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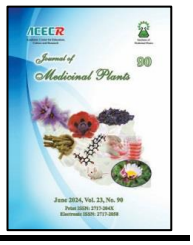

Research Article

Optimizing polyphenol extraction from *Anacyclus pyrethrum* var. *depressus* (Ball) Maire roots: a simplex-centroid mixture design approach

Oumaima Chater[1,*], Smail Aazza[2], Hiba Bouzaid[3], Lahsen El Ghadraoui[1]

[1] *University of Sidi Mohamed Ben Abdellah, Faculty of Science and Technology, Laboratory of Functional Ecology and Environmental Engineering, Fez, Morocco*

[2] *Mohammed 1st University, Oujda, Nador Multidisciplinary Faculty (FPN), OLMANBGPE, Nador, Morocco*

[3] *University of Sidi Mohamed Ben Abdellah, Faculty of Science and Technology, Laboratory of Organic Chemistry, Fez, Morocco*

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ABSTRACT

Background: *Anacyclus pyrethrum* var. *depressus* (Ball) Maire is a medicinal plant whose roots have been reported to possess medicinal properties in traditional and modern medicine systems. **Objective**: The present work aims to maximize the extraction of polyphenols and compounds with antioxidant properties by applying the Simplex-Centroid Mixture Design. **Methods:** Eleven solvents were tested for their affinities with *A. pyrethrum* bioactive molecules, and the three best solvents (water, methanol, and ethanol) were subjected to mixing modeling for optimization, and different models were developed to study the binary and ternary combined effects. Extract fractionation was performed using four solvents namely Chloroform, dichloromethane, ethyl acetate, and hexane. The evaluation of the antioxidant activity of the fractions was performed using various tests (TAC, DPPH, FRAP, ABTS, chelating power, and nitric oxide scavenging activity). **Results:** The developed models and all their parameters were significant. The analysis of the response surfaces generated by the models indicated that the quantity of phenolic compounds extracted in the binary mixtures and the total antioxidant activity of the extracts increase with the increasing water percent in the solvent mixture using both ethanol or methanol. While, the binary "water-ethanol", and "water-methanol" and the ternary mixture proved to be the most effective combinations for extracting anti-radical compounds. Fractionation results showed that the aqueous fraction exhibited the highest antioxidant activity, due to its higher content of phenolic compounds. **Conclusion:** With this type of research, it would be easier to treat and prevent human damage from free radicals and also replace synthetic antioxidants in industry.

Abbreviations: AAE, ascorbic acid equivalent; ABTS, 2, 2-azinobis 3-ethylbenzothiazoline 6-sulfonate; AQF, aqueous fraction; CHF, chloroform fraction; DCMF, dichloromethane fraction; DPPH, 2, 2-dipheny-1-picrylhydrazyl; E, ethanol; EAF, ethyl acetate fraction; FRAP, Ferric Reducing-Antioxidant Power; GAE, gallic acid equivalent; HF, hexane fraction; M, methanol; NO, Nitric oxide; TAC, total antioxidant activity; TPC, total phenolic content; W, water

*Corresponding author: oumaima.chater@usmba.ac.ma

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1. Introduction

Natural phenolic phytochemicals derived from plants are garnering substantial interest for their potential health benefits, particularly in preventing coronary heart disease and cancer. This interest is primarily due to their antioxidant properties, which are critical in inhibiting or delaying the oxidation of other molecules and curtailing the oxidative chain [1].

Antioxidants have a pivotal role in protecting the body from the detrimental effects of free radicals and reactive oxygen species (ROS), thereby slowing the progression of numerous chronic diseases and lipid peroxidation [2, 3]. Additionally, their use as food additives helps guard against oxidative degradation, preserving food quality and safety [4].

Beyond their antioxidative capacity, natural antioxidants also show a wide range of biological activities, including anti-inflammatory, antibacterial, antithrombotic, antiviral, anti-allergic, and vasodilator effects, underscoring the need for their exploration and application in disease prevention [5].

Our research focuses on *A. pyrethrum*, an endemic species of the Asteraceae family found in Morocco and Algeria. This perennial plant thrives in the Middle Atlas region, particularly in the forests of Timehdit. Recent studies have highlighted various pharmacological effects of *A. pyrethrum* extracts, such as enhancing memory, antibacterial properties, anti-depressive actions, and androgenic and spermatogenic effects in adult males [6, 7]. The primary active compound in *A. pyrethrum*, pyrethrin, is a colorless crystalline acid amide known as pellitorin. Additional phytoconstituents identified include N-isobutyldienediynamide and various polysaccharides [8]. Notably, the roots of this plant have been recognized for their anti-inflammatory [9], immunostimulatory [10], anabolic, and aphrodisiac properties [11].

In our research, we investigated the antioxidant activity and phenolic content of fractions obtained utilizing n-hexane, dichloromethane, chloroform, ethyl acetate, and water extract of *A. pyrethrum* using in vitro testing methods, we evaluated the scavenging activities on ABTS, DPPH, and NO, as well as reducing power, chelating capacity, and the phospho-molybdenum complex formation.

2. Materials and methods

2.1. Plant samples

A. pyrethrum specimens were gathered in the Timehdit district of the Middle Atlas in Morocco in May 2018. The plant was identified by Botanist Dr. Helena Silva in the Herbarium of the Department of Biology at the University of Aveiro, Portugal (AVE), and the voucher specimen was deposited by AVE 7897 code number. The roots were desiccated in a shaded and arid environment. Subsequently, the specimen was pulverized into a fine particulate and preserved in opaque containers for subsequent examination.

2.2. Extraction

2.2.1. Extraction by several solvents

Samples were prepared by mixing 50 mg of powdered roots with 1,000 µL of the solvent (either pure or in mixture) in triplicate. The resulting combination was then submitted to 30 minutes of sonication in an ultrasonic bath at ambient temperature. After sonication, the mixture was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was collected and stored in a dark environment at 4 °C. Using a variety of polarity solvents, such as acetone, butanol, chloroform, diethyl ether, dichloromethane, ethyl acetate, ethanol, hexane,

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methanol, petroleum ether, and water, the first extraction step entailed screening. This screening was conducted to determine the most suitable solvent for further extraction steps, which would involve using the chosen pure solvent and its combinations.

2.2.2. Fractionation

An optimum mixture of water, methanol, and ethanol in the ratio (50:10:40) was used to extract the powdered root (20 g) by sonication for 30 minutes. The resulting extract was filtered using Whatman filter paper. Subsequently, the filtrate was concentrated under vacuum at 40 °C using a rotary evaporator to reduce its volume. The concentrated extract was then combined with 200 ml of distilled water. The extract was then partitioned using a separating funnel with (× 3) 200 mL n-hexane, dichloromethane, chloroform, and ethyl acetate, respectively. The three aliquots from each fractionation were mixed and evaporated under low pressure using a rotary evaporator. The resulting residue was then dissolved in ethanol. This process yielded fractions labeled as HF (hexane), DCMF (dichloromethane), CHF (chloroform), EAF (ethyl acetate), and AQF (aqueous).

2.3. Total phenolic content (TPC)

This assay was determined by using a spectrophotometric method, which followed a modified colorimetric approach with the Folin-Ciocalteu reagent [12]. In this method, 50 µL of the sample was mixed with 450 µL of a 10-fold diluted Folin-Ciocalteu reagent. The mixture was left to incubate at ambient temperature for 5 minutes, after which 450 µL of a sodium carbonate solution (75 g L^{-1}) was added. The samples were then incubated for two hours in the dark at ambient temperature. After the incubation period, the absorbance was measured at 760 nm using a spectrophotometer. The calibration curve, with the equation $y = 2.838x + 0.056$ and an R^2 of 0.9994, was constructed using an ethanolic solution of gallic acid, covering a concentration range from 0.062 to 1 mg mL^{-1}. The results are expressed as milligrams of gallic acid equivalents (GAE) per gram of dry plant material. All experiments were performed in triplicate.

