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Extractive optimization of antioxidants and phenolic compounds from *Anacyclus pyrethrum*

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Abstract

Anacyclus pyrethrum (L.) Lag. is a Moroccan endemic species well appreciated as a remedy against toothache, digestive disorders, and as a tonic agent for the nervous system. This work aims to select the best solvents for extracting antioxidants and optimize their extraction using a surface mixture design. In this study, eleven solvents with different polarities were screened for their efficiency to extract total phenolic compounds and other molecules endowed with antioxidant activity. The antioxidant activity was measured using 2,2diphenyl-1-picrylhydrazyl (DPPH) scavenging and total antioxidant activities. The selected solvent was subjected to response surface methodology using a simplex axial mixture design to optimize the extraction of polyphenols and antioxidants. The results showed a significant influence of solvent nature on extraction. Water, ethanol, chloroform, and methanol were the most effective solvents to achieve good polyphenol yields. The best yield is obtained using the tertiary mixture "water-methanol-ethanol". The anti-radical activity in A. pyrethrum was significantly influenced by the extraction solvent's nature and the mixture's nature. The percentage of inhibition of DPPH was higher in both extracts obtained with the ternary mixture and binary mixtures "water-methanol" and "water-ethanol". the best total antioxidant capacity was observed for pure water, followed by binary mixtures including water. This study revealed a good synergetic effect between water and both ethanol and methanol on extraction efficiency. Furthermore, the ternary mixture with the following proportions: water 75%; ethanol 11%; methanol 14% was the most efficient.

Keywords: African pyrethrum; antioxidant activity; polyphenols; solvent extraction; surface design methodology

Introduction

Morocco is a country of culinary flavors, aromas, scents, and medicinal know-how inspired by an extraordinary floristic diversity, which finds its richness thanks to a Mediterranean climate shaped by oceanic

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Interest in the therapeutic use of plants is beginning to draw its foundations from pharmacopeia. The African pyrethrum or pyrethrum chamomile (*Anacyclus pyrethrum* L.) is one of the emblematic aromatics and medicinal plants of Moroccan pharmacopeia and whose virtues continue to arouse the scientific curiosity of several research groups around the world. This species belonging to the family of Asteraceae is a perennial herbaceous plant, endemic to Morocco and Algeria (Amine *et al.*, 2017). Most often used as a tonic, stimulant, revitalizer, and rubefacient (Kumar and Choudhary, 2016). The plant displays a range of effects, including antidiabetic, insecticidal, immunostimulatory, antimicrobial, analgesic, aphrodisiac, and antioxidant potentials, in addition to various other important medicinal properties (Elazzouzi *et al.*, 2022).

A. pyrethrum is known to contain a diverse range of chemical constituents, including steroids, alkaloids, flavonoids, saponins, and tannins (Subasri and John, 2015). Molecules that have caused a lot of ink to flow thanks to their antioxidant and antibacterial potential (Bouaziz *et al.*, 2009). Furthermore, studies have identified various chemical constituents in *A. pyrethrum*, such as triterpenes and bio-active N-alkylamides, which are responsible for its aphrodisiac properties. Notably, alkaloids are prevalent in the plant's roots, while tannins and flavonoids are more abundant in the aerial parts, with the highest levels of flavonoids and polyphenols being found in the flowers(Pandey *et al.*, 2018)

Extraction is a crucial step in the isolation, identification, and use of phenolic compounds (Yahyaoui and Ghazouani, 2017), however, the extraction technique is influenced by several parameters, among others, the nature of the solvents used for this purpose (Naczk and Shahidi, 2004). It should therefore be noted that the extraction procedure and solvent used to obtain a polyphenol-enriched extract can greatly influence biological activity. To this end, ultrasound-assisted extraction (UAE) is a cost-effective technique that provides a simple and effective alternative to conventional extraction techniques due to its low instrumental requirements (Ghafoor *et al.*, 2009). While, the ultrasonic bath is commonly employed for phenolic extraction due to its cost-effectiveness, availability, and the ability to process multiple samples simultaneously (*Chemat et al.*, 2017). The use of UAE can prevent the possible chemical degradation of the target compounds due to the lower chemical involvement and shorter extraction time (Wang and Weller, 2006). Response Surface Methodology (RSM) is a set of statistical and mathematical techniques that are often successfully used to determine the effects of different variables (ultrasonic time, solvent content, temperature...) on the total polyphenol yield and to optimize the process of extraction of phenolic compounds from plant processes (Amami *et al.*, 2017). Its main advantage is that it reduces the number of experimental trials required to evaluate several variables and their interactions (Ma *et al.*, 2010).

While it is true that several efforts are being made to optimize the extraction of phenolic compounds via the UAE technique, the fact remains that little attention is generally paid to the selection of the appropriate solvent or solvent system for extraction, Despite all the extraction applications reported in the literature, few systematic studies study the effect of altered solvent proportions in binary and ternary mixtures (DiCiaulaa *et al.*, 2014), and even less attention is generally paid to the selection of an appropriate solvent or solvent system for the extraction of phenolic compounds. Faced with a promising but increasingly demanding national and international market, a growing concern for clean green chemistry, and an awareness of the concept of

sustainable development, scientific bodies require moving more and more towards the strands of this innovative research component working in the optimization of the extraction of phenolic compounds from this heritage plant of Moroccan flora.

This research is directed toward two primary objectives. Firstly, it involves assessing the ability of solvents with different polarities to extract phenolic compounds and to develop a highly effective mixture of three or two solvents to understand their interactions using the mixture design methodology. Notably, the extraction was performed using an ultrasonic bath apparatus. Subsequently, the second objective is to optimize the antioxidant profile of the extracts from the aerial part of *A. pyrethrum*.

