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Chemometric Optimization of Functional Compound Extraction from *Artemisia annua* L. and Assessment of Its Antioxidant and Antimicrobial Properties

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ABSTRACT: *Artemisia annua* L. is a medicinal plant valued for its bioactive compounds, especially artemisinin. This study focused on improving the extraction of these compounds using a CCRD design, combining ethanol/water (0−100%) and extraction times (60−300 s), with a mechanical homogenizer (ultradisperser). The obtained extracts were analyzed by using spectrophotometric methods (UV−vis and HPLC-DAD) to determine the presence of phenolic compounds, flavonoids, and sesquiterpenes. Antioxidant and antimicrobial capacities were evaluated. Ethanol concentrations between 50 and 60% increased the extraction of phenolics with antioxidant potential, and concentrations greater than 70% were more effective for sesquiterpenes. Extracts with a higher artemisinin content showed strong antimicrobial activity, especially against Gram-positive bacteria. Multivariate analyses validated the relationships between the extracted compounds and their biological effects. The overall extraction condition was 50% (ethanol/water, v/v) for 180 s, highlighting the biological potential of *A. annua* L. and the importance of optimizing extraction processes.

KEYWORDS: *medicinal plants, artemisinin, response surface methodology, central composite design (CCD), statistical modeling*

1. INTRODUCTION

Since ancient times, aromatic medicinal plants have been used as alternatives for the prevention, treatment, and prophylaxis of various health problems. However, their effects on human health have been better understood and studied in greater detail in recent years, as analytical and pharmacological advances have allowed for a more precise understanding of which bioactive compounds are responsible for their biological effects.[1] Plants such as *Artemisia annua* L have their potential maximized through the use of appropriate technologies for extracting bioactive compounds.

A. annua L., which belongs to the Asteraceae family, is commonly used in traditional Chinese medicine to treat different kinds of health problems. This species has an abundance of bioactive compounds from diverse chemical classes, including phenolics, flavonoids, coumarins, sesquiterpenoids, steroids, amino acids, and vitamins.[2,3] This wide range of compounds and chemical profile are directly influenced by the extraction method, as the extent to which these compounds are recovered determines the biotechnological potential of the resulting extracts.

Recent studies have shown that *A. annua* L. exhibits various biological activities, including antioxidant, anti-inflammatory, immunomodulatory, antimicrobial, leishmanicidal, antifungal, antiviral, antitumor, antipyretic, and antimalarial activities.[4,5]

Many of these biological activities are associated with artemisinin ($C_{15}H_{22}O_5$), a cadinane-type sesquiterpene lactone that contains an endoperoxide ring in its structure.[6] Besides artemisinin, the plant also contains other bioactive molecules such as artemisinic acid, dihydroartemisinic acid, flavonoids, and phenolic acids, which can synergistically contribute to the therapeutic potential of the species.[7,8]

Maximizing the therapeutic potential of medicinal plants depends directly on the efficiency of extracting bioactive compounds. The choice of ideal extraction conditions not only affects the yield of individual metabolites but also the overall phytochemical profile of the extracts.[9] Despite growing interest in this species, there is still a lack of studies in the scientific literature that establish extraction parameters capable of maximizing the recovery of different groups of bioactive compounds.

By lowering the amount of energy and solvent used and encouraging the use of environmentally friendly solvents like ethanol and water, optimizing extraction conditions can also be good for the economy and the environment.[10] Optimized extraction methods can broaden the range, quantity, and availability of bioactive compounds and may even eliminate the need for additional extraction and purification steps. These methodologies speed up the procedure, making it more efficient, especially when the aim is to obtain extracts suitable for biological and food applications.[11] In this context, statistical

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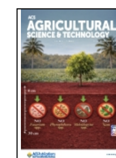