2.4. Solvent effects evaluation using Simplex Axial Design

An examination was conducted to enhance the extraction of polyphenols by employing a solvent mixing method. The Simplex-Lattice Design and the Simplex-Centroid Design are two regularly employed standard designs in extraction investigations that involve solvent combinations. The two methods assess the triangle response surface by analyzing the maximum points and the geometric centers.

In the Simplex-Centroid Design, a triangle is formed by the different conditions tested, with the pure solvents placed at the vertices, each indicating a 100% concentration of the respective solvent. The midpoint along each side of the triangle corresponds to binary solvent mixtures (1/2: 1/2: 0; 1/2: 0: 1/2; 0: 1/2: 1/2), while the centroid of the triangle represents a ternary mixture (1; 1; 1).

A mixing model was devised to improve the extraction process's efficiency. The Simplex-Centroid Design, incorporating three repetitions at the axial points, was employed to determine the optimal combination of the three solvents: methanol (M), ethanol (E), and water (W). The choice of these three solvents was made deliberately based on their proven effectiveness in extracting phenolic compounds, as well as their different polarity that affect the solubility

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of certain phytochemicals. After doing an initial screening using eleven different solvents, it was found that water, which is the most polar, removed the most amount of phenolic compounds. These compounds were mainly hydrophilic in nature. Methanol and ethanol, which have moderate polarity, were closely observed. This characteristic allows for the extraction of a broader range of phytochemicals, including substances that are moderately lipophilic. In order to enhance the accuracy of our extraction method, we utilized the Simplex-Centroid Mixture Design model. This model was employed to assess the synergistic effects of these solvents in different ratios, with the goal of optimizing the extraction process and increasing the yield of phenolic components.

2.5. Assessment of antioxidant activity:

2.5.1. Total Antioxidant Capacity (TAC)

The phospho-molybdenum complex's production served as a proxy for the samples' total antioxidant activity [13]. One milliliter (mL) of the reagent solution, which included 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate, was mixed with an aliquot of the sample solution. Using a blank as the reference, the absorbance was measured at 695 nm using a spectrophotometer after incubation in a water bath at 95 °C for 90 minutes. The calibration curve was created using an ascorbic acid aqueous solution, yielding the equation y = 1.74x + 0.24, with an R^2 value of 0.9999. The ascorbic acid concentration in the solution ranged from 0.0625 to 1.00 mg mL^{-1}. The experiment was conducted in triplicate, and the results represent the average values of antioxidant activity reported in grams of ascorbic acid equivalents per gram of dry plant material.

2.5.2. FRAP: Ferric reducing-antioxidant power

The reducing power of the samples was evaluated following the method described by [14]. Each sample or standard was combined with 2,500 µL of phosphate buffer (0.2 M, pH 6.6) and 2,500 µL of potassium ferricyanide [K3Fe(CN)6] solution (1 %). The mixture was incubated at 50 °C for 20 minutes. Afterward, 2,500 µL of a 10 % trichloroacetic acid solution was added, and then the mixture was centrifuged for 10 minutes at 3,000 rpm. A 2,500 µL aliquot from the upper layer was taken and mixed with 2,500 µL of distilled water and 500 µL of FeCl3 (0.1 %). The absorbance of the resulting solution was measured at 700 nm using a spectrophotometer.

2.5.3. DPPH Free radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was conducted following the method described by [15]. A 60 µM ethanolic solution of DPPH (1 mL) was mixed with 25 µL of various concentrations of the samples or standards. After incubating the mixtures at room temperature for 1 hour, the absorbance was measured at 517 nm. A negative control was performed by using a blank solution containing only DPPH and methanol. The following formula was used to measure the percentage inhibition of each extract's scavenging activity against free radicals throughout the three experiments:

$$\% \text{ inhibition} = \left(\frac{Abs\ control - Abs\ sample}{Abs\ control}\right) \times 100 \qquad \textbf{Eq. 1}$$

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A graph was created to compare the percentage of inhibition with the content of the sample or standard. The IC_{50} value, which represents the concentration of the extract needed to capture 50 % of the DPPH free radicals, was determined.

2.5.4. ABTS radical scanning activity

The ABTS test was performed using the methodology described by [16,17], with certain alterations. The stock solutions consisted of a 7 mM ABTS solution and a 2.4 mM potassium persulfate solution. The operational remedy was thereafter concocted by combining the two pre-existing solutions in equal proportions and permitting them to undergo a chemical reaction for a duration of 14 hours at ambient temperature in the absence of light. To dilute the solution, 1 mL of ABTS solution was mixed with 60 mL of methanol. This resulted in an absorbance of 0.832 ± 0.01 at 734 nm when measured using a spectrophotometer. A new ABTS solution was produced for each test. The plant extracts (50 µl) were mixed with 1 ml of the ABTS solution and the absorbance was measured at 734 nm after 7 minutes using a spectrophotometer. The percentage of inhibition, estimated as the radical scavenging activity, was determined by applying Equation 1. The absorption of the ABTS radical in methanol is referred to as "Abs control". Conversely, "Sample Abs" denotes the absorbance of the ABTS after it has been combined with either the standard or the sample extract.

2.5.5. Metal ions chelating

The percentage of ferrous ion chelation by the fractions was determined using the method described by [18]. In this procedure, 100 µL of the sample was mixed with 200 µL of $FeCl_2 \cdot 4H_2O$ (2 mM) and 0.5 mL of water, followed by incubation. The reaction was initiated by adding 200 µL of a 5 mM ferrozine solution, and the absorbance was measured at 562 nm after 10 minutes. A control sample, which did not receive any treatment, was used for comparison. The chelating activity was calculated as a percentage using Equation 1.

2.6. Nitric oxide scavenging activity: Anti-inflammatory activity

This approach relies on the fundamental idea that sodium nitroprusside, when dissolved in water at a pH level found in the human body, naturally produces nitric oxide. Nitric oxide subsequently undergoes a reaction with oxygen to generate nitrite ions. The presence of nitrite ions can be detected by the Greiss reagent, composed of 1 % sulfanilamide, 2 % phosphoric acid, and 0.1 % naphthyl ethylene diamine dihydrochloride (NED). Compounds possessing the ability to scavenge free radicals lead to a reduction in the generation of nitric oxide radicals. In this experiment, a solution containing SNP (10 mM) in phosphate buffered saline (PBS, pH 7.4) was combined with various quantities of each sample fraction. The solution was kept at ambient temperature for a duration of 2 hours under illuminated conditions. After the incubation period, Griess reagent was introduced. The absorbance of the ensuing pink chromophore, which was generated by the reaction between nitrite ions and sulfanilamide followed by coupling with NED, was measured at a wavelength of 550 nm using a blank [19]. The tests were conducted three times each. The percentage of inhibitory activity was determined using Equation 1.

