the Umbellifereae family; it contains a wide range of bioactive substances. In Moroccan traditional medicine, *Ammi visnaga L* is empirically practiced to treat numerous ailments (Jouad et al., 2002). Herb presents different pharmacological effects including antihyperglycemic effect, antispasmodic effect, antiheadache, antinephritic colic, and used widely for dental hygiene (Bellakhdar et al., 1991; Ziyyat et al., 1997).

The bioactive ingredients possessed high antioxidant potential that makes them suitable for maintaining different physiological functions (Laaroussi et al., 2020; Ousaaid et al., 2020; Pejin et al., 2013). The extraction yield is profoundly associated with numerous factors such as pedoclimatic conditions, maturity of fruits, variety, extraction process, and polarity of extractor solvent.

The efficacy of the extraction method and suitability of extractor solvents affect the composition of the obtained extract, which impacts its biological properties. A successful extraction technique makes a mixture of solvents with suitable proportions. To select an extraction method, it is necessary to assess the accuracy, the stability of the extracted substances, the availability of resources, and processing costs leading towards a biological application of the extract (Oliveira et al., 2001). Within this frame, numerous techniques are coming in the forefront to reduce the amount of waste solvents and benchwork developing inventory tools including optimization studies (Cavalcanti et al., 2021). Optimized extraction constitutes a good tool to study the effect of one variable, especially solvent, on the recovered bioactive compounds and antioxidant ability (Aazza, 2021). Response surface methodology (RSM) is an accurate instrument for improving the extraction procedure (Saravana Pandian et al., 2022). In addition, it is a promoting technique for elaborating, enhancing, and optimising procedures. It can evaluate the effect of different variables, factors, and their interactions (Farris and Piergiovanni, 2009). The experimental combination design has been largely used in a variety of fields, including the development of new biofilms and for the extraction of secondary metabolites from natural products

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(Munhoz et al., 2014). The main aim of the experimental methodology is to assess how the responses are influenced by the fluctuation in the proportions of the mixture components.

Ammi visnaga L. is considered as a member of Apiaceae family. It is well known for its numerous therapeutic properties due to its high amount of biological active compounds (Kamal et al., 2022). Phytochemical analysis of *Ammi visnaga* extract using HPLC revealed the presence of numerous phenolic compounds including coumarin, apignin, kaempferol, caffeic acid, rutin, quercetin, visnagin and ferulic acid (Al-Zaidi and Khorsheed, 2021). In the same context, Ahmed et al. (2021) listed different bioactive compounds of *Ammi visnaga* such as flavonoids, coumarins and furocoumarins, isobensofurans, sesquiterpenes, phthalides, and miscellaneous. These molecules possess different pharmacological activities including antibacterial, anticancer, antidiabetic, and antihyperlipidemic activities (Afzal et al., 2021; Ahmed et al., 2021).

The current work was designed to screen the ability of the solvent to extract phenolic compounds from *Ammi visnaga* inflorescence. Secondly, we used statistical mixture design beside a simplex centroid model with acetone, methanol, and water as extractor solvents, in order to boost the extraction of the compounds and increase the phenolic activity and other antioxidant composites.

2. Material and methods

2.1. Chemicals and standards

Folin–Ciocalteu reagent, sodium carbonate (Na_2CO_3), sulphuric acid, sodium phosphate, ammonium molybdate, acetone, methanol, ethyl acetate, chloroform, dichloromethane, toluene, hexane, petroleum ether, gallic acid, and ascorbic acid were purchased from Sigme Aldriche.

2.2. Extraction procedure and sample preparation

The extractions were carried out in triplicate conferring to the following formula: 50 mg of dried and pulverized inflorescence of *Ammi visnaga* was extracted for 20 minutes by sonication with 1 mL of solvents mixture. The extracts were centrifuged for 15 minutes at 6000 rpm, and the supernatants were recuperated and stored at 4ºC (Ousaaid et al., 2020b).

2.3. Total phenolic content (TPC)

The TPC was quantified using the Folin–Ciocalteu method as previously described by (Lamuela-ravents, 1999). In brief, 50 µL of extract was mixed with 450 µL of Folin–Ciocalteu reagent (0.2 N), after 5 minutes, 450 µL of a Na_2CO_3 solution (75 g L^{-1}) was added to the mixture. All samples were incubated in the dark environment for 2 hours, and their absorbance was read at 760 nm in a Jenway 6505 UV/visible, scanning spectrophotometer. The concentration of the calibration curve (y = 1,6021x + 0,0683, R^2 = 0,997) ranged from 0.008 to 1 mg/mL of gallic acid. The experiment was prepared in triplicates, and the results are expressed as mg equivalent of gallic acid (mg EGA/g) of dried plant.

