Application of response surface methodology for bioactive compounds extraction and the variability in phytochemical profile from roots of *Ammi visnaga (L.) Lam.*

Zineb El Jabboury[1], *Smail* Aazza[2], *Meryem* Benjelloun[1], and *Lahsen* El Ghadraoui[1]

[1] Laboratory of Functional Ecology and Environmental Engineering, Faculty of Science and Technology, USMBA - Fez, Morocco.
[2] OLMANBGPE, Nador Multidisciplinary Faculty, Mohammed 1st University, Oujda, Morocco.

Abstract: *Ammi visnaga L. Lam.* is the most popular therapeutic plant in the Mediterranean region. Umbel is a traditional Moroccan remedy for diabetes, and oral care. The main goal of this study was to determine which extractor solvents had a particular affinity for the total phenolic content (TPC) found in the roots of *Ammi visnaga*. Using solvents chosen from the first step, the phenolic contents were maximized using the mixture design response surface methodology. The optimal solvent mixes of 10% methanol, 50% water, and 40% ethanol were used to prepare the root extracts. This study was designed to examine the phenolic profile of Moroccan *Ammi visnaga L.* roots using UHPLC/DAD–MS/MS Assessing the antioxidant capacities with the use of four complimentary tests (TAC, TPC, CUPRAC, and HCA). The obtained results showed the peak of antioxidant activity TAC (10.17±1.4 mg/g AAE) and TPC (15,99±0,4 mg GAE/g)).Various bioactive compounds (21 components) with different concentrations were identified, including isorhamnetin 3-O-glucoside (25.8%),and isorhamnetin 3-O-rutinoside (22.3%), which are the three most abundant individual phenolic compounds. The findings revealed the presence of numerous bioactive substances with important biological capabilities in the roots of *A. visnaga L.,* emphasizing the need for further experimental model validation of the data.

Keywords: Total phenolic content, antioxidant activity, phenolic profile, Ammi visnaga. L., roots

1. Introduction

Ammi visnaga (L.) Lam. is a native of North Africa, Europe, and North America, and a member of the Umbellifereae family [1]. Humans have had a very long history of learning about pharmacognosy and phytochemistry, which are really regarded as the foundation of both traditional and modern medicine. Actuality, a variety of uses for medicinal herbs are commonly practiced, including the extraction of bioactive substances with biological interest. The plant has been used to treat renal colic, vitiligo, psoriasis, arthritis, and rheumatoid arthritis in traditional medicine [2]. The beneficial properties of *Ammi visnaga (L.)* are attributed to its dense chemical composition. The phytochemical compounds of Ammi visnaga (L.) include khellinol, khellol, pyrones coumarins, khellin, 4-norvisnagin, visnagin, ammiol and visamminol, [3,4]. Considered an endless supply of phenolic compounds with well-established biological effects, medicinal plants [5]. Roots of *A. visnaga* are found to be effective ineradicating different bacterial strains and cancer cells [6]. An investigation into the phytochemistry of *A. visnaga* roots showed the existence of many bioactive substances, including docosanolide, angecin and 2,5-Dimethyl-5-Nitrohexanal [6]. Importantly, the appropriate extraction procedure using different solvents constitutes A crucial stage in obtaining the maximum concentrations of powerfully active bioactive chemicals.

Recent studies have looked at effective extraction methods to greatly reduce the extraction time, energy use, extraction cost, and organic solvent consumption [7].The extraction optimization using different extractor solvents mixture could provide promising findings. Design of experiments has highlighted its importance for extraction optimization [8]. Response surface methodology (RSM) is extensively used as a new avenue to optimize the phenolic compounds extraction to predict the most appropriate extractor solvent combination in vision to avoid wasting time, solvents, and reducing benchwork [8].

In this optic, the purpose of the current study was to determine the suitable extractor solvent combination for bioactive compounds extraction from roots *of Ammi visnaga.* In addition, the antioxidant ability of different optimized extracts was evaluated.

2. Material and methods

2.1. Plant material

The fresh plant roots were collected in the Taounate region of Morocco in April 2020 (34°33'47'N, 4°39'34'W). Before being extracted, the roots were finely powdered and dried at 40 ºC.