Materials and Methods

Solvents

Acetone, hexane, VWR, Leuven, Belgium. Chloroform, LAB-SCAN, Dublin, Germany. Ethanol, methanol, ethyl acetate, dichloromethane, Fisher Scientific, New Jersey, USA. Petroleum ether, diethyl ether, butanol, Merck, Darmstadt, Germany.

Chemicals

L(+)-Ascorbic acid, Folin Ciocalteu's phenol reagent, Sodium carbonate, gallic acid, Sulfuric acid 95-97%, Merck, Darmstadt, Germany. DPPH, Acros Organics, New Jersey, USA. Ammonium molybdate, Sodium phosphate, Panreac, Barcelona, Spain.

Plant material

Samples of *A. pyrethrum* were collected at the flowering stage during May 2018 in the Timehdit region of the Moroccan Middle Atlas. Once brought back to the laboratory, the aerial part of the plant was dried in the shade in a dry place. The sample was then ground into a fine powder and stored in dark pillboxes for further analysis.

Solvent Extraction using ultrasonic bath

Extracts were prepared in triplicate by adding 1 ml of the solvent (pure solvent and mixtures) to 50 mg of plant powder (aerial part) and sonicating in an ultrasonic bath (Bandelin, Sonorex, RK52, Berlin, Germany) at room temperature for 30 min. The extract is obtained after centrifugation at 10,000 rpm for 10 minutes and stored in the dark at 4 °C. The first step of the extraction consists of screening with solvents of different polarity, namely water, ethanol, methanol, acetone, ethyl acetate, dichloromethane, chloroform, hexane, petroleum ether, di-ethyl ether, and butanol to select the best solvent for the next step. The second step consists of the extraction of the pure selected solvents and their mixtures.

Total phenolic compounds (TPC)

Total phenolic content (TPC) was determined spectrophotometrically using the colorimetric method based on the Folin-Ciocalteu reagent (Libbey and Walradt, 1968) with modifications.

A 50 μ L sample uptake was mixed with 450 μ L of a 10-fold diluted Folin-Ciocalteu reagent solution. After 5 minutes of incubation at room temperature, 450 μ L of a Na₂CO₃ solution (75 g L⁻¹) was added. All samples were then incubated for 2 hours at room temperature in the dark, and their absorbance was read at 760 nm in a Jenway 6505 UV/visible scanning spectrophotometer. Depending on the equation of the calibration curve (y = 2.8388x + 0.0556 R² = 0.9994), the concentration varied from 0.062 to 1 mg mL⁻¹ in an ethanolic solution of gallic acid. The results are expressed in gallic acid equivalent (GAE) g⁻¹ dry mg of the plant.

DPPH free radical scavenging activity

The free radical elimination activity of the extracts of the studied plant was evaluated using the dark purple DPPH (2.2-dipphenyl-1-picrylhydrazyl) according to the procedure described by (Brand-Williams *et al.*, 1995). In this part of the study, a volume of 25 μ L of the samples was added to 1 ml of an ethanol solution containing 60 μ M DPPH. Absorbance measurements were performed at 517 nm after an incubation period of 60 min at room temperature. The absorbance of a blank sample containing the same amount of ethanol and DPPH solution served as a negative control. Percent inhibition of free radical scavenging activity in each extract was calculated as follows:

% inhibition = [(Abs control - Abs sample / Abs control) \times 100] (1)

Three measurements were performed for each sample and the results were expressed as average \pm standard error.

Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) of the samples is determined by the phosphomolybdenum method. This technique is based on the reduction of molybdenum Mo (VI), which is in the form of MoO42-molybdate-molybdenum ions MoO_2+ - in the presence of the extract to form a green phosphate/Mo(V) complex with acidic pH (Prieto *et al.*, 1999). To a volume of 50 µL of the sample solution, 1 mL of the reagent solution (sulfuric acid 0.6 M, sodium phosphate 28 mM, and ammonium molybdate 4 mM) was added. After 90 minutes of incubation in a double boiler at 95 °C. The absorbance of the mixture was measured at 695 nm against white in a spectrophotometer. The aqueous solution of ascorbic acid, which served as the calibration curve (y = $1.7355x + 0.235 R^2 = 0.9999$), covered a concentration range from 1.0 to 0.0625 mg ml⁻¹. The results reported (antioxidant activity in ascorbic acid equivalents) are average values expressed in g ascorbic acid equivalents per g dry plant.

Evaluation of solvent effects by simplex axial design

A study to optimize the extraction of polyphenols was conducted using a mixing plan. Two types of standard designs are commonly used for extraction experiments with solvent mixtures: Simplex-Lattice Design and Simplex-Centroid Design. Both designs will evaluate the triangular response surface at the tops and the centroids (Montgomery, 2012).

In the simplex centroid design, the various conditions tested form a triangle with the pure components at the top representing 100% of each solvent. The central points on each side represent the permutations of binary mixtures (1/2: 1/2: 0; 1/2: 0: 1/2; 0: 1/2: 1/2), and the focal point is a ternary mixture (1: 1: 1) (Sampaio et al., 2015).

The Simplex-Centroid Design with axial points in three repetitions was selected to determine the combination of solvents: water (W), ethanol (E), and methanol (M). This design allows the evaluation of linear models: water, ethanol, and methanol (W, E, and M), quadratics: water-ethanol, water-methanol, and ethanol-methanol (WE, WM, and EM) and special cubic: water; ethanol; methanol (WEM) for study response.