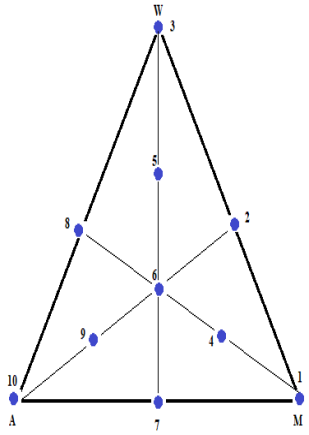
2.4. Total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) of all samples was determined using the phosphomolybdenum method according to (Prieto et al., 1999). An aliquot of 25 µL of the sample solution was mixed principally with 1 mL of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate) in a falcon of 15 mL. The falcon tubes were covered and incubated in a water bath at 95°C temperature for 90 minutes. Then, the absorbance of the mixture was measured at 695 nm, against a blank, in a Jenway 6505 UV/visible, scanning spectrophotometer. Calibration curve was prepared using aqueous ascorbic acid solution with doses varying between 5,0 to 0,0039 mg/mL (y = 0,7889x + 0,0492, R^2 = 0,996). The results are expressed as g of equivalent ascorbic acid (g EAA/g) of dried plant.

2.5. Evaluation of solvent impact by simplex axial design

Two dissimilar categories of standard designs are usually used for extraction experimentations with combinations: (i) simplex-centroid design, and (ii) simplex-lattice design. Both designs will assess the triangular reply surface at the vertices (i.e., the corners of the triangle) and then the centroids (sides of the triangle) (Montgomery, 2012).

In the simplex-centroid design, different conditions tested form a triangle, with pure components in the vertex, representing 100% of each single solvent. Central points on every side expressing permutations of the binary blends (1/2: 1/2: 0; 1/2: 0: 1/2; 0: 1/2: 1/2) and the medium point as a ternary mixture (1: 1: 1). This scheme is from time to time increased with internal points (axial ones) expressing 2/3 of one of the targeted solvents and 1/6 for the others (Figure1), also known as a Simplex Axial Design (SAD)



	Acetone	Water	Methanol
1	0,00	0,00	100,00
2	0,00	50,00	50,00
3	0,00	100,00	0,00
4	16,67	16,67	66,67
5	16,67	66,67	16,67
6	33,33	33,33	33,33
7	50,00	0,00	50,00
8	50,00	50,00	0,00
9	66,67	16,67	16,67
10	100,00	0,00	0,00

(Sampaio et al., 2015).

Figure 1. Simplex axial design (SAD).

To boost the extraction procedure, a mixture design was developed as presented in Figure 1. The simplex-centroid design coupled with axial points in three replicates was chosen to determine the solvent combination of water (W), methanol (M), and acetone (A) on the basis of preliminary results obtained using different solvents as presented in Table 1.

Figure 1 presents all tested conditions. This design permitted the evaluation of linear (W, E and A), quadratic (WE, WA, and EA), and special cubic (WEA) models for the response under study.

2.6. Statistical analysis

All the experiments for selection of the solvent proportion, as well as total phenolic content and total antioxidant capacity were performed in triplicate, and the results were reported as mean ± standard deviation.

Variance analysis (ANOVA) was applied to determine the fittest of the multiple regression model (p <0.05) to evaluate the significant effects of the variables and their interactions. From the regression coefficients, the response and contour surface graphs of the model were generated. The analyses were executed via the free version of STATISTICA version 10 software (StatSoft, 2011).

3. Results and discussions

3.1. Total phenolic compounds (TPC)

Different solvents of different polarities ranging from (0.1 to 10,2) have been screened for their ability to extract a high amount of TPC, and the obtained results are presented in Table 1 and Figure 2. The analysis of data revealed that the water, methanol, acetone are the suitable extractor solvents to recover the high amounts of TPC, while the lowest values were registered in petroleum ether and hexane extracts (Table 1). Our findings are in agreement with those reported by (Aourabi et al., 2019). It has been proved that the mixture of different solvents with different polarities is an effective procedure to extract the high amounts of bioactive compounds (Aazza, 2021; Aourabi et al., 2019; Saravana Pandian et al., 2022).