2.7. Experimental design and optimization

The research used the augmented simplex centroid design to examine the effects of various extracting solvents on Total Phenolic Content (TPC) and antioxidant activities. The objective

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was to optimize the extraction conditions by comprehending the interactions among the solvents. The experiments using mixture design were executed and evaluated with a complimentary version (10) of STATISTICA. A total of 10 combinations being utilized. The data were presented as the average value ± standard deviation (SD) and were evaluated using analysis of variance (ANOVA). Tukey's test was used to ascertain significant differences, with a significance threshold of $p < 0.05$. The polynomial equation of function Xi was calibrated for each component evaluated at every experimental point. The equation has the anticipated response Y and constant coefficients β1, β2, β3, β12, β13, and β23 for each linear and non-linear interaction component. The equation is expressed as:

$$Y = \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{123}X_1X_2X_3 \quad \textbf{Eq. 2}$$

Principal component analysis (PCA) was used to elucidate the connections among the variables and to examine the impact of extraction solvents on the antioxidant chemicals.

2.8. Statistical analysis

Every experiment was run in triplicate, including the solvent percentage selection. IC50 values were computed and the findings are shown as the mean ± standard error. One-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used for statistical studies. For every test, a significance criterion of $P < 0.05$ was established. The STATISTICA version 10 program, available for free download (STATSOFT, INC., 2011), was used to perform the statistical analysis.

3. Results

3.1. Extraction solvents screening

It is widely known that several factors can influence the yield and efficiency of the extraction, among the most crucial aspects of this process is the nature of the solvent used. Eleven solvents with varying polarity were employed in the first section of this study to extract phenolic compounds from *A. pyrethrum* roots.

Figure 1 illustrates how the polyphenol levels in *A. pyrethrum* roots vary depending on the extraction solvent. Water shows the highest TPC (17.68 ± 0.04 mg GAE /g dr), indicating it is the most effective solvent for extracting phenolics, followed by methanol, which also yields a high phenolic content. Ethanol, acetone, and ethyl acetate exhibit progressively lower TPC values, while non-polar solvents such as hexane, petroleum ether, and dichloromethane show negligible extraction efficiency. These results demonstrate the significant impact of solvent extraction potency on the production of total phenolic compounds. The results also highlight the importance of the solvent in determining the concentrations of phenolic compounds, determining that the best solvents for increasing polyphenol yields are ethanol, methanol, and water.

3.2. Extraction using solvent mixtures

The total phenolic compounds (TPC) value was used to select the top three solvents. When eleven different solvents were evaluated, water yielded the highest amounts of phenolic compounds (17.68 ± 0.04 mg GAE / g dr), followed by methanol (12.1 ± 0.01 mg GAE / g dr) and ethanol (5.36 ± 0.03 mg GAE / g dr).

After evaluating the outcomes of extracting solvents individually, we opted to use a

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combination of the 3 solvents (water, methanol, and ethanol) that showed the highest yields of polyphenols. We conducted experiments using the simplex-centroid mixture design approach to alter the quantities of solvents in the mixture.

The examination of the findings displayed in Table 1 clearly demonstrates that the highest output is achieved when utilizing the binary "water-ethanol" combination, with pure water being the next most effective. In contrast, the use of pure ethanol resulted in a reduced level of total phenolic compounds (TPC). Furthermore, when ethanol was combined with methanol, the extraction of phenolic compounds was further decreased. However, when ethanol was combined with water, significant amounts of TPC were extracted.

Table 1. TPC, TAC, and DPPH obtained from different combinations

Crude extract	Extract (solvent proportions)	TPC mg Gallic acid / g dr	TAC mg Ascorbic acid / g dr	DPPH (%)
1	W (1)	14.55 ± 0.19	106.24 ± 0.05	44.68 ± 0.08
2	M (1)	8.41 ± 0.03	48.25 ± 0.07	37.94 ± 0.22
3	E (1)	3.50 ± 0.03	20.00 ± 0.09	31.26 ± 0.22
4	W: M (1/2:1/2)	13.03 ± 0.21	80.95 ± 0.08	67.98 ± 0.11
5	W: E (1/2:1/2)	15.75 ± 0.13	102.21 ± 0.05	73.48 ± 0.23
6	E: M (1/2:1/2)	4.88 ± 0.05	29.80 ± 0.07	25.65 ± 0.11
7	W: E:M (1/3:1/3:1/3)	12.31 ± 0.17	91.34 ± 0.00	70.56 ± 0.13

3.3. Evaluation of mixture optimization using response surface approach

Figure 2 displays the contour diagram of the special cubic model, which illustrates the interaction effects and the varying proportions of pure solvents and their mixtures in relation to TPC values.

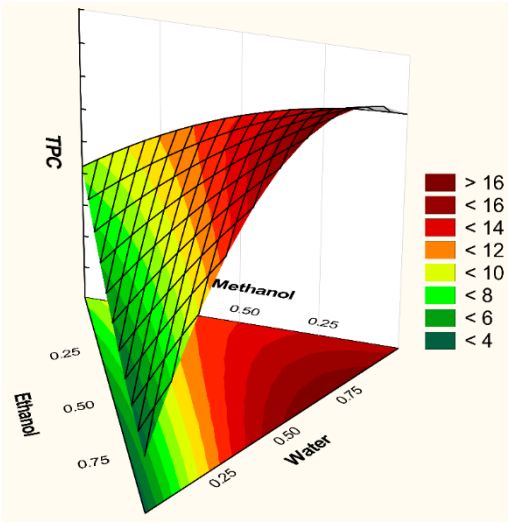

Fig. 2. 3D response surface contour plot of the special cubic model for TPC

Linear, quadratic, and cubic models were assessed, with their adequacy evaluated through ANOVA. The cubic model, represented by Equation (Eq. 3), establishes a correlation between the variables (W: water, M: methanol, E: ethanol). The analytical solution is as follows:

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TPC = + 14.55 * (%W) + 8.41 * (%M) + 3.50 * (%E) + 6.22 * (%W * %M) + 26.89 * (%W* %E) - 4.29 * (%M * %E) + 7.67 * (%W * % M * %E) + 0. **Eq. 3**

Equation 3 indicates that the extraction of TPC was primarily and favorably influenced by the binary mixture "water-ethanol". The water linear coefficient is significantly greater compared to the other solvents, suggesting a more pronounced favorable impact on TPC extraction. Conversely, the "water-ethanol" mixture exhibited the most significant positive impact in binary interactions. While the combination of ethanol and methanol had the least favorable impact.

Fig. 2 illustrates the 3D response surface of the specialized cubic model for extracting TPC. Based on this graph, it can be shown that 100 % ethanol and 100 % methanol had the lowest levels of total phenolic compounds (TPC) extraction, but pure water led to higher levels. The peak on the contour plot, representing the highest value, identifies the optimal solvent composition for extracting phenolic compounds. This optimal composition lies between the areas where equal proportions of ethanol and water, and 100 % water are found.

3.4. Evaluation of antioxidant and antiradical activities

Table 1 displays the variations in antiradical activity and total antioxidant activity of *A. pyrethrum*, depending on the specific solvent mixes employed. The findings indicate that the antiradical activity in *A. pyrethrum* was considerably affected by both the kind of solvent used for extraction and the composition of the combination.

It is important to highlight that the ternary mixture shows the highest total antioxidant capacity (TAC), the mixture "water-ethanol", and 100% water comes after. The DPPH inhibition percentage is greater in the extracts obtained from the ternary (water, ethanol, methanol) mixture as well as the mixtures "water-ethanol" and "water-methanol".

3.5. Response Surface Analysis of Antioxidant and Antiradical Activity

3.5.1. Assessment of the total antioxidant activity

Response surfaces were established to evaluate the total antioxidant activity (TAC) by varying the proportion of the solvents used in the extraction. Linear, quadratic, and cubic models were evaluated (Fig. 3).

Water in its pure form yields an extract with a significant level of total antioxidant activity, whereas ethanol (100 %) and methanol (100 %) yield extracts with a low level of antioxidant activity. The surface response and contour graph show that the highest antioxidant capacity values are found in water; methanol (50 %; 50 %), water; ethanol (50 %; 50 %), and water (100 %). Thus, the best combination to extract phenolic compounds for this plant species is using the hydroalcoholic mixture 70 %.