Statistical analysis

All experiments for solvent fraction selection, as well as total phenolic compound content, free radical capture activity (DPPH), and total antioxidant capacity, were performed in triplicate, and results were expressed as mean \pm standard error.

To validate the multiple regression model ($p \le 0.05$), an analysis of variance (ANOVA) was performed to evaluate the significant effects of the variables and their interactions. The response and contour area plots of the model were generated from the regression coefficients. Statistical analyses were performed using the free version of STATISTICA version 1StatSoftOFT, INC., 2011).

Results and Discussion

Extraction solvent screening

It is widely accepted that extraction yield is dependent on several factors that can influence extraction efficiency, and the nature of the solvent is one of the most preponderant in this process. In the first part of this study, eleven increasing polarity solvents were employed to extract phenolic compounds from the aerial part of *A. pyrethrum*.

The variability of polyphenol levels in the aerial part of *A. pyrethrum* depending on the extraction solvent are shown in Figure 1. The yield of total phenolic compounds ranged from $(16.37 \pm 0.15 \text{ mg GAE} / \text{g} dry \text{ plant})$ recorded for water to $(0.59 \pm 0.02 \text{ mg GAE} / \text{g} dry \text{ plant})$ obtained for diethyl ether extract. This difference was very large, up to 27-fold. Polyphenolic compounds typically possess polar characteristics, and the high polarity of water as a solvent makes it particularly efficient for extracting such polar compounds. In contrast, both butanol and ethyl ether have lower polarity, which can limit their efficacy in extracting these polar phenolic compounds (Kaczorová *et al.*, 2021). The results depicted in this figure reveal a great significant influence of the solvent extraction power on TPC yield. Thus, confirming that the extractability of phenolic compounds is highly dependent on the nature of the solvent. Consequently, water, ethanol, and chloroform were the most effective solvents to extract a good yield of polyphenols. Thereby, water and ethanol were chosen to perform the mixture design optimization. Whereas, methanol which comes in the 4th position was chosen instead of chloroform, due to its lack of miscibility with water.



Figure 1. Total phenolic compounds based on different solvents used

Extraction using solvent mixtures

Many studies suggest that using aqueous mixtures of organic solvents is the preferred method for extracting phenolic compounds from plant sources (Venkatesan *et al.*, 2019). The selection of the best three solvents was based on the values of total phenolic compounds (TPC) of the eleven solvents used, in which water extracted the highest amount of phenolic compounds ($16.37 \pm 0.15 \text{ mg GAE}$ / g dry plant) compared to other solvents, followed by chloroform $13.97 \pm 0.36 \text{ mg GAE}$ / g dp) and ethanol ($12.68 \pm 0.34 \text{ mg GAE}$ / g dp). We subsequently exchanged chloroform by the 4th solvent; methanol ($7.00 \pm 0.10 \text{ mg GAE}$ / g dp), because the water-chloroform-ethanol mixture did not give good results.

Based on the results of single solvent extraction at a time, we opted for the blending of the three solvents that yielded the best polyphenol yields, namely water, methanol, and ethanol, and varied the percentages of the solvents that make up the mixtures according to the simplex centroid design.

The results obtained in Table 1 unquestionably reveal that the best yield was obtained by the use of the equi-proportional tertiary solvent mixture "water-methanol-ethanol" followed by the binary mixture "water-methanol". On the other hand, pure methanol extracts presented a low amount of TPC, and its combination with ethanol lowered even more phenolic extraction indicating an antagonistic interaction between the two solvents. Whereas pure water extracted good amounts of TPC.

Crude extract	Extract (solvent	TPC mg Gallic acid / g dry plant	DPPH (%)	TAC mg Ascorbic acid / g dry plant	
1	Water $(W)(1)$	16.99 ± 0.07	36.83 ± 0.22	97.01 ± 0.05	
2	Methanol (M) (1)	6.60 ± 0.14	17.45 ± 0.44	37.66 ± 0.05	
3	Ethanol (E) (1)	11.19 ± 0.01	3.93 ± 0.11	27.51 ± 0.12	
4	W: M (1/2:1/2)	17.65 ± 0.06	78.29 ± 0.23	81.24 ± 0.17	
5	W: E (1/2:1/2)	11.08 ± 0.02	77.27 ± 0.19	82.67 ± 0.05	
6	E: M (1/2:1/2)	4.27 ± 0.01	11.15 ± 0.19	27.04 ± 0.12	
7	W: E: M (1/3:1/3:1/3)	19.06 ± 0.13	78.29 ± 0.23	73.91 ± 0.08	

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

Analysis of mixture optimization by response surface methodology

Linear, quadratic, and special cubic models were tested. The lack of fit of these surfaces was evaluated using an ANOVA test. The cubic model is given by equation (2), which correlates the three variables and is the analytical response:

 $\begin{array}{l} \textbf{TPC} = 16.99 * (\% \ \text{Water}) + 6.60 * (\% \ \text{Methanol}) + 11.186 * (\% \ \text{Ethanol}) + 23.43 * (\% \ \text{Water}) * (\% \ \text{Methanol}) + 7.98 * (\% \ \text{Water}) * (\% \ \text{Ethanol}) + 1.49 * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 192.86 * (\% \ \text{Water}) * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 1.49 * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 192.86 * (\% \ \text{Water}) * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 1.49 * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 192.86 * (\% \ \text{Water}) * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 1.49 * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 192.86 * (\% \ \text{Water}) * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 1.49 * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 192.86 * (\% \ \text{Water}) * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 1.49 * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 192.86 * (\% \ \text{Water}) * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 1.49 * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) + 192.86 * (\% \ \text{Water}) * (\% \ \text{Methanol}) * (\% \ \text{Methanol}) * (\% \ \text{Ethanol}) = 100 \times 10^{-10} \times 10$

Overall, according to equation 2, all the solvents and their mixtures had a positive effect on TPC extraction which was mostly and positively affected by the ternary mixtures. The linear coefficient for water is much higher than the other pure solvents indicating its bigger positive effect on TPC extraction followed by ethanol. Meanwhile, in the binary interactions, the mixture of methanol-water had the greatest positive effect followed by ethanol. Whereas, mixing the two organic solvents resulted in the lowest positive effect.