Table 1 .polarity index

Solvant	Polarity Index (P·)	TPC mg acid galic eq./g dry plant
Petroleum ether	0.1	0,01±0,01
Hexane	0.1	0,47±0,14
Toluene	2.4	3,80±0,58
Dichloromethan	3.1	8,10±0,37
Chloroform	4.1	10,05±0,24
Ethanol	4. 3	18,90±1,50
Ethyl Acetate	4.4	8,91±0,00
Methanol	5.1	27,06±2,37
Acetone	5.1	24,55±0,63
Water	10.2	20,74±0,64

Subtitle: W = Water, M = Methanol, A = Acetone.

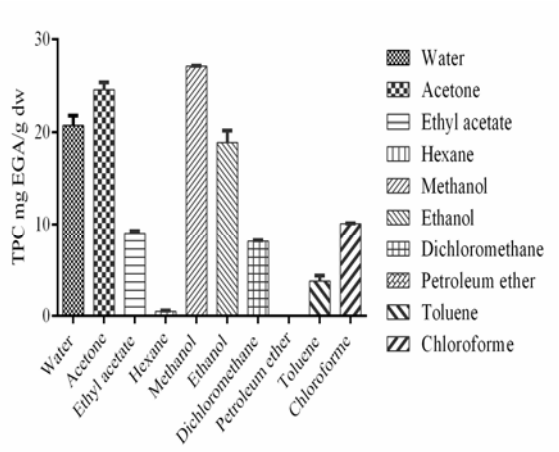


Figure 2: Total phenolic compounds based on different solvents used

3.2. Mixture design optimization of the Extraction Process

Dissimilar levels of water, methanol, and acetone have been usually used to extract phenolic content, essentially from medicinal herbs (Wang et al., 2008). In this work, we have identified the optimal values of the water, methanol, and acetone considered as independent variables, to attain the extreme response for the extraction of total phenolic content from dried inflorescence of *Ammi visnaga*. The recapture of phenolic mixtures is founded on the polarity of the solvent applied; thus, we choose methanol, water, and acetone as the most appropriate mixture which recovered the highest amount of TPC in this experiment.

The response surfaces obtained for TPC using mixture design as a function of the percentage composition of water, methanol, and acetone are illustrated in Figure 3A and contour plots are illustrated in Figure 3B.

Figure 3 A and B showed that the pure acetone has low extractor power and presented the lowest amounts of total phenolic content, followed by pure methanol, while the highest phenolic content was established in water extract.

The amount of extracted phenolic compounds is proportional to the water concentration in the solvent mixture using either both methanol and acetone. The same results have been reported by (Santos Felix et al., 2018) for *Spondias mombin* L. apple bagasse agroindustrial residues, where the lowest contents of phenolic compounds were obtained using 100% acetone, 100% alcohol or mixture of two extractor solvent (acetone (50%) and ethanol (50%), while the highest amounts were obtained only with water or mixture of three studied solvents (water, methanol, and acetone). Maximum total phenols contents can be expected from mixtures mostly rich in water. This shows that the extraction yield is highly related to the polarity of the extractor solvent. Our finding are in accordance with those reported by Rajha et al., (2014).

The highest values on the contour graph (Figure 3B) occurred between the following positions: water (100%), water/ acetone (1/2: 1/2) and water (100%), and water/methanol (1/2: 1/2). The proportions were optimized according to the response surface were (Water 70%), methanol (%5) and acetone (25%) as being the maximum inside the experimental domain, yielding maximum predicted amount of TPC (29,9197 mg EGA/g dry plant).

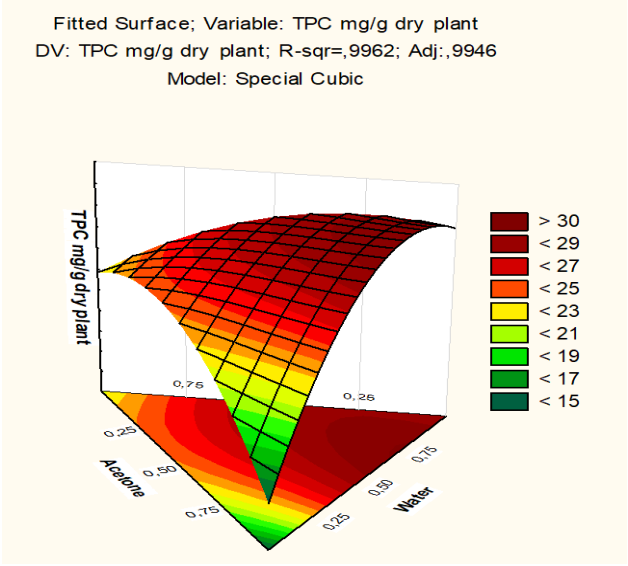


Figure 3A : Response surface contour plot of the special cubic model predicted TPC as a function of the acetone, water and methanol proportions(3D).