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Table 1. Phenolic Compounds, Total Flavonoids, Antioxidant Activity, and Phytochemical Composition of Extracts Obtained under Different Ethanol Concentrations and Extraction Times Based on a Central Composite Rotatable Design (CCRD)[a]

treatment	ethanol (%)/ level	time (s)/ level	TPC (g GAE 100 g^{-1})	TFC (g QE 100 g^{-1})	ABTS (mmol TE g^{-1})	DPPH (mmol TE g^{-1})	FRAP (mM Fe^{2+} g^{-1})
T1	15 (−1)	95 (−1)	9.80 ± 0.25^{cd}	1.79 ± 0.14^{def}	82.27 ± 4.37^{bc}	337.17 ± 4.83^{d}	2729.33 ± 41.63^{d}
T2	15 (−1)	265 (1)	10.11 ± 0.07^{bcd}	1.61 ± 0.05^{ef}	83.60 ± 5.21^{bc}	340.39 ± 3.54^{d}	2958.22 ± 59.75^{d}
T3	85 (1)	95 (−1)	7.94 ± 0.48^{d}	2.58 ± 0.14^{cd}	76.71 ± 3.91^{c}	276.94 ± 6.41^{e}	5684.89 ± 103.57^{abc}
T4	85 (1)	265 (1)	10.00 ± 0.11^{abcd}	2.05 ± 0.64^{de}	89.16 ± 3.67^{b}	371.20 ± 9.39^{e}	5749.33 ± 112.15^{ab}
T5	0 (−1.414)	180 (0)	7.96 ± 0.16^{d}	1.00 ± 0.07^{fg}	73.82 ± 4.68^{c}	208.90 ± 1.38^{f}	1131.56 ± 73.43^{e}
T6	100 (1.414)	180 (0)	4.01 ± 0.19^{e}	0.62 ± 0.27^{g}	56.04 ± 1.02^{d}	111.43 ± 2.87^{g}	5769.33 ± 93.33^{ab}
T7	50 (0)	60 (−1.414)	13.51 ± 0.16^{ab}	3.08 ± 0.02^{bc}	112.49 ± 1.92^{a}	646.30 ± 27.77^{a}	4599.33 ± 3.33^{bc}
T8	50 (0)	300 (1.414)	14.46 ± 0.89^{a}	4.49 ± 0.09^{a}	113.38 ± 4.34^{a}	664.69 ± 32.44^{a}	6087.11 ± 795.36^{a}
T9 (C)	50 (0)	180 (0)	14.15 ± 0.88^{a}	3.31 ± 0.19^{bc}	112.04 ± 2.69^{a}	546.53 ± 21.43^{b}	4362.67 ± 1091.32^{c}
T10 (C)	50 (0)	180 (0)	14.39 ± 0.18^{a}	3.63 ± 0.23^{ab}	115.38 ± 4.68^{a}	473.89 ± 29.66^{c}	4907.11 ± 60.12^{abc}
T11 (C)	50 (0)	180 (0)	14.34 ± 0.35^{a}	3.88 ± 0.56^{ab}	118.71 ± 4.54^{a}	678.02 ± 15.87^{a}	4758.22 ± 336.80^{bc}