The response surface plot of the special cubic model for TPC extraction is depicted in Figure 2.





Figure 2. The response surface of the Special Cubic Model predicts TPC based on the proportions of Water, Ethanol, and Methanol. Two-dimensional (a) and three-dimensional (b).

According to this figure, pure ethanol and methanol extracted the lowest amounts of TPC, while pure water yielded higher amounts. The dark red area with the highest value on the contour plot, which is between the position of equal proportions of all three solvents and pure water, indicates the best solvent composition for the extraction of the phenolic compounds. We can also conclude that increasing the water content in binary mixtures containing both methanol and ethanol increases their ability to extract total phenolic compounds, which is in agreement with the results obtained for the extraction of phenolic compounds from *Cannabis sativa* waste (Aazza, 2021). Furthermore, the ternary mixtures were more efficient for TPC extraction. According to (Mohammedi and Atik, 2011), the use of mixed solvents leads to a strong enrichment of polyphenolic extracts. It is believed that the superiority of mixed solvents is due to the higher solubility of phenolic compounds in the extracts obtained with mixed solvents compared to those obtained with pure solvents (Trabelsi *et al.*, 2010). Several studies have confirmed that the use of water in combination with organic solvents contributes to the creation of a moderate polar environment that ensures the extraction of phenolic compounds (Liyana-Pathirana and Shahidi, 2006). According to the work presented by (Aazza, 2021) and (Ksibi *et al.*, 2015), on the extractive capacity of solvents concerning polyphenols, the addition of water to the solvent improves the extraction rate, but too high a water content resulted in increased simultaneous extraction of other compounds

and, consequently, a decrease in phenolic concentrations in the extracts. Conversely, Turkmen *et al.* (Turkmen *et al.*, 2007) reported that aqueous solvents were more effective in extracting total phenolic compounds, and Santos *et al.* (Santos Felix *et al.*, 2018), stated that the highest phenolic compound contents and antioxidant capacity of the extracts from *Spondias mombin* L. were obtained with pure water or with a mixture of water and acetone/ethanol, while (Liyanapathirana and Shahidi, 2005) reported that water-ethanol mixtures were most effective in extracting phenolic compounds from whole wheat and soft and durum wheat bran. Thus, indicating that the ability of solvents to extract phenolic compounds also depends on the plant material. Another possible reason for the increased efficiency in the presence of water could be the increase in swelling of the plant material by water, which increases the contact area between the plant matrix and the solvent (Xiao *et al.*, 2008).

Evaluation of antioxidant activities

Anti-radical activity /DPPH free radical scavenging activity

The main objective of this study was to assess the antioxidant and antiradical activity of the phenolic compounds and other compound-endowed antioxidant activity present in the *A. pyrethrum* extracts and also to check whether there is efficacy between the different mixtures of the studied solvents.

The results of the antiradical activity assessed by DPPH free radical scavenging activity and the total antioxidant activity in *A. pyrethrum* extracts based on the used pure and solvent mixtures are shown in Table 1. The results reveal that the anti-radical activity of *A. pyrethrum* extracts was significantly influenced by both the nature of the extraction solvent and the composition of the mixture.

As stated in Table 1, the percentage of inhibition of the DPPH radical is much higher in both extracts obtained with the ternary mixture and the binary mixtures "water-methanol" and "water-ethanol". As for total antioxidant capacity (TAC), the best activity was observed for pure water, followed by binary mixtures of "water-ethanol" and "water-methanol". whereas, pure ethanol exhibited the lowest activity, besides its combination with methanol.

To better understand the effect of solvent concentrations on antioxidant extraction, solvent mixtures at different levels were set by response surface methodology (RSM).

Response surface analysis of the antioxidant activities

Like total phenolic compounds, response surface technology would be the appropriate tool for a more judicious assessment of the percentage of DPPH inhibition and the total antioxidant capacity (TAC) based on extraction solvent compositions.

• Evaluation of DPPH free radical trapping

Response surfaces were determined for extracts of antioxidant power by DPPH based on extraction solvent compositions. The linear, quadratic, and special cubic models were tested. The response surface for DPPH is shown in Figure 3 based on the proportions of water, methanol, and ethanol.

In general, pure solvent extracts presented the lowest DPPH free radical scavenging activities compared to solvent mixtures. Among the pure solvents, water extracts exhibited the best activity followed by the ones of methanol. It is noteworthy that, increasing the water percentage in ethanol and methanol mixtures increases their DPPH free radical scavenging capacity, which reaches its maximum when the water percentage is between 50 and 75%. This scavenging capacity starts to lower when mixtures contain over 75% of water. The same results were reported by (Aazza, 2021).

It is noted that the highest value on the contour graph occurs with the ternary interactions of the threesolvent followed by the binary interactions of pure water with ethanol and methanol. So, the binary effects of water-ethanol and water-methanol were synergistic while methanol: ethanol binary effects were antagonistic. Accordingly, numerous prior studies have unequivocally indicated that the incorporation of aqueous mixtures of organic solvents, such as ethanol, methanol, alongside water for extraction significantly enhances the



antioxidant effectiveness of the majority of botanical products, as opposed to using water alone (Venkatesan *et al.*, 2019).