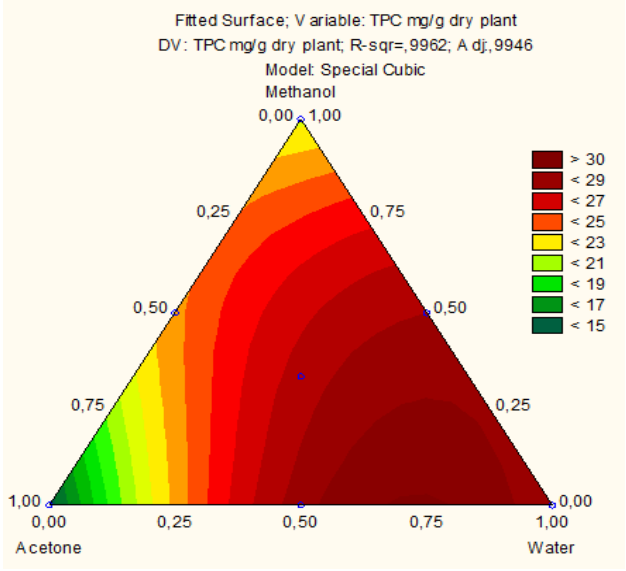


Figure 3B : Response surface plot of the special cubic model predicted TPC as a function of the acetone, water and methanol proportions(2D).

3.3. Analysis of variance (ANOVA)

In order to investigate the fitness and significance of the model, the analysis of variance (ANOVA) was performed. This analysis also shows the effects of individual parameters and interaction of variables of the mixture on response parameters as presented in Table 2.

The F-value compares the dissimilarity of the variances in the average responses at the design points and the equivalent assessed responses using the linear model (linear regression) with the predicted experimental variation as assessed from replicated design points (pure error). The model F-value of 349.14 implies that the selected model is statistically important (Table 3).

Table 2 :simplex axial design and results for mixture tested of TPC and TAC

	Acetone	Water	Methanol	TPC mg/g dry plant	TAC mg/g dry plant
1	0,00	0,00	100,00	21,69±0,26	168,09±1,23
2	0,00	50,00	50,00	27,99±0,31	94,18±1,56
3	0,00	100,00	0,00	28,01±0,13	69,87±1,16
4	16,67	16,67	66,67	21,68±0,31	111,10±1,93
5	16,67	66,67	16,67	11,59±0,00	83,76±1,31
6	33,33	33,33	33,33	27,43±0,25	130,69±1,89
7	50,00	0,00	50,00	23,71±0,75	78,26±1,09
8	50,00	50,00	0,00	28,57±0,10	142,72±2,00
9	66,67	16,67	16,67	29,62±0,60	153,43±1,30
10	100,00	0,00	0,00	14,44±0,29	45,57±1,94
All Runs				23,47±6,02	168,09±1,23

Table 3 . Analysis of variance (ANOVA) results for the mixture models

	SS	DF	MS	F	P
Model	476,04	5	95,20	349,14	0,00
Total Error	4,0904	15	0,27		
Lack of Fit	2,2870	1	2,28	17,75	0,000867
Pure error	1,8034	14	0,12		
Total adjusted	480,13	20	24,00		

The p-value (or Prob> F) is the likelihood of attaining the F-value. A value less than 0.05 designates that there is a statistically significant dissimilarity among the calculated means. A value larger than 0.10 defines that there is no dissimilarity among the calculated means, whereas the p-value (or Prob> F) is the probability of achieving the F-value. A value less than 0.05 specifies that there is a statistically significant dissimilarity among the means. A value superior than 0.10 designates that there is no difference between the calculated means (Sahu et al., 2009). Hence, in our study, the overall model p-value (probability of error value) being less than 0.0001 toughly confirms that the model is very significant.