The cubic model is represented by equation (Eq. 4), which establishes a correlation between the 3 variables. The analytical response is:

TAC = + 106.24 * (%W) + 48.25 * (%M) + 20.00 * (%E) + 14.82 * (%W * %M) + 156.37 * (%W * %E) - 17.28 * (%M * %E) + 434.21 * (%W * %M * %E) + 0. **Eq. 4**

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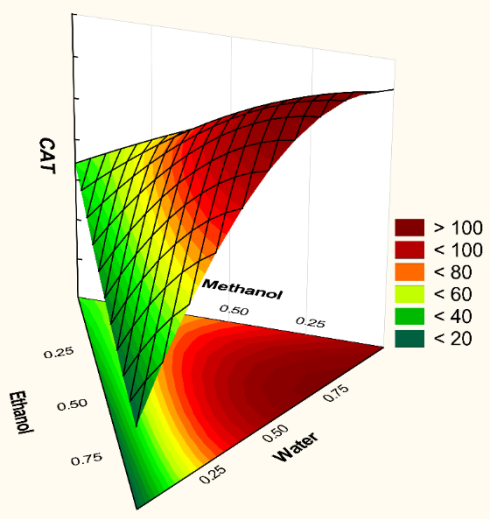

Fig. 3. 3D response surface contour plot of the special cubic model for TAC

The water linear coefficient is significantly higher compared to pure ethanol and methanol, leading to extracts with substantially greater overall antioxidant activity, with methanol being a close second. Binary mixtures that contained water showed notable positive coefficients, indicating a strong synergistic interaction between water and ethanol or methanol. Conversely, the binary interaction between ethanol and methanol was found to be antagonistic. Overall, the ternary combinations exhibited the highest positive coefficients, demonstrating their effectiveness in extracting a large amount of molecules with antioxidant activity.

3.5.2. Assessment of free radical scavenging

Response surfaces were established to assess the antioxidant capacity of DPPH based on the compositions of the extraction solvents. Linear, quadratic, and cubic models were evaluated. Figure 4 displays the response surface of DPPH, which is determined by the ratios of water, ethanol, and methanol.

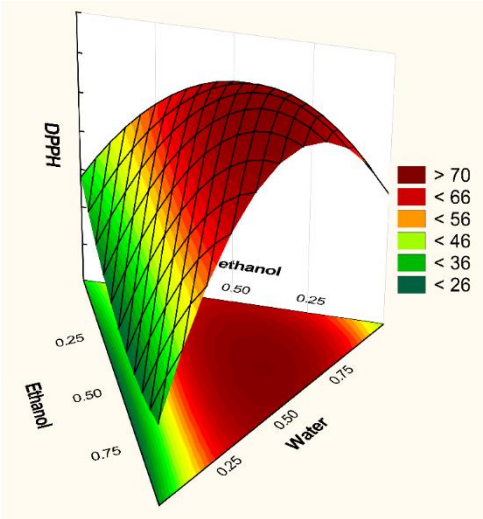

Fig. 4. 3D response surface contour plot of the special cubic model for DPPH

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Generally, solvents that are pure extracts showed lower levels of DPPH free radical scavenging capabilities when compared to combinations of solvents. Water extracts demonstrated the highest activity among the pure solvents, with methanol and ethanol following in terms of effectiveness. It is important to note that the DPPH free radical scavenging capability of ethanol and methanol mixtures increases as the water percentage increases. The highest scavenging capacity is achieved when the water percentage is between 50 and 75 %. The scavenging capability begins to decrease when mixes contain more than 75 % water. The contour graph shows that the highest value is observed when the three solvents interact together, next comes the interactions of pure water with ethanol and with methanol. The binary effects of "water-ethanol" and "water-methanol" were found to be synergistic, while the binary impact of "methanol-ethanol" was determined to be antagonistic.

The cubic model is represented by equation (Eq. 5) which establishes a correlation between the three variables and provides an analytical response. The equation is as follows:

DPPH = + 44.68 * (%W) + 37.94 * (%M) + 31.26 * (%E) + 106.67 * (%W* %M) + 142.04 * (%W* %E) - 35.81 * (%M* %E) + 241.64 * (%W* % M* %E) + 0. **Eq. 5**

Based on the polynomial equation, water had the most significant positive impact on extracting compounds with free radical scavenging activity among pure solvents, followed by methanol. The binary mixtures demonstrated substantial synergistic effects between the two organic solvents (methanol, ethanol) and water. In contrast, the ternary interaction among all three solvents had the most pronounced impact.

3.6. Statistical analysis for validation of experimental models

To assess the appropriateness and importance of the model, a variance analysis (ANOVA) was conducted. P-values are utilized to assess the significance of the corresponding coefficient, with lesser p-values indicating more significance.

The independent and dependent variables were fitted to linear, quadratic, and cubic models. The extraction solvent used on the roots of the plant investigated was evaluated for its efficiency in terms of total phenolic component content and antioxidant activity (Table 2). The quadratic model was selected due to its excellent predictive ability, accurately describing 100% of the total variance (determination coefficient R2 = 1.000, R2 adjusted = 1.000). This indicates that all response functions effectively fit the experimental data, and the models can be reliably used to predict the extraction of polyphenols and other antioxidant molecules using various solvent mixtures.

Based on the data shown in Table 3, all models had a high level of significance, as evidenced by the very low p-values of 0.000000 and the high F-values of 3643.37, 925668.6, and 43108.73 for TPC, TAC, and DPPH, respectively. Based on the analysis of the estimated F values and probability values, it can be concluded that the models below are adequately adjusted and very suitable and significant.

To assess the significance of the main effects and their interactions, a graph of standardized Pareto was employed, with a significance level set at $P < 0.05$. Based on Figures 5, 6, and 7, the primary components that had a significant

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impact over the baseline level of 0.05 were water (A), methanol (B), ethanol (C), as well as their binary interactions (AB, AC, and BC) and ternary interaction (ABC).

Table 2. Results of variance analysis for several statistical models

Essay	Models	SS	df	MS	F	p	R^2	R^2 adj
TPC	Linear	300.31	2	150.16	23.48	0.000010	0.72	0.69
	Quadratic	114.69	3	38.23	1384.35	0.000000	1.00	1.00
	Special Cubic	0.15	1	0.15	7.81	0.014317	1.00	1.00
	Total Adjusted	415.42	20	20.77				
TAC	Linear	17347.81	2	8673.90	30.4	0.000002	0.77	0.75
	Quadratic	4668.10	3	1556.03	49.0	0.000000	0.98	0.97
	Special Cubic	476.11	1	476.11	117568.0	0.000000	1.00	1.00
	Total Adjusted	22492.08	20	1124.60				
DPPH	Linear	1721.39	2	860.69	2.80	0.087055	0.24	0.15
	Quadratic	5376.55	3	1792.18	181.84	0.000000	0.98	0.97
	Special Cubic	147.45	1	147.45	5263.75	0.000000	1.00	1.00
	Total Adjusted	7245.77	20	362.29				