Figure 3. The response surface of the Special Cubic Model predicts DPPH based on the proportions of water, ethanol, and methanol. 2D (a) and 3D (b)

The cubic model is given by the following equation (3) correlating the three variables and the analytical response:

DPPH = 36.83 * (% Water) + 17.45 * (% Methanol) + 3.93 * (% Ethanol) + 204.59 * (% Water) * (% Methanol) + 227.54 * (% Water) * (% Ethanol) + 1.82 * (% Methanol) * (% Ethanol) + 287.98 * (% Water) * (% Methanol) * (% Ethanol). (3)

According to the polynomial equation, regarding pure solvents, water exhibited the highest positive effects on extracting molecules endowed with free radical scavenging activity followed by methanol. The binary mixtures showed significant synergetic effects between the two organic solvents (methanol, ethanol) and water. Meanwhile, the ternary interaction between the three solvents exhibited the greatest positive effect. Overall, the use of an ethanol + water mixture leads to a general improvement in extraction efficiencies of antioxidants.

• Evaluation of the total antioxidant activity

Response surfaces were determined for total antioxidant activity (TAC) based on extraction solvent compositions. Accordingly, the linear, quadratic, and special cubic models were tested (Figure 4).

Pure water produced extracts with high antioxidant activity, while pure ethanol and methanol generated extracts with low antioxidant activity. The highest antioxidant capacity values on the contour graph appear between the following positions: water (100%), water; ethanol (50%; 50%), water; methanol (50%; 50%).



Figure 4. The response surface of the Special Cubic Model predicts TAC based on the proportions of Water, Ethanol, and Methanol. 2D (a) and 3D (b)

The cubic model is given by equation (4) correlating the three variables and the analytical answer: **TAC** = 97.01 * (% Water) + 37.66 * (% Methanol) + 27.51 * (% Ethanol) + 55.64 * (% Water) * (% Methanol) + 101.66 * (% Water) * (% Ethanol) - 2.18 * (% Methanol) * (% Ethanol) + 160.74 * (% Water) * (% Methanol) * (% Ethanol). (4) According to equation 4, the linear coefficient of water was much higher than the other pure solvents, thus yielding extracts with much higher total antioxidant activity, followed by methanol. The binary mixtures including water presented high positive coefficients indicating good synergetic interaction between water and the two organic solvents, in contrast to the binary interaction between ethanol and methanol which was antagonistic. Overall, the ternary mixtures showed the highest positive coefficient indicating the capacity of these mixtures to extract a high amount of molecules endowed with total antioxidant activity.

Optimization of extraction conditions by the response surface has been reported in several studies (Ouafae, 2012; Pradal, 2016; Rajeha, 2013). Several subsequent studies confirm the effectiveness of solvents in extracting phenolic compounds and extracts with strong antioxidant and antiradical activities (Ksouri *et al.*, 2008; Nawaz *et al.*, 2006; Trabelsi *et al.*, 2012). The results obtained during this investigation converge on the same deductions.

Validation of experimental models by statistical analysis

An analysis of variance (ANOVA) was performed to examine the fit and significance of the model. The p-values are used to test the significance of the corresponding coefficient and the smaller the p-values are, the greater the significance of the corresponding coefficient (De Lima Da Silva *et al.*, 2009).

The model's significance was inspected using the F-test the goodness of fit of the model justifying its robustness was assessed by the coefficient of determination (R^2) and the adjusted correlation coefficient (Adj- R^2).

The independent and the response variables were fitted to linear, quadratic, and special cubic models. The total phenolic compound content and antioxidant activity were determined to evaluate the efficiency of the extraction solvent used for the aboveground part of the studied plant (Table 2). The particular cubic model was chosen because it had a perfect predictive capacity describing 100% of the total variance (coefficient of determination $R^2 = 1.000$, adjusted $R^2 = 1.000$), indicating that all response functions adequately fit the experimental data and the models can used for predictive purposes in the extraction of polyphenols and other molecules with antioxidant capacity using different solvent mixtures.

Assay	Models	SS	df	MS	F	Р	R ²	R² adj
ТРС	Linear	617.51	2	308.75	18.49	0.000043	0.673	0.636
	Quadratic	206.59	3	68.86	10.99	0.000451	0.898	0.863
	Special Cubic	93.92	1	93.92	14492.15	0.000000	1.000	1.000
	Total Adjusted	918.11	20	45.91				
DPPH	Linear	5891.62	2	2945.81	3.58	0.048968	0.285	0.205
	Quadratic	14585.78	3	4861.93	346.73	0.000000	0.990	0.986
	Special Cubic	209.42	1	209.42	3229.84	0.000000	1.000	1.000
	Total Adjusted	20687.73	20	1034.39				
TAC	Linear	15475.19	2	7737.59	60.99	0.000000	0.871	0.857
	Quadratic	2218.08	3	739.36	169.63	0.000000	0.996	0.995
	Special Cubic	65.24	1	65.24	6712.86	0.000000	1.000	1.000
	Total Adjusted	17758.65	20	887.93				

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

Abbreviations: SS-the sum of the square, df-degrees of freedom, MS-mean of the square, F-F-values for $p \le 0.05$, R^2 -coefficient of determination, R^2 adj.-coefficient of determination adjusted.