The linear model explained that the variance in TPC content at the level of R^2 with a frequency of 62,02% and 57,87% for R^2adj. whereas, the quadratic and special cubic model explained better the variance in TPC content at the level of R^2 with an of ($R^{2:}$ 99, 15 %; R^2 Adj: 98, 86%) and ($R^{2:}$ 99,62 % 15; R^2 Adj: 99, 46%) respectively, indicating better how well fit of the model previously analysed is. Thus, the quadratic and cubic models were better in predicting the behaviour of the mixture.

The cubic model is given by the equation correlating the three variables. Hence, the analytical response is:

TPC = + 17,08 * acetone + 24,11 * water + 21,56 * methanol + 26,85 * acetone * water + 4,50 * acetone * methanol + 27,59 * water * methanol - 113,28 * acetone * water * methanol + 0,

To analyse the validity of the regression model, a raw residue analysis was performed.

A normal distribution was observed by comparing raw residue results to the expected normal values Figure 4.

The statistical analysis was carried out with the experimental values. The analysis of the main effects and their interactions in the form of analysis of variance (ANOVA) are presented in (Table 2) at the 95% confidence level ($p < 0.05$). Although the lack of fit was significant ($p < 0.05$) for the total content of polyphenols in the three extraction times, the cubic model was adjusted with significant probability ($p < 0.05$).

In order to examine the relative importance of the main effects and their interactions with statistical significance ($p < 0.05$), a standardized Pareto chart was employed (Figure 5). The main factors of different mixtures which extend beyond the reference line were significant at the level of 0.05.

Water (B) represented the most significant effect on phenolic compounds extraction followed by methanol (C) and acetone (A), and finally by the binary interaction of pure acetone with both methanol and water. Thus, the application of the mixture design was effective to establish the best proportion among the solvents for the extraction of phenolic compounds.

The desirability profile for TPC was generated with the values of 12.123 mg EGA/g – low (0.00); 21,404 mg EGA /g – intermediate (0.05); and 28.685 mg EGA /g – high (1.00). A graphic representation of the predicted values and desirability profile are shown in Figure 6. A predicted composition of the EO reference mixture and experimental data is presented in Table 4.

Table 4 .Approximate and experimentally determined percentage of inhibition (n = 8) for values obtained from desirability profile evaluation.

Data	Values measured experimentally			TPC	TAC
	Acetone	Water	Methanol		
Predicted values	33.33	33.33	33.33	27,43	130.69
Values measured experimentally				48.68	108.84

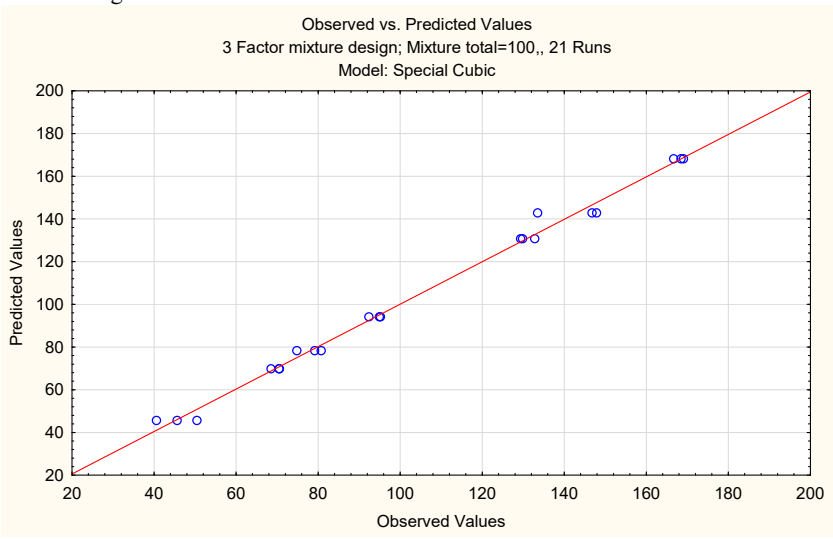


Figure 4: Predicted values (from the model) versus actual values (from experiments).