2.2. Choice of extraction solvents

Initially, we used sonication to do a solid-liquid extraction utilizing a variety of polar solvents, such as methanol, ethanol, water, and acetone.

2.3. Extraction procedure and sample preparation

The extraction was done in triplicates using three pure solvents, in accordance with the protocol that follows: 50 mg of the dried and ground roots of Ammi visnaga were extracted using a solvent combination in 1 mL of sonication for 20 minutes. Following a 15-minute, 6000 rpm centrifugation of the extracts, the supernatants were recovered and kept at 4ºC.

2.4. Evaluation of solvent impacts by simplex axial design

The vertex of the triangle formed by the various circumstances examined in the simplex-centroid design represents 100% of each individual solvent, and is composed of pure components. The median point, represented by a ternary mixture of 1: 1: 1, and the central points on either side representing permutations of the binary blends (1/2: 1/2: 0; 1/2: 0: 1/2; 0: 1/2: 1/2) are shown in Table 1. Known as Simplex Axial Design (SAD), this scheme is periodically expanded with internal points (axial ones) indicating 1/6 for the remaining solvents and 2/3 for one of the targeted solvents (Figure 1) [9].

Table 1: Different solvent mixtures.

	Ethanol	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

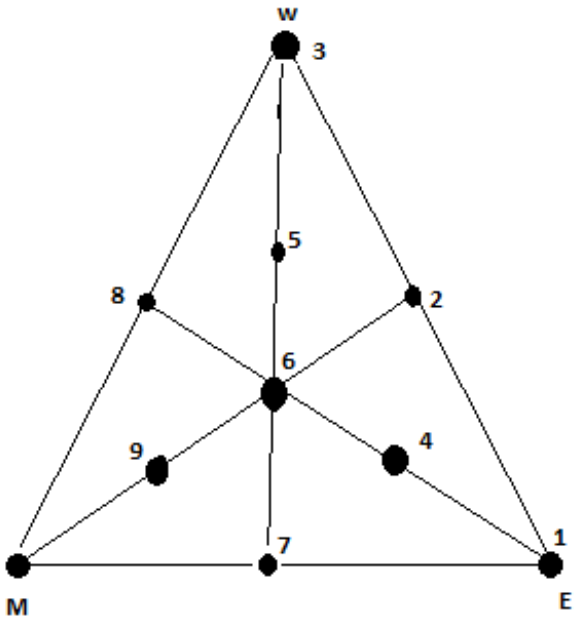

Figure1:*Simplex axial design* (SAD).
Subtitle: W = Water, M = Methanol, E = Ethanol

2.5. Total phenolic content (TPC)

The Folin-Ciocalteu technique, as described by [10], was used to calculate the total phenol concentration. To put it briefly, 450 µL of a Na2CO3 solution (75 g L-1) was added after 50 µL of the extract and 450 µL of Folin-Ciocalteu reagent (0.2 N) were combined for five minutes. After two hours of dark incubation at room temperature, the absorbance of each sample was measured at 760 nm using a Jenway 6505 UV/visible scanning spectrophotometer. The calibration curve concentration in an ethanol solution of gallic acid ($y = 1,6021x + 0,0683$, $R^2 = 0,997$). The findings are presented as mg Gallic acid equivalents (GAE) g-1 of dried plant, and the experiment was run in triplicate.

2.6. TAC assay

The TAC test was carried out using the procedure described by Prieto et al. (1999) [11]. Using ascorbic acid as the standard, a calibration curve was created to determine the antioxidant capacity. A measure of ascorbic acid equivalents (AAE) per g of DW was used to express the results.

2.7. CUPRAC assay

The methodology for this test was followed in accordance with Yilar et al. (2020) [12]. The findings were represented as mg of ascorbic acid equivalents (AAE) per g of DW after a calibration curve was created using various ascorbic acid concentrations as a reference.

2.8. Total dihydroxycinnamic acid derivative content (HCA):

The technique outlined by Didier et al. (2011) [13]was used to estimate the total HCA content.. Using chlorogenic acid

(CGA) as a reference, the calibration curve was used to calculate the extract's total HCA concentration. The findings were given as milligrams of CGA equivalents (CGAE) per gramme of DW.