According to Table 3, all the special cubic models were highly significant as indicated by the low p-values of 0.000000 and the high F-values: 23607.69; 53173.99, and 304524.6 for TPC, DPPH, and TAC, respectively. Considering the calculated F-values and probability values, the models below did not suffer from a lack of fit and are very reasonable and meaningful.

Assay	Source	SS	df	MS	F	Р
ТРС	Model	918.02	6	153.00	23607.69	0.000000
	Total Error	0.09	14	0.01		
	Lack of Fit	0.00	0	0.00		
	Pure Error	0.09	14	0.01		
	Total Adjusted	918.11	20	45.91		
DPPH	Model	20686.82	6	3447.80	53173.99	0.000000
	Total Error	0.91	14	0.06		
	Lack of Fit	0.00	0	0.00		
	Pure Error	0.91	14	0.06		
	Total Adjusted	20687.73	20	1034.39		
TAC	Model	17758.51	6	2959.75	304524.6	0.000000
	Total Error	0.14	14	0.010		
	Lack of Fit	0.00	0	0.000		
	Pure Error	0.14	14	0.010		
	Total Adjusted	17758.65	20	887.93		

Table 3. Statistical parameters using response surface methods

Pareto chart analysis

To examine the relative importance of the main effects and their interactions with statistical significance ($p \le 0.05$), a plot of standardized Pareto (Figures 5, 6, and 7) was used. According to Figures 5, 6, and 7, the main factors of water (A), methanol (B), ethanol (C) and their binary interaction (AB, AC, and BC) and ternary integration (ABC) that exceeded the baseline were significant at the 0.05 level.



Figure 5. Pareto's Graph analysis of standardized effects for TPC



Figure 6. Pareto's graph of standardized effects for DPPH



Figure 7. Pareto's Graph of Standardized Effects for TAC

Figure 5, shows that water (A) has the most positive significant effect on the extraction of phenolic compounds, followed by methanol (B), and the ternary mixture. Whereas, the binary mixture presented the lowest positive effect. Meanwhile, regarding the DPPH assay (Figure 6), the binary interaction of pure water with ethanol primarily and positively influenced the extraction of antioxidant compounds, followed by the binary combination of water and methanol. Water was the third important parameter that showed a significant positive effect on the extraction of antioxidant compounds. Concerning the total antioxidant activity, the standardized Pareto diagram (Figure 7) shows that all solvents and their interactions were significant, except the binary methanol-ethanol combination. We can also note that water is the solvent that has most influenced the extraction of antioxidant compounds, followed by pure methanol and the binary interaction of pure ethanol with water.

According to the Pareto analysis, pure water presented the highest positive impact on both TPC extraction and the antioxidant capacity of the extracts. Whereas, its interaction with ethanol yielded extracts with the highest DPPH free radical scavenging ability.

Desirability analysis

The extraction optimization using the desirability function was assessed to maximize both TPC and the antioxidant capacity (DPPH and TAC) in the extracts. The graphic representation of the predicted values and

the desirability profile is presented in Figure 8. The desirability profile for TPC and antioxidant activities were generated with values of 1.17 mg / g - low (0.00); 10.19 mg / g intermediate (0.05); and 19.2 mg / g - high (1.00). For DPPH 3.8 mg / g - low (0.00); 41.2 mg / g intermediate (0.05); and 78.5 mg / g - high (1.00). The TAC desirability profile was generated with the following values 17.3 mg / g - low (0.00); 57.2 mg / g intermediate (0.05); and 97.1 mg / g - high (1.00). The optimal solvent mixture for yielding extracts with an optimal value of TPC and antioxidant activities was: 75 % water, 14 % methanol, and 11 % ethanol.



Figure 8. Desirability profile for optimizing solvent reference mixture

PCA analysis

To provide an overview of the complete data set., data were subjected to PCA to evaluate the correlation between the different variables. The first factor (factor 1) explained 63.64% of the total variance, while the second Factor (factor 2) explained a further 25.35 %, indicating that the two first factors were able to explain 88.99 % of the total variance. The correlation circle or graph of variables (Figure 9) shows the correlations between components and initial variables. According to Figure 9, water has a positive correlation with total polyphenol content and antioxidant activity measured by the two assays. Moreover, water subtended a very small angle to TAC assays, indicating their strong correlation. So, increasing the water in the extraction solvent increases its ability to extract phenolic compounds and molecules endowed with antioxidant power. The same conclusion was established using Pareto analysis.



Figure 9. Principal Component Analysis of TPC, TAC, DPPH, and extracting solvents (ethanol, methanol, and water)

Discussion

The use of a solvent mixture containing both ethanol and methanol in water for extraction presents several advantages, aligning with findings from various scientific works. Ethanol efficiently decreases polarity in hydroethanolic solutions, increasing the solubility of phenolic compounds; this is consistent with ethanol's overall effectiveness in extracting polyphenols (Shi et al., 2005). Furthermore, the inclusion of methanol in the mixture further complements the extraction process, considering methanol's reported efficiency in extracting lower molecular weight polyphenols (Dai and Mumper, 2010). Aqueous methanol has been shown in the literature to be an excellent solvent for polyphenol extraction (Iloki-Assanga et al., 2015). The specific solvent composition highlighted in the study, consisting of 75% water, 11% ethanol, and 14% methanol, aligns with findings suggesting that a 75 wt.% ethanol/water solvent may yield the highest extraction yield and exhibit the strongest antioxidant properties (Sun et al., 2015). The Principal Component Analysis (PCA) findings in our study, indicating a significant correlation between water usage in extraction, Total Phenolic Content (TPC), and antioxidant activity, further align with the larger understanding of solvent composition influencing extraction efficiency and bioactive properties. The concordance between our findings and the literature underscores the robustness of the chosen solvent mixture in extracting phenolic compounds with potent antioxidant activity, providing a valuable contribution to the field of natural product extraction and highlighting the versatility of hydroethanolic mixtures in optimizing extraction outcomes.