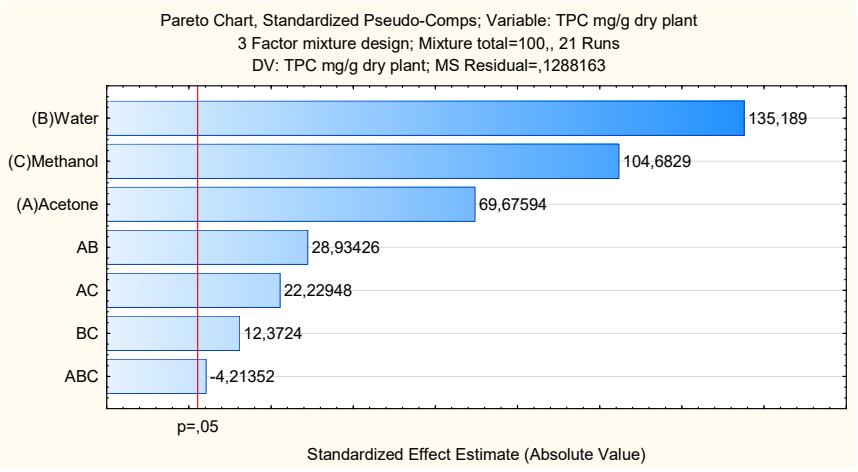


Figure 5: standardized Pareto's chart analysis of standardized effect for TPC

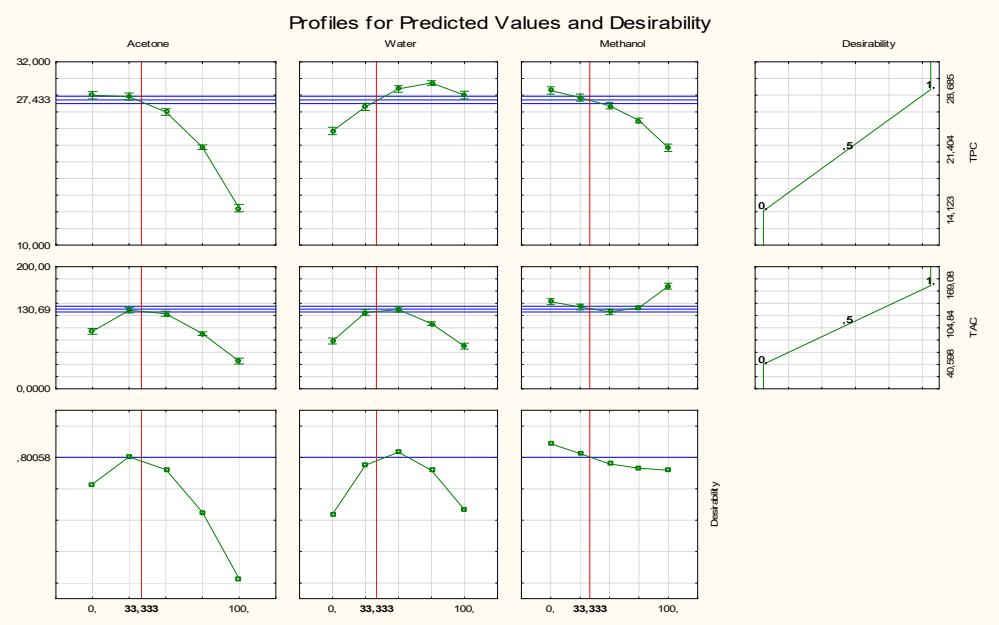


Figure 6: Desirability profile for optimization of references mixture of solvents

3.4. Total Antioxidant capacity (TAC):

Recently, the natural substances with high antioxidant ability have had a paramount importance due to their pharmacological properties (Laaroussi et al., 2020). Natural products constitute a dense source of antioxidants and are well-known for their efficacy and safety with regard to chemicals (Baj et al., 2018). Hence, the main objective of the current study was to examine the efficacy of the simplex-centroid mixture design method for the design of the most suitable extractor solvents mixture to recover high amounts of bioactive compounds with potent antioxidant properties.

Assays to measure TAC can be direct, which are founded on the capability to prevent the oxidation of a substance. On the foundation of the chemical reactions convoluted, TAC assays can also be separated into two classifications: hydrogen atom transfer (HAT) based procedures or on single electron transfer (SET) based approaches (Apak et al., 2016). The HAT-based techniques measure the aptitude of an antioxidant to quench free radicals by hydrogen contribution (Rubio et al., 2016). Response surfaces were identified for antioxidant activity as a meaning of the extraction solvent configurations. The linear, special cubic and quadratic models were experienced.