2.9. Statistical analysis

Analysis of variance (ANOVA) was carried out to look at the developed mixture design's significance and fitness. The effects of individual factors and variable interactions on the TPC and mixture are also displayed using ANOVA.
The multiple regression model's fittest ($p < 0.05$) was found using variance analysis (ANOVA), which was then used to assess the variables' significant effects and their interactions. The model's contour and response surface diagrams were produced using the regression coefficients. The STATISTICA version 10 free version was used to conduct the analysis.

3. Results and Discussion

3.1. TPC/CUPRAK/HCA/TAC

The results of total phenolic content obtained for extracts of the roots of *Ammi visnaga* are given in Table 2.

Table 2: Total phenolic contents obtained for the roots of *Ammi visnaga* extract

	Ethanol	Methanol	Water	Roots
1	0.00	50.00	50.00	10.61±0.29
2	0.00	100.00	0.00	13.65±0.86
3	16.67	16.67	66.67	6.82±0.22
4	16.67	66.67	16.67	12.91±0.57
5	33.33	33.33	33.33	12.89±0.30
6	50.00	0.00	50.00	15.53±1.62
7	50.00	50.00	0.00	15.99±0.43
8	66.67	16.67	16.67	6.71±0.51
9	100.00	0.00	0.00	12.75±0.39
10	0.00	0.00	100.00	4.37±0.07

Results showed that the total phenolic content (TPC) ranged from 4,37±0,07 to 15,99±0,43 mg GAE/g, for a dry roots. This highlights the influence of solvent extraction. The obtained results of antioxidant assays are presented in table 3. The results obtained showed significant antioxidant power. Accordingly, based on the data obtained, the root extract had the greatest level of antioxidant activity as determined by the CUPRAC assay, with a value of 0.37±0.0009 mg AAE/g. While, the results found using TAC and HCA were 10.17±1.46 mg GAE/g and 3.58±0.11 mg CGAE/g, respectively. The study's results are better than those that El Karkouri et al. reported [14]. In the same line, Aziz et al. found that the TPC of seed extract was the most interesting than that of root extract with TPC values of 366.57 ± 2.86 and 270.78 ± 2.86 mg GAE/g dry weight of the dry extract, [6]. It has been found that binary combination of water and other organic solvent constitutes the suitable combination for recovering the highest amount of phenolic content [15,16].

Table 3: The antioxidant activity of the extracts of *A. visnaga* roots.

	TAC mg/g GAE	**CUPRAC mg/g AAE**	**HCA mg/g CGAE**
Root	10.17±1.46	0.37±0.009	3.58±0.11

3.2. Analysis of variance (ANOVA)

In the table 4, Also at the model which shows the significant effect of the processing variables on the TPC ($p<0.001$).

Table 4: Analysis of variance results for different statistical models

Model	**SS Effect**	**df Effect**	**MS Effect**	**F**	**P**	**R-Sqr**	**R-Sqr adjuster**
Linear	112,60	2,00	56,30	4,70	0,01	0,25	0,20
Quadratic	277,59	3,00	92,53	48,89	0,00	0,89	0,87
SpecialCubic	6,16	1,00	6,163	3,611	0,001	0,91	0,88
TotalAdjusted	435,61	29,00	15,02				

The special cubic model yielded the greatest fit and enhanced coefficients of determination. It also explained 91% (R2 91%; 88% R2adj) of the variance and corrected for roots. Comparable outcomes have been reported by [17]. A statistically significant interaction between three-component systems could be shown by this examination. This might be explained by interactions between the three chosen solvents and the model's increased complexity.
With a frequency of (R2 25%; 20% R2adj), the linear model provided an explanation of the variance at the R2 level for roots. The regression analysis's fit was enhanced by expanding the linear model into a quadratic one (Table 5).

Table 5: Coefficients of the overall fit for the regression model ($p < .05$).