Conclusions

This work presents the first report on phenolic compounds and antioxidant extraction from *A. pyrethrum*. The second-order polynomial model provided a satisfactory mathematical description for the TPC and antioxidant extraction from this species. Thus, pointing out that the precious plant *A. pyrethrum* exhibits a high antioxidant and anti-radical activity, highlighted by an optimization of the extraction of phenolic compounds, using the optimal extraction mixture was the ternary mixture (water; ethanol; methanol) with the following proportions: water 75%; ethanol 11%; methanol 14%. The optimal condition was validated and found to be consistent with experimental values. In addition, the plant is rich in mainly phenolic compounds.

The design of the statistical mixture has been successfully applied to obtain an optimized set of extraction conditions to maximize the extraction of polyphenols with high antioxidant activity. These conditions can be chosen to exploit the higher concentration of natural phenolic compounds reducing free radicals.

Authors' Contributions

OC: Software, Methodology, Writing – original draft, Formal analysis, Data curation, Conceptualization, Validation. LEG: Supervision, Project administration, Formal analysis, review, Validation. AH: Investigation Software, Validation, Formal Analysis. SA: Supervision, Project administration, Writing – review & editing, Formal analysis, Methodology, Conceptualization, Validation.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

- Aazza S (2021). Application of multivariate optimization for phenolic compounds and antioxidants extraction from Moroccan *Cannabis sativa* waste. Journal of Chemistry 1-11. *https://doi.org/10.1155/2021/9738656*
- Amine D, Mohamed B, Jamal I, Laila N (2017). Antibacterial activity of aqueous extracts of *Anacyclus pyrethrum* (L) Link and *Corrigiola telephiifolia* Pourr. from the Middle Atlas region, Morocco. European Scientific Journal 13(33):116. https://doi.org/10.19044/esj.2017.v13n33p116
- Bouaziz M, Dhouib A, Loukil S, Boukhris M, Sayadi S (2009). Polyphenols content, antioxidant and antimicrobial activities of extracts of some wild plants collected from the south of Tunisia. African Journal of Biotechnology 8(24):7017-7027. https://doi.org/10.5897/AJB2009.000-9545
- Brand-Williams W, Cuvelier ME, Berset C (1995). Use of a free radical method to evaluate antioxidant activity. LWT -Food Science and Technology 28(1):25-30. *https://doi.org/10.1016/S0023-6438(95)80008-5*
- Chemat F, Rombaut N, Sicaire A-G, Meullemiestre A, Fabiano-Tixier A-S, Abert-Vian M (2017). Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. Ultrasonics Sonochemistry 34:540-560. https://doi.org/10.1016/j.ultsonch.2016.06.035
- De Lima Da Silva N, Batistella ĆB, Filho RM, Maciel MRW (2009). Biodiesel production from castor oil: Optimization of alkaline ethanolysis. Energy and Fuels 23(11):5636-5642. https://doi.org/10.1021/ef900403j