Table 3. Response surface approach statistical parameters

Essay	Source	SS	df	MS	F	p
TPC	Model	415.16	6	69.19	3643.37	0.000000
	Total Error	0.27	14	0.02		
	Lack of Fit	0.0000	0	0.00000		
	Pure Error	0.27	14	0.02		
	Total Adjusted	415.42	20	20.77		
TAC	Model	22492.02	6	3748.67	925668.6	0.00
	Total Error	0.06	14	0.004		
	Lack of Fit	0.00	0	0.000		
	Pure Error	0.06	14	0.004		
	Total Adjusted	22492.08	20	1124.604		
DPPH	Model	7245.8	6	1207.56	43108.73	0.000000
	Total Error	0.39	14	0.028		
	Lack of Fit	0.000	0	0.000		
	Pure Error	0.39	14	0.028		
	Total Adjusted	7245.77	20	362.29		

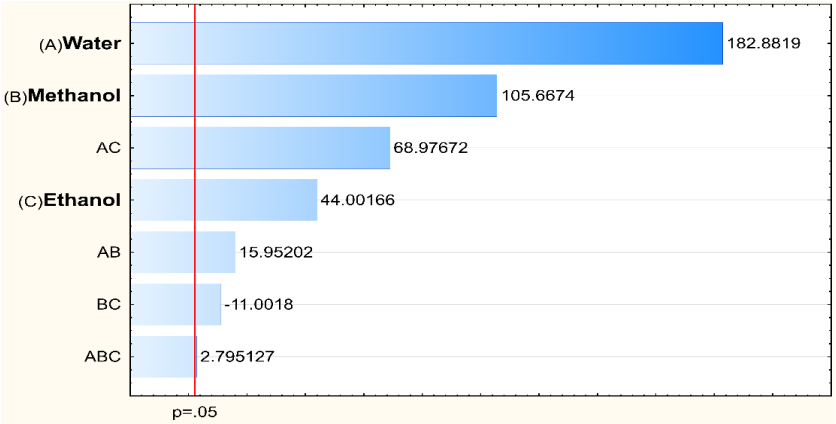

Fig. 5. Pareto chart analysis of standardized effects for TPC

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According to Figure 5, water (A) has the greatest impact on the extraction of phenolic chemicals, with methanol (B) having a slightly lesser influence. Therefore, the application of the combination has determined the optimal ratio of solvents for extracting phenolic chemicals.

The Pareto diagram, standardized and depicted in Figure 6, clearly indicates the significance of all solvents and their interactions. It is worth mentioning that water has had the greatest impact on the extraction of antioxidant chemicals, followed by methanol and the combined effect of pure ethanol with water.

In the case of DPPH (Fig.7), the extraction of antioxidant chemicals was mainly and positively affected by pure water, with methanol following suit. Among the parameters examined, ethanol shown a notable and favorable impact on the extraction of antioxidant chemicals, ranking third in terms of significance.

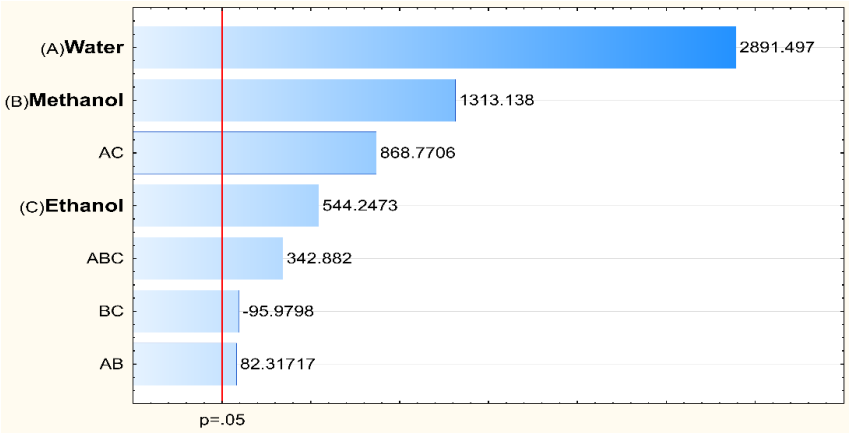

Fig. 6. Pareto chart analysis of standardized effects for TAC

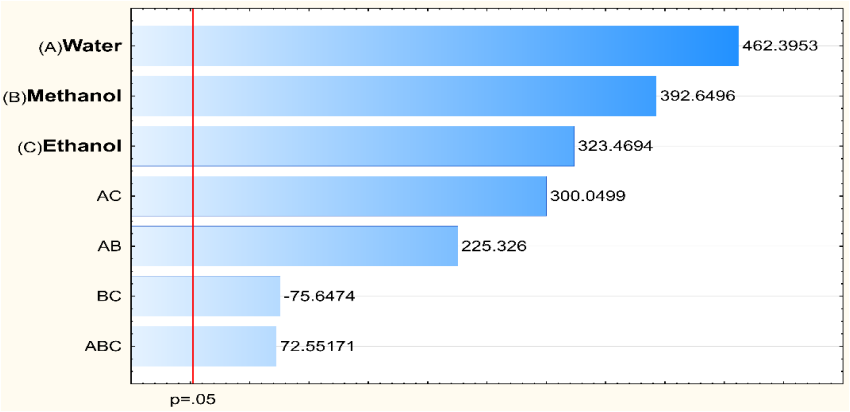

Fig. 7. Pareto chart analysis of standardized effects for DPPH

Figure 8 illustrates a graphical depiction of the anticipated values and the desirability profile.

A desirability profile was created for TPC and antioxidant activity, with values of 3.5 mg/g classified as low (0.00), 9.7 mg/g as intermediate (0.05), and 15.8 mg/g as high (1.00). The TAC desirability profile was created using the values of 19.9 mg/g for low desirability (0.00), 63.1 mg/g for intermediate desirability (0.05), and 106.3 mg/g for high desirability (1.00). The DPPH concentration is

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25.6 mg/g, which is considered low (0.00). The concentration of 49.6 mg/g is classified as moderate (0.05), while the concentration of 73.7 mg/g is considered high (1.00). The most effective combination of solvents for producing extracts with the highest value of total phenolic content (TPC) and antioxidant activity was found to be composed of 50 % water, 10 % methanol, and 40 % ethanol.

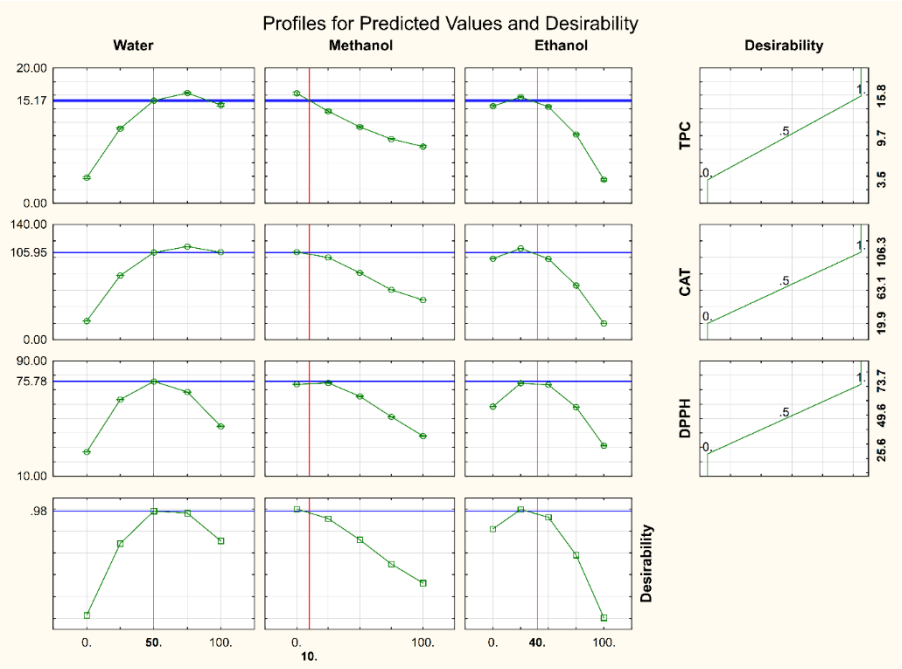

Fig. 8. Desirability profile for the optimization of the solvent reference combination

3.7. Fractionation

3.7.1. Total phenolic content (TPC)

Phenolic compounds are a class of antioxidant agents which constitute a major group of compounds which contribute to the antioxidant activities of plant materials due to their ability to scavenge free radicals due to their hydroxyl groups [20].