	SS	**DF**	**MS**	**F**	**P**
Model	396,35	6,00	66,06	38,70	0,00
Total Error	39,26	23,00	1,71		
Lack of Fit	30,23	3,00	10,08	22,32	0,00
Pure error	9,03	20,00	0,45		
Total adjusted	435,61	29,00	15,02		

A unique cubic model was selected because of its better coefficient of determination in order to assess the statistical impact of the solvent mixture composition on the total phenolic content of plant root extracts. The regression models for the experiment are shown below: (X: ethanol, Y: methanol, Z: water)
TPC-roots=+4,72*x+7,20*y+9,82*z+5,98*x*y+33,12*x*z+18,90*y*z+45,98*x*y*z+0,

In the roots, the TPC was positively and linearly influenced by methanol (y) and water (z), respectively. The obtained results show that ethanol (x) has the lowest coefficient; and the smallest proportion of TPC.
In the group of the binary interactions, the use of ethanol reduced the extraction ability of methanol (xy), without affecting the extracting power of water (z). The ternary interaction (xyz) showed a synergistic effect between the components of the mixture for roots.

4. Surface analysis

4.1. TPC from roots

The response surface for TPC from roots, shown in figure 2, is a role played by water, methanol, and ethanol. The highest TPC value on the contour graph is seen to occur near a focal point with equivalent proportions of all four solvents. While, the methanol/ethanol solvents are antagonistic, binary effects are all synergic. The ternary model coefficients contribute synergistically to the total polyphenol content. Both extractions of compounds with antioxidant activity and total polyphenol content have shown similar results to those of *Trichiliacatigua* [18].

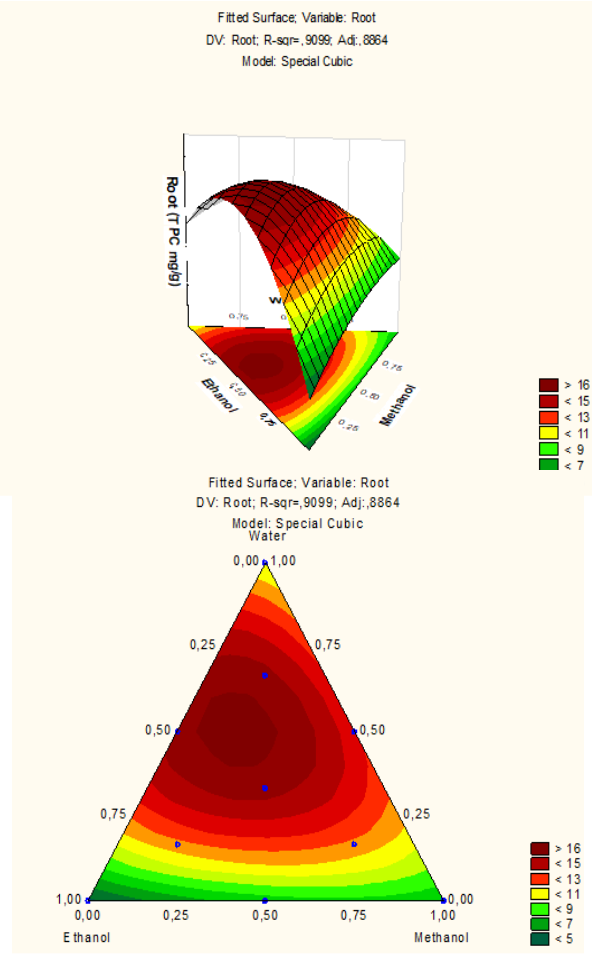

Figure 2: The special cubic model's response surface contour plot predicted TPC as a function of the ratios of methanol, water, and ethanol.

4.2. Pareto chart analysis

In Pareto charts, each effect and its combination is represented by a bar with a decreasing significance level. This aids in the visual representation of the influencing variables and the extent of their effect from a graphical perspective. With a p-value significance criterion of 5%, the Pareto diagrams provide a ranking of the most important components to the least significant ones. A component that has a negative impact has a low level value that is greater than a high level value [7]. Figure 3 show the Pareto chart of the effects of the studied variables on the polyphenol content. Water was the solvent that influenced mostly and positively phenolic extraction, followed by methanol. Ethanol was the third parameter that showed a significant positive effect for the extraction from roots while the binary integration between water and ethanol (AC) comes in the third place, also with a positive effect for roots.

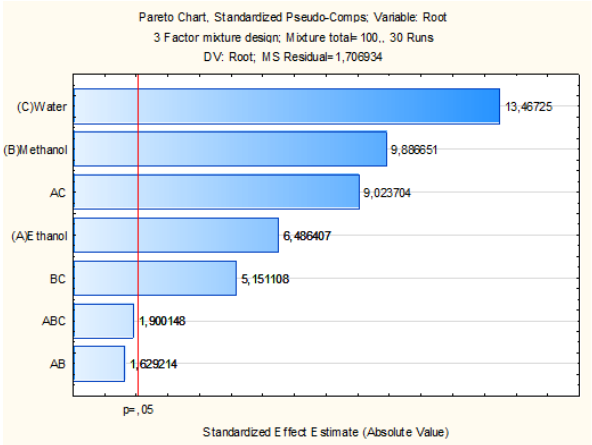

Figure 3: Pareto charts of the standardized effects on TPC from roots.

5. Phenolic profile of optimized extraction of *Ammi visnaga roots*

The consequences of determining the phenolic profile of *A. visnaga* root optimized extracts are displayed in Figure 4. The phenolic profile was determined using HPLC to provide a scientific basis of the phytochemistry of roots of Moroccan *Ammi visnaga* to show on the one hand the richness of the roots and also to encourage their use in the traditional pharmacopeia. The quantification and determination of the phenolic profile of 0,007 g yield roots revealed 21 compounds with different levels.
Isomesterin 3-O-glucoside is the most prevalent individual phenolic component seen in large concentrations (5,51 µg/mg) followed by isorhamnetin_3-O-rutinoside (4,77µg/mg), p-coumaric acid (2,47µg/mg), chlorogenic acid (1,62µg/mg), and caffeic acid (1,09µg/mg).The obtained results match those that have been reported by Bencheraiet et al. [16].
A collection of chemically altered metabolites, such as apiumetin-O-glucosid, junipediol A 4-O-glucoside, and junipediol A 8-O-glucoside, describe the metabolomics of the bioactive components of *A. visnaga* L. roots [17]. The most intriguing bioactive substances are flavonoids, which have a

broad spectrum of biological activities that include immunostimulant, antibacterial, anticancer, antidiabetic, and antioxidative actions [21].

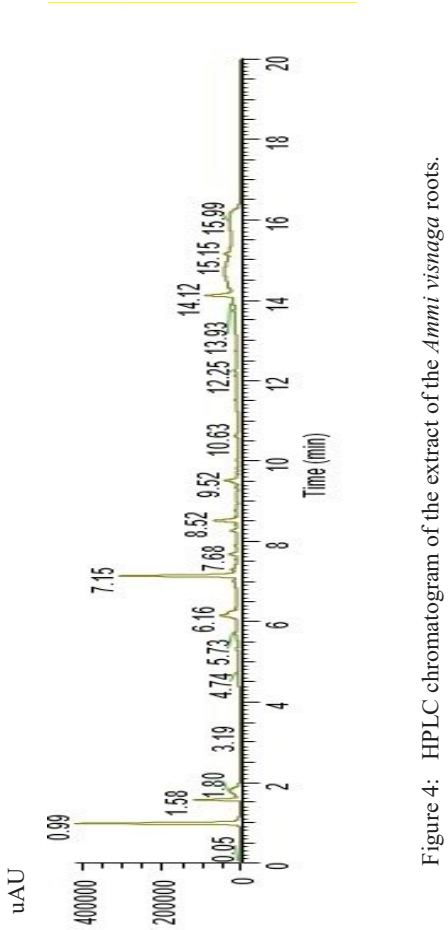

Figure 4: HPLC chromatogram of the extract of the *Ammi visnaga* roots.

6. Conclusion

The study demonstrated the impact of extractor solvents on phenolic extraction using simplex-centroid mixture design in order to determine the most appropriate combination for TPC extraction. The obtained findings indicate that the binary combinations of water and ethanol or ethanol and methanol with equal proportions were the most suitable mixture for extraction of phenolics. The optimized extract revealed the presence of wide range of bioactive components, including isorhamnetin_3-O-glucoside, isorhamnetin_3-O-rutinoside, p-coumaric acid, chlorogenic acid, caffeic acid. Through analysis of model-derived response surfaces, this study indicated that linear and binary mixtures had a synergistic effect on the extraction of TPC from roots of the plants.

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