treatment	ART (mg·g^{-1})	DHAA (mg·g^{-1})	AA (mg·g^{-1})	gallic acid (mg·g^{-1})	catechin (mg·g^{-1})	chlorogenic acid (mg·g^{-1})	caffeic acid (mg·g^{-1})	*p*-coumaric acid (mg·g^{-1})	ferulic acid (mg·g^{-1})
T1	1.47 ± 0.10^{bc}	<LD	<LD	0.47 ± 0.13^{b}	<LD	209.70 ± 9.75^{ab}	87.67 ± 2.10^{b}	4.10 ± 0.28^{b}	17.62 ± 0.56^{b}
T2	2.14 ± 0.45^{c}	<LD	<LD	3.61 ± 0.52^{a}	<LD	209.75 ± 4.59^{ab}	92.05 ± 6.26^{b}	4.45 ± 0.27^{b}	16.88 ± 0.61^{b}
T3	5.99 ± 0.11^{a}	2.23 ± 0.06^{ab}	0.75 ± 0.02^{ab}	<LD	0.26 ± 0.16^{c}	178.40 ± 6.71^{ab}	3.50 ± 0.39^{c}	1.24 ± 0.24^{d}	0.70 ± 0.17^{c}
T4	6.47 ± 0.03^{a}	2.45 ± 0.07^{a}	0.84 ± 0.02^{a}	<LD	0.46 ± 0.27^{c}	229.94 ± 9.41^{a}	3.36 ± 0.34^{c}	2.74 ± 0.09^{c}	0.71 ± 0.06^{c}
T5	0.70 ± 0.58^{c}	<LD	<LD	<LD	0.26 ± 0.16^{c}	69.96 ± 4.77^{bc}	107.20 ± 2.74^{a}	5.73 ± 0.19^{a}	46.44 ± 2.53^{a}
T6	6.13 ± 0.20^{a}	2.23 ± 0.04^{ab}	0.77 ± 0.01^{ab}	<LD	<LD	20.75 ± 1.06^{c}	1.77 ± 0.40^{c}	1.75 ± 0.18^{d}	<LD
T7	5.63 ± 0.35^{a}	1.95 ± 0.16^{b}	0.62 ± 0.06^{c}	<LD	4.11 ± 0.38^{a}	298.91 ± 11.43^{a}	5.78 ± 0.07^{c}	<LD	1.25 ± 0.06^{c}
T8	6.22 ± 0.22^{a}	2.24 ± 0.08^{a}	0.73 ± 0.04^{b}	<LD	3.82 ± 0.06^{ab}	289.95 ± 8.64^{a}	4.93 ± 0.25^{c}	<LD	1.11 ± 0.12^{c}
T9 (C)	6.09 ± 0.24^{a}	2.21 ± 0.12^{ab}	0.72 ± 0.04^{bc}	<LD	4.18 ± 0.25^{a}	298.91 ± 11.43^{a}	5.61 ± 0.11^{c}	<LD	1.25 ± 0.06^{c}
T10 (C)	6.24 ± 0.30^{a}	2.24 ± 0.14^{a}	0.72 ± 0.04^{bc}	<LD	3.24 ± 0.11^{b}	269.24 ± 6.76^{a}	4.91 ± 0.08^{c}	<LD	0.89 ± 0.03^{c}
T11 (C)	6.33 ± 0.45^{a}	2.23 ± 0.13^{ab}	0.71 ± 0.05^{bc}	1.08 ± 1.88^{b}	3.57 ± 0.39^{ab}	200.52 ± 158.27^{ab}	5.37 ± 0.19^{c}	<LD	0.87 ± 0.02^{c}

[a]Different letters in the same column indicate a significant difference according to the Bonferroni test ($p < 0.05$). TPC: Total phenolic compounds; TFC: Total flavonoids compounds; ART: artemisinin; DHAA: dihydroartemisinic acid; AA: artemisinic acid; GAE: gallic acid equivalent; QE: quercetin equivalent; TE: Trolox equivalent; LD: limit of detection; (C): center point.

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approaches have been frequently used to identify the most suitable settings.

For obtaining optimized extracts, statistical tools widely used in modeling and process optimization involving multiple independent variables include experimental designs such as the Central Composite Rotational Design (CCRD), combined with Response Surface Methodology (RSM). Using polynomial models, three-dimensional response surface plots, and effect estimates, these optimization tools allow for more precise identification of optimized conditions for extracting bioactive compounds.[12,13]

In this study, we applied a Central Composite Rotational Design (CCRD) to optimize the extraction of *A. annua* L. It evaluated the effects of the ethanol concentration and extraction time on the levels of artemisinin, artemisinic acid, dihydroartemisinic acid, phenolic compounds, and flavonoids. The design also helped identify the factors that shape the antimicrobial and antioxidant activities of the extracts (ABTS, DPPH, and FRAP), enabling us to define the optimal experimental parameters to maximize both biological activity and bioactive compound content.

2. MATERIALS AND METHODS

2.1. Chemicals and Microorganisms

99.5% ethanol and the Folin-Ciocalteu phenol reagent were purchased from Xodo Científica Ltd. (Sumaré, SP, Brazil). The 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), quercetin 98%, gallic acid, and the standards used for HPLC analysis were obtained from Sigma-Aldrich Co. (St. Louis, MO). Aluminum nitrate was supplied by Reatec (Colombo, PR, Brazil), and potassium acetate was supplied by Dinâmica Química (Indaiatuba, SP, Brazil). Brain Heart Infusion (BHI) was acquired from IonLab (Araucária, PR, Brazil); nutrient agar from Himedia (Curitiba