Figure 9 depicts the content of total phenolic compounds in the aqueous fraction and different solvent fractions. Among the 5 different solvent extracts, TPC showed significant differences ($P \leq 0.05$). Ranging from 0.49 to 5.06 mg GAE / g dry roots, for chloroform extract and ethyl acetate extract, respectively. While the greatest amount of TCP was found in the remaining water fraction with 14.57 ± 0.01 mg GAE / g dry roots.

3.7.2. Antioxidant activity

The antioxidant activity of *A. pyrethrum* fractions was observed in the present study by in vitro assays; reducing potency assays to assess the free radical scavenging activity and antioxidant capacity of each fraction.

3.7.2.1. Total antioxidant capacity (TAC)

This test relies on the reduction of the phosphomolybdate ion in the presence of an antioxidant, leading to the creation of a green phosphate / MoV complex. The concentration of this complex is then determined using spectrophotometry [21].

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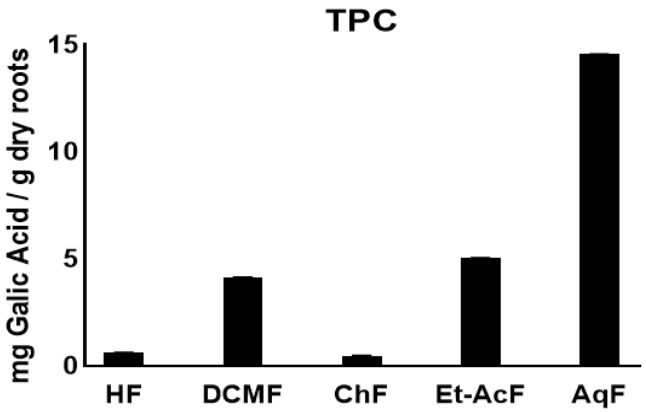

Fig. 9. Total phenolic contents from various fractions

The results of Fig. 10 showed that the aqueous fraction was the most active fraction (18.48 ± 0.02 mg EAA / g of dry roots), the other fractions have the order of DCMF (5.59 ± 0.02 mg EAA / g dr) > EtAcF (4.57 ± 0.02 mg EAA / g dr) > CHF (3.72 ± 0.01 mg EAA / g dr) > NHF (2.17 ± 0.02 mg EAA / g dr). Therefore, these results suggest that phenolic compounds could be the main contributors to antioxidant potential and inhibitory actions towards oxidative reactions.

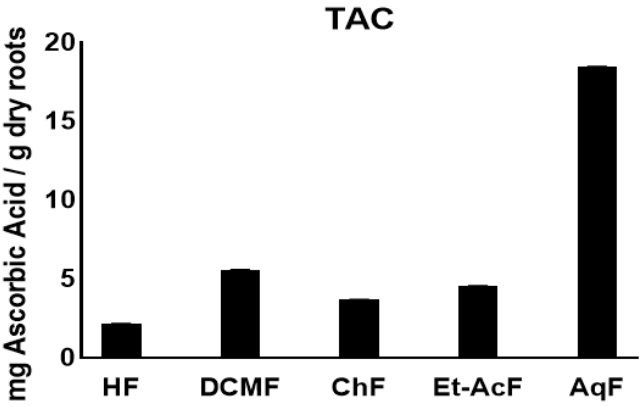

Fig. 10. Total antioxidant capacity of different fractions

3.7.2.2. Ferric reducing-antioxidant power (FRAP)

The reducing property is commonly linked to the existence of reducing agents. The antioxidant effect of reducing substances is derived from their ability to disrupt the radical chain reaction by the donation of a hydrogen atom. Reducing agents also undergo reactions with specific peroxide precursors, hence inhibiting the generation of peroxide. The data presented here suggests that the extracts' significant reducing activity is attributed to the presence of polyphenols. These compounds function as reducing agents by donating electrons and reacting with free radicals, converting them into more stable products and terminating the radical chain reaction [1].

Figure 11, shows the reducing power of the fractions which increased with increasing concentration. The ethyl acetate fraction of *A. pyrethrum* showed the highest reducing ability than all the other fractions tested. The

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dichloromethane, hexane, and chloroform fractions also showed significant activity indicating its reductive power. A much lower reducing ability was observed for the aqueous fraction. findings revealed a significant correlation between total phenolic compounds and reducing power in *A. pyrethrum*.

Several plant extracts have been found to exhibit a decrease in potency due to the presence of polyphenolic components [1, 22]. The findings indicate that the polyphenolic content of the sample extracts functions as effective electron and hydrogen donors. Consequently, it has the ability to halt the radical chain reaction by transforming free radicals into more stable substances.

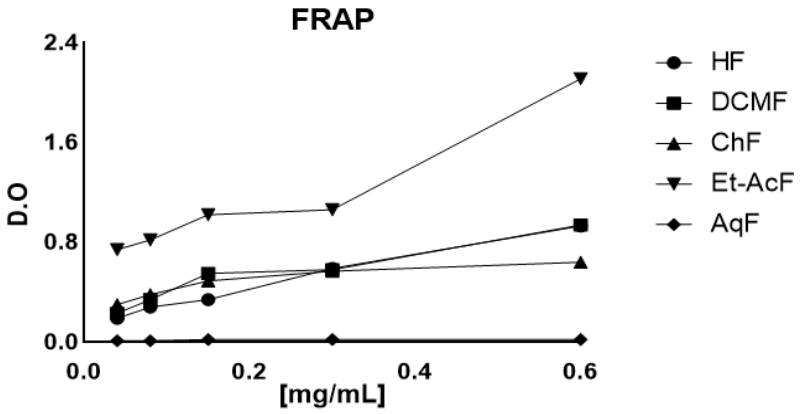

Fig. 11. Ferric Reducing-Antioxidant Power of fractions

3.7.2.3. Free radical scavenging activity: DPPH

The DPPH Free Radical Scavenging Test is a highly effective method commonly employed to evaluate the ability of individual or combined substances to remove free radicals. When the antioxidants interact with the DPPH (purple hue), a transfer of one or more hydrogen atoms occurs from the antioxidants to the DPPH. This leads to the oxidation of the antioxidants and the reduction of the DPPH radicals, resulting in the formation of yellow-colored DPPH-H [23]. The DPPH test utilized various doses of extract to quantify the anti-free radical activity. The antioxidant activity of the fractions is depicted in Figure 12.