The highest antioxidant capacity values on the surface response and the contour graph are seen to occur with methanol or with equivalent proportions of water and acetone (Figure 7). The Highest antioxidant activity was achieved using the pure methanol and followed by a mixture of the studied extractor solvents (66.67/16.67/16.67). In contrary, the both solvents, water and acetone, were achieved the lowest antioxidant activity. This capacity reaches its maximum mixing the equal proportions of both solvents. The obtained results are in concordance with those reported by (Ribeiro et al., 2013). Statistical analysis indicated that the phenolic compounds are considered as the main contributors to antioxidant ability (Ousaaid et al., 2020c). The obtained results revealed that the extracts preformed using ternary solvent mixture with equal proportion and those with high proportion of methanol present high content of TPC. The combination of water and acetone exert their extraction effect synergistically, but the introduction of methanol to the mixture increases the extractable ability of the optimized procedure.

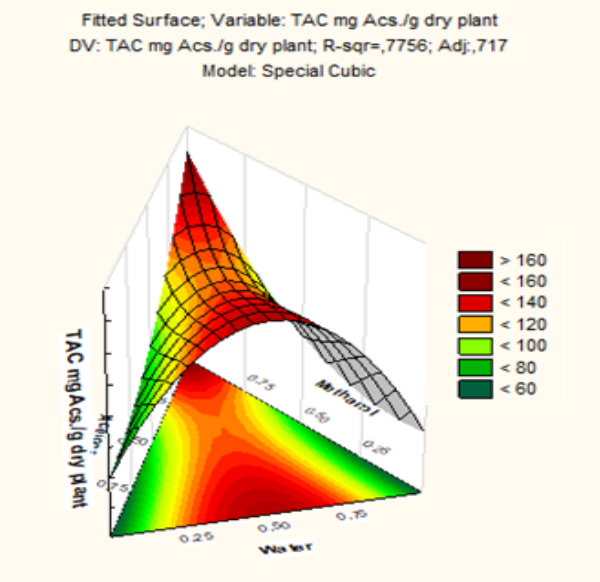


Figure 7 A: Response surface contour plot of the special cubic model predicted CAT as a function of the acetone, water and methanol proportions (3D).

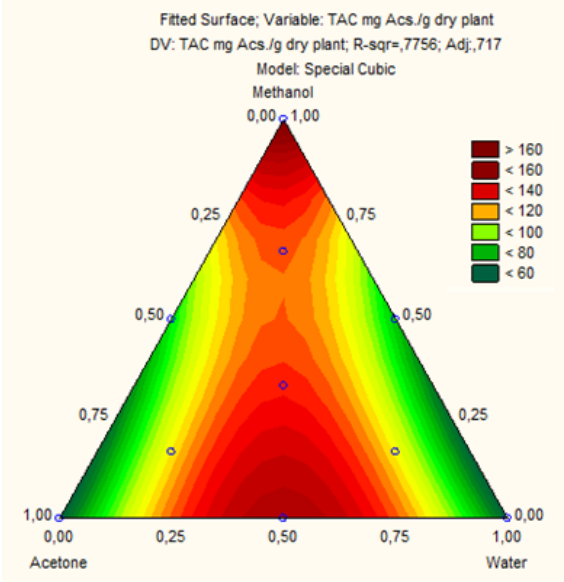


Figure 7B: Response surface plot of the special cubic model predicted CAT as a function of the acetone, water and methanol proportions (2D).

3.5. Analysis of variance (ANOVA)

ANOVA is applied to the linear, quadratic, and even special cubic models showed significant lack of fit at the 95% confidence level for total antioxidant capacity. The special cubic model is given by the following polynomial equation:

TAC = + 59,21 * acetone + 60,85 * water + 163,46 * methanol + 349,26 * acetone * water - 96,27 * acetone * methanol - 126,51 * water * methanol + 597,12 * acetone * water * methanol + 0,

In the statistical experimental design, the full cubic model, including linear and interaction terms, was chosen to describe the mixture composition. The responses and the consistent factors are optimized and modelled using ANOVA test to assess the statistical parameters via means of response surface technique (Table 5) and in order to evaluate which terms have more influence on the response variables using the *p* value (significance probability), always checking if the model was well adjusted to data by R^2 (coefficient of determination).

Table 5 : analysis of variance (ANOVA) results for the mixture models

	SS	DF	MS	F	P
Model	33727,77	6	5621,29	13,24	0,000002
Total Error	9759,45	23	424,32		
Lack of Fit	9531,47	3	3177,15	278,72	0,000000
Pure error	227,98	20	11,39		
Total adjusted	43487,23	29	1499,55		

According to Table 5, the model was significant since *p* value was lower than 0.01, and F value was high (F= 13,25). We also found a high correlation between observed and predicted values for antioxidant activity (Figure 8).