- DiCiaulaa MC, Lopesa GC, Scarminiob IS, Melloa JCP de (2014). Optimization of solvent mixtures for extraction from bark of *Schinus terebinthifolius* by a statistical mixture-design technique and development of a uv-vis spectrophotometric method for analysis of total polyphenols in the extract. Quim. Nova 37(1):158-163.
- El Alami A (2022). Biodiversity loss in the Moroccan central High Atlas, its impact on local ecosystems and national economy, and wildlife conservation strategy: Findings from 20 years of research. Journal of Analytical Sciences and Applied Biotechnology 4(2):81-96. https://doi.org/10.48402/IMIST.PRSM/jasab-v4i2.35045
- Elazzouzi H, Fadili K, Cherrat A, Amalich S, Zekri N, Zerkani H, Tagnaout I, Hano C, Lorenzo JM, Zair T (2022). Phytochemistry, biological and pharmacological activities of the *Anacyclus pyrethrum* (L.) Lag: A systematic review. Plants 11(19):2578. https://doi.org/10.3390/plants11192578
- Fennane M, Rejdali M (2016). Aromatic and medicinal plants of Morocco: Richness, diversity and threats. Bulletin de l'institut Scientifiue, Rabat, Section Sciences de La Vie 38:27-42.
- Ghafoor K, Choi YH, Jeon JY, Jo IH (2009). Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from grape (*Vitis vinifera*) seeds. Journal of Agricultural and Food Chemistry 57(11):4988-4994. https://doi.org/10.1021/jf9001439
- Iuchi K, Hatano Y, Yagura T (2008). Heterocyclic organ bismuth (III) induces apoptosis of human promyelocytic leukemic cells through activation of caspases and mitochondrial perturbation. Biochemical Pharmacology 76(8):974-986. https://doi.org/10.1016/j.bcp.2008.07.038
- Kaczorová D, Karalija E, Dahija S, Bešta-Gajević R, Parić A, Ćavar Zeljković S (2021). Influence of extraction solvent on the phenolic profile and bioactivity of two *Achillea* species. Molecules 26(6):1601. https://doi.org/10.3390/molecules26061601
- Ksibi I El, Slama R Ben, Faidi K, Ticha M Ben, M'henni MF (2015). Mixture approach for optimizing the recovery of colored phenolics from red pepper (*Capsicum annum* L.) by-products as potential source of natural dye and assessment of its antimicrobial activity. Industrial Crops and Products 70:34-40. https://doi.org/10.1016/j.indcrop.2015.03.017
- Ksouri R, Megdiche W, Falleh H, Trabelsi N, Boulaaba M, Smaoui A, Abdelly C (2008). Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes. Comptes Rendus Biologies 331(11):865-873. https://doi.org/10.1016/j.crvi.2008.07.024
- Kumar V, Choudhary AK (2016). Akarkara: a versatile medicinal plant a review. Journal of Ayurveda and Holistic Medicine 1-14.
- Libbey LM, Walradt JP (1968). 3,5-di-Tert-butyl-4-hydroxytoluene (BHT) as an artifact from diethyl ether. Lipids 3(6):561-561. https://doi.org/10.1007/BF02530903
- Liyana-Pathirana CM, Shahidi F (2006). Antioxidant properties of commercial soft and hard winter wheats (*Triticum aestivum* L.) and their milling fractions. Journal of the Science of Food and Agriculture 86(3):477-485. https://doi.org/10.1002/jsfa.2374
- Liyanapathirana C, Shahidi F (2005). Optimization of extraction of phenolic compounds from wheat using response surface methodology. Food Chemistry 93(1):47-56. *https://doi.org/10.1016/j.foodchem.2004.08.050*
- Ma T, Wang Q, Wu H (2010). Optimization of extraction conditions for improving solubility of peanut protein concentrates by response surface methodology. LWT - Food Science and Technology 43(9):1450-1455. https://doi.org/10.1016/j.lwt.2010.03.015
- Mohammedi Z, Atik F (2011). Impact of solvent extraction type on total polyphenols content and biological activity from *Tamarix aphylla* (L.) Karst. International Journal of Pharma and Bio Sciences 2:609-615.
- Montgomery DC (2012). Design and analysis of experiments Eighth Edition. Design 2:757. https://doi.org/10.1198/tech.2006.s372
- Naczk M, Shahidi F (2004). Extraction and analysis of phenolics in food. Journal of Chromatography A 1054(1-2):95-111. https://doi.org/10.1016/j.chroma.2004.08.059
- Nawaz H, Shi J, Mittal GS, Kakuda Y (2006). Extraction of polyphenols from grape seeds and concentration by ultrafiltration. Separation and Purification Technology 48(2):176-181. https://doi.org/10.1016/j.seppur.2005.07.006
- Pandey S, Rani Kushwaha G, Singh A, Singh A (2018). Chemical composition and medicinal uses of *Anacyclus pyrethrum*. Pharma Science Monitor 9(1):551-560.

- Prieto P, Pineda M, Aguilar M (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Analytical Biochemistry 269(2):337-341. https://doi.org/10.1006/abio.1999.4019
- Sampaio CRP, Anastácio LMC, De Francisco TMG, Ribani RH (2015). Anthocyanins and phenolic compounds in five ripening stages of *Byrsonima ligustrifolia* after extraction optimization. Journal of Food and Nutrition Research 54(4):365-378.
- Santos Felix AC, Novaes CG, Pires Rocha M, Barreto GE, do Nascimento BB, Giraldez Alvarez LD (2018). Mixture design and Doehlert matrix for the optimization of the extraction of phenolic compounds from Spondias mombin L Apple bagasse agro-industrial residues. Frontiers in Chemistry 5:1-8. https://doi.org/10.3389/fchem.2017.00116
- Subasri G, John SA (2015). Screening of phytochemical compounds, trace metals and antimicrobial activity of *Anacyclus* pyrethrum. International Journal of Advances in Scientific Research 1(08):322-328. https://doi.org/10.7439/ijasr
- Trabelsi N, Falleh H, Jallali I, Daly A Ben, Hajlaoui H, Smaoui A, Abdelly C, Ksouri R (2012). Variation of phenolic composition and biological activities in *Limoniastrum monopetalum* L. organs. Acta Physiologiae Plantarum 34(1):87-96. https://doi.org/10.1007/s11738-011-0807-8
- Trabelsi N, Megdiche W, Ksouri R, Falleh H, Oueslati S, Soumaya B, Hajlaoui H, Abdelly C (2010). Solvent effects on phenolic contents and biological activities of the halophyte *Limoniastrum monopetalum* leaves. LWT - Food Science and Technology 43(4):632-639. https://doi.org/10.1016/j.lwt.2009.11.003
- Turkmen N, Velioglu Y, Sari F, Polat G (2007). Effect of extraction conditions on measured total polyphenol contents and antioxidant and antibacterial activities of black tea. Molecules 12(3):484-496. https://doi.org/10.3390/12030484
- Venkatesan T, Choi Y-W, Kim Y-K (2019). Impact of different extraction solvents on phenolic content and antioxidant potential of *Pinus densiflora* Bark extract. BioMed Research International 2019:1-14. *https://doi.org/10.1155/2019/3520675*
- Wang L, Weller CL (2006). Recent advances in extraction of nutraceuticals from plants. Trends in Food Science and Technology 17(6):300-312. https://doi.org/10.1016/j.tifs.2005.12.004
- Xiao W, Han L, Shi B (2008). Microwave-assisted extraction of flavonoids from Radix Astragali. Separation and Purification Technology 62(3):614-618. https://doi.org/10.1016/j.seppur.2008.03.025
- Yahyaoui M, Ghazouani N (2017). Comparison of the Effect of various extraction methods on the phytochemical composition and antioxidant activity of *Thymelaea hirsuta* L. aerial parts in Tunisia. Biosciences, Biotechnology Research Asia 14(3):997-1007. https://doi.org/10.13005/bbra/2534



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