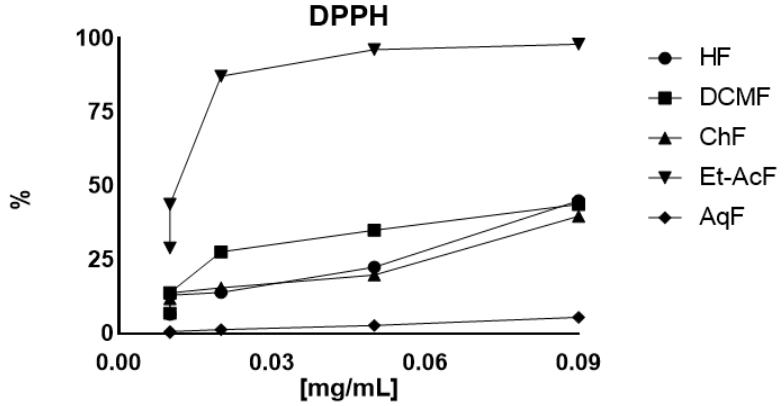

Fig. 12. Fractions capacity to scavenge free radicals: DPPH

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Based on the results, all fractions exhibited dose-dependent antioxidant activity. From the results of this study, the ethyl acetate fraction showed the highest antioxidant activity (98.02 ± 0.13 %), followed by the hexane, dichloromethane, and chloroform fractions (45.05 ± 0.23, 43.75 ± 0.17, and 39.82 ± 0.13 %, respectively), whereas the aqueous fraction exhibited the lowest antioxidant activity.

3.7.2.4. ABTS radical scanning activity

The ABTS test involves the ABTS • + radical cation, which has a greenish-blue color. When this radical cation reacts with antioxidants, it has the ability to donate an electron to the body. The study investigated the relationship between the concentration of antioxidants and the ABTS radical cation and color discharge [24]. The ABTS radical possesses a notable advantage in terms of its high reactivity, which enables it to effectively interact with a broader spectrum of antioxidants. However, the process of preparing the ABTS reagent is more challenging and it lacks stability compared to the DPPH, resulting in more accurate outcomes [25].

According to the findings in Figure 13, the chloroform fraction exhibited the maximum antioxidant activity at a level of 64.89 ± 0.15 %. Comes next the ethyl acetate, dichloromethane, and hexane fractions, respectively. The aqueous fraction exhibited the lowest ABTS• + radical scavenging activity.

3.7.2.5. Chelation capacity

Ferrozine is known to form quantifiable complexes with Fe^{2+} ions in the presence of chelators, however, this complex formation is disrupted, resulting in a reduction of the complex's characteristic red coloration. This decrease in color intensity allows for the indirect quantification of the chelating activity of any coexisting chelators [14]. The impact of chelating agents from *A. pyrethrum* fractions on the interaction between Fe^{2+} and ferrozine is depicted in Fig. 14.

The chloroform fraction demonstrated the most potent chelating effect, achieving 82.04 ± 0.10 %, followed by ethyl acetate at 69.43 ± 0.06 %, dichloromethane at 50.13 ± 0.12 %, and hexane fractions at 47.73 ± 0.18%. The aqueous fraction exhibited the least effectiveness in chelation. This variation in metal chelating ability among the different solvent extracts can largely be attributed to the nature of the solvent used during the extraction process. Non-polar solvents such as chloroform and ethyl acetate are particularly effective in extracting compounds with strong chelating properties, thereby enhancing their capacity to bind metal ions and reduce metal-induced oxidative stress. These findings underscore the importance of selecting an appropriate solvent to optimize the extraction of specific bioactive compounds, particularly those that contribute to metal chelation. Such insights are crucial for developing targeted antioxidant therapies and suggest that the strategic choice of solvent can significantly enhance the functional properties of plant-derived extracts, potentially leading to more effective applications in mitigating oxidative damage in biological systems.

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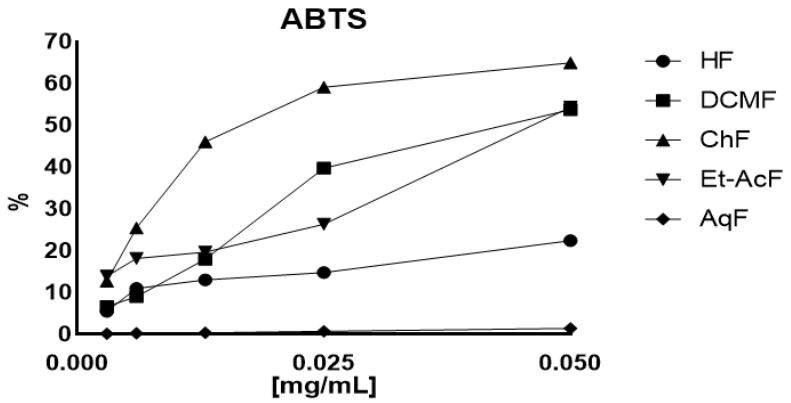

Fig. 13. Activity of ABTS radical scanning on fractions

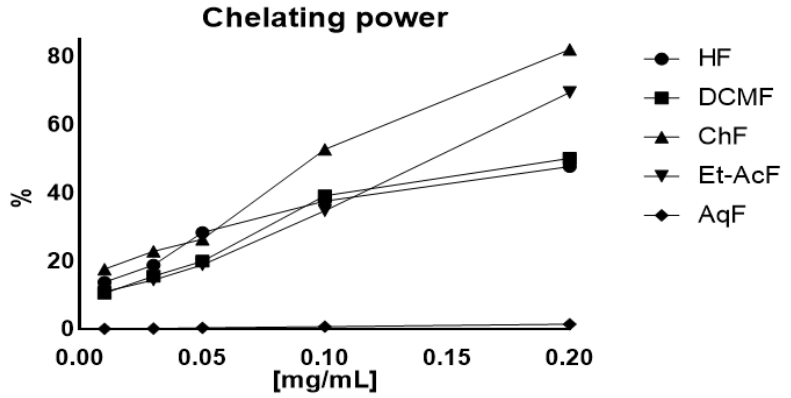

Fig. 14. Chelating power for the fractions

3.7.2.6. Nitric oxide scavenging activity

The current study assessed the nitrite-free radical scavenging activity of five fractions at varying concentrations using an in vitro model. The sodium nitroprusside produces NO, which then combines with oxygen to create nitrite. The nitrite ions are then diazotized with sulfanilamide acid and combined with naphthyl ethylene diamine, resulting in the formation of a pink color. This color is measured at a wavelength of 550 nm.

As Fig. 15 shows, the NO radical scavenging capacity of the fractions was dependent on extract concentration; with the most effective inhibition 96.7 ± 0.06 % at 0.20 mg/ml concentration for chloroform fraction, followed by the ethyl acetate, hexane, and dichloromethane fractions (64.73 ± 0.06, 49.21 ± 0.16 and 42.69 ± 0.10 %, respectively) whereas the AqF present the lowest nitrite free radical scavenging activity. The results obtained for all the fractions tested were statistically significant ($P \leq 0.05$). The result indicates that the extracts contain compounds capable of inhibiting NO and offer scientific evidence for the use of the plant as an anti-inflammatory.