The standardized Pareto chart (Figure 9) shows that all extractor solvents and some of their interactions were significant except the binary combination of acetone-methanol and the ternary combination of the three solvents. It is worthy to note that the methanol solvent affects the extraction of antioxidant compounds from *Ammi visnaga*.

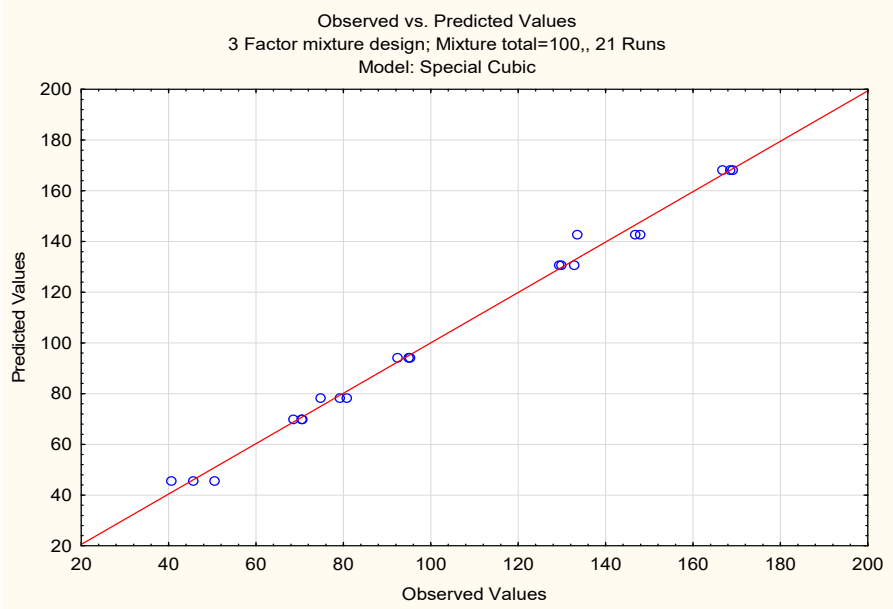


Figure 8: Predicted values (from the model) versus actual values (from experiments).

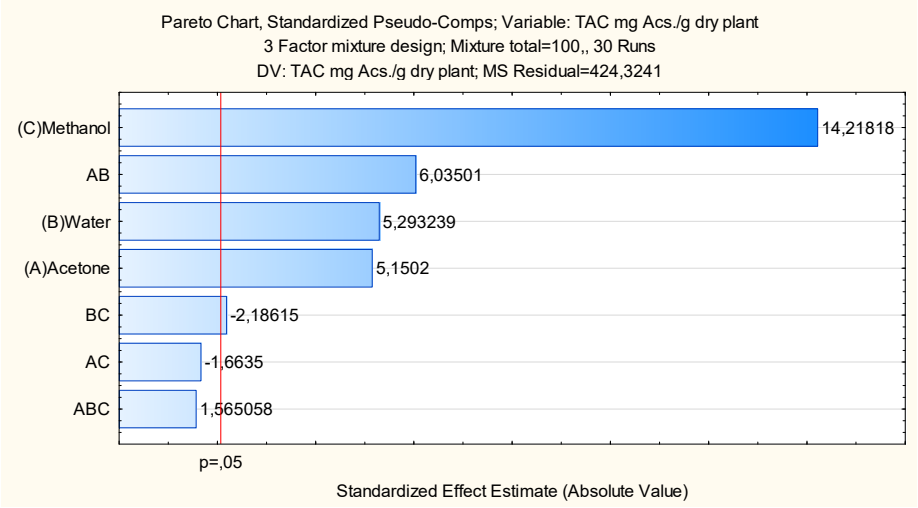


Figure 9: Analysis of Pareto chart of the standardized effects for TPC

4. Conclusion

In this work, the extraction with different solvents using simplex-centroid mixture design in order to find the optimal solvents mixture for extraction was studied. This study indicated that ternary mixture of water-acetone-methanol (66.67/16.67/16.67) was the most appropriate solvent mixture for extraction of phenolic compounds. While, the highest antioxidant activity occurs with methanol and ternary interaction between acetone, water, and methanol (66.67/16.67/16.67). Meanwhile, the pure methanol appeared to be the best extractor solvent of antioxidant compounds from *Ammi visnaga*.

5. Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of interest

There are no conflicts of interest.

Funding Statement

No funding.

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