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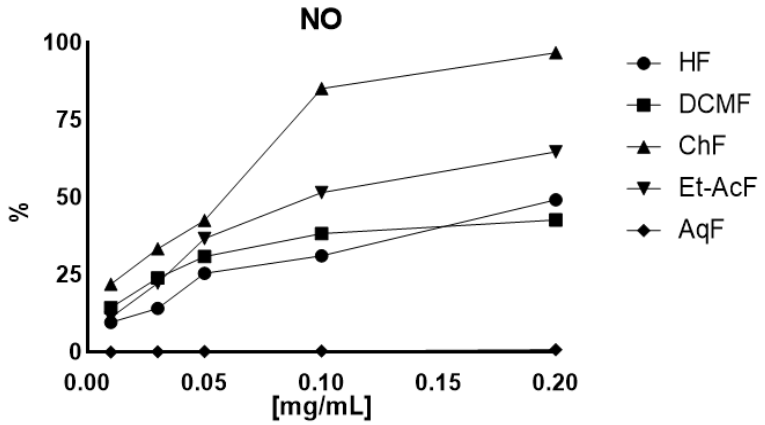

Fig. 15. Fractions NO scavenging activity

4. Discussion

Recent advancements in the search for potent natural antioxidants, especially those derived from herbal sources, have emphasized the crucial role of extraction techniques in isolating bioactive compounds. Among these, Ultrasonic-assisted extraction (UAE) has been demonstrated to be an efficient method due to its rapid action and strong cavitation effects. Concurrently, Response Surface Methodology (RSM) has been instrumental as a statistical tool for optimizing these extraction parameters and elucidating their interactions [26]. Our findings indicate that the selection of extraction solvents significantly impacts the phenolic content and antioxidant capacity of *A. pyrethrum* root extracts. This observation is consistent with reports by Chater et al. (2023) [27] and Aazza (2021) [28], which identified water, ethanol, and methanol as particularly effective for high polyphenol yield. Specifically, our results highlight the binary water-ethanol mixture, with 70 % alcohol, as the most effective, suggesting a synergistic effect that enhances phenolic extraction more than when these solvents are used individually. This synergy aligns with Aazza (2021) who reported optimal phenolic yields at a similar solvent ratio [28], and is supported by Muñiz-Márquez (2013), who found that a 70 % ethanol concentration provided the best yields due to the polarity alignment with the phenolic compounds [29]. Moreover, different studies, such as Şahin and Şamlı (2013), have pinpointed alternative optimal solvent mixtures for maximizing phenolic extraction, further demonstrating the variability in solvent efficacy based on specific extraction goals [30].

In terms of total antioxidant capacity (TAC), our analysis revealed that ternary mixtures generally outperformed binary and single-solvent systems. Notably, pure water extracts exhibited significant antioxidant activity, overshadowing those obtained from pure ethanol and methanol. This trend was also evident in DPPH scavenging activity, where water-rich mixtures (50 to 75 % water) showed enhanced free radical scavenging capacity, diminishing with higher water content. The contour plots and surface response analyses confirmed these findings, pinpointing high antioxidant capacities near water-dominated mixtures, which underscores the critical influence of solvent composition on the effectiveness of antioxidant extraction.

5. Conclusion

This study comprehensively assessed the total phenolic content and antioxidant capacities of *A. pyrethrum* root fractions establishing them as

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a potent source of natural antioxidants. The investigations revealed a strong correlation between the antioxidant properties and phenolic content across various extracts, underscoring the significant role of phenolics in mediating antioxidant activities. The robust antioxidative potential observed in the root fractions indicates promising applications for *A. pyrethrum* in pharmaceutical formulations aimed at combating oxidative stress.

Furthermore, the study highlights the critical influence of methodological parameters, such as solvent choices and extraction techniques, on the efficacy of bioactive compound isolation. Our findings suggest that optimizing these parameters can significantly enhance the extraction and therapeutic potential of the extracts. Given the positive correlations observed, it is imperative that further in vivo clinical research be conducted to validate these findings and explore their practical applications in medicine.

Author contribution

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

Conflicts of interest

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

Acknowledgment

None

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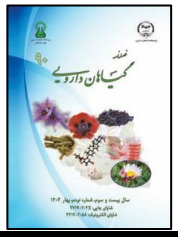

مقاله تحقیقاتی

بهینه‌سازی استخراج پلی‌فنل از ریشه‌های *Anacyclus pyrethrum* var. *depressus*: یک رویکرد طراحی مخلوط سیپلکس مرکزی

اومیما چاتر[۱،*]، اسماعیل اعزا[۲]، هیبه بوزید[۳]، لاحسن ال قدراوی[۱]

[۱] دانشگاه سیدی محمد بن عبدالله، دانشکده علوم و فناوری، آزمایشگاه اکولوژی کاربردی و مهندسی محیط زیست، فاس، مراکش

[۲] دانشگاه محمد اول، اوجدا، دانشکده چند رشته‌ای نادور (FPN)، OLMANBGPE، نادور، مراکش

[۳] دانشگاه سیدی محمد بن عبدالله، دانشکده علوم و فناوری، آزمایشگاه شیمی آلی، فاس، مراکش

اطلاعات مقاله

گل‌واژگان:

Anacyclus pyrethrum

ظرفیت آنتی‌اکسیدانی

ترکیبات فنلی

طراحی مخلوط Simplex-Centroid

بهینه‌سازی حلال

چکیده

مقدمه: *Anacyclus pyrethrum* var. *depressus* یک گیاه دارویی است که ریشه‌های آن دارای خواص دارویی در سیستم‌های طب سنتی و مدرن گزارش شده است. **هدف:** این مطالعه با هدف بهینه‌سازی استخراج پلی‌فنول‌ها و ترکیبات با خواص آنتی‌اکسیدانی با استفاده از طراحی مخلوط Simplex-Centroid انجام شده است. **روش بررسی:** یازده حلال برای بررسی تمایل آنها به مولکول‌های زیست‌فعال *A. pyrethrum* آزمایش شدند و سه حلال برتر (آب، متانول و اتانول) برای مدل‌سازی مخلوط‌سازی به منظور بهینه‌سازی انتخاب شدند. مدل‌های مختلفی برای مطالعه اثرات ترکیبی دوگانه و سه‌گانه توسعه یافتند. جداسازی عصاره‌ها با استفاده از چهار حلال شامل کلروفرم، دی‌کلرومتان، اتیل استات و هگزان انجام شد. ارزیابی فعالیت آنتی‌اکسیدانی این فراکسیون‌ها با استفاده از تست‌های مختلف (TAC، DPPH، FRAP، ABTS، قدرت کی لیت و فعالیت مهاراکسید نیتریک) انجام شد. **نتایج:** مدل‌های توسعه یافته و تمام پارامترهای آنها معنی‌دار بودند. تحلیل مدل‌های ایجاد شده با روش پاسخ سطح نشان داد که مقدار ترکیبات فنولی استخراج شده در مخلوط‌های دوگانه و همچنین فعالیت کل آنتی اکسیدانی عصاره‌ها با افزایش درصد آب در مخلوط حلال با استفاده از هر دو حلال متانول یا اتانول افزایش می‌یابد. ترکیب‌های دوگانه "آب-اتانول" و "آب-متانول" و مخلوط سه‌گانه مؤثرترین ترکیبات برای استخراج ترکیبات ضد رادیکال بودند. نتایج جداسازی نشان داد که فراکسیون آبی بالاترین فعالیت آنتی‌اکسیدانی را به دلیل محتوای بالاتر ترکیبات فنولی دارد. **نتیجه‌گیری:** با این نوع تحقیقات، می‌توان به راحتی به پیشگیری و درمان آسیب‌های انسانی ناشی از رادیکال‌های آزاد پرداخت و همچنین آنتی‌اکسیدان‌های مصنوعی را در صنعت جایگزین کرد.

مخففها: AAE، معادل اسید اسکوربیک؛ ABTS، ۲، ۲-آزینوبیس ۳-اتیل بنزوتیازولین ۶- سولفونات؛ AQF، کسر آبی؛ CHF، کسر کلروفرم؛ DCMF، کسر دی کلرومتان؛ DPPH، ۲، ۲-دی فنیل-۱-پیکریل هیدرازیل؛ E، اتانول؛ EAF، کسر اتیل استات؛ FRAP، قدرت آنتی‌اکسیدانی کاهش دهنده آهن؛ GAE، معادل اسید گالیک؛ HF، کسر هگزانی؛ M، متانول؛ NO، اکسید نیتریک؛ TAC، فعالیت آنتی‌اکسیدانی کل؛ TPC، محتوای فنلی کل؛ W، آب

* نویسنده مسؤول: oumaima.chater@usmba.ac.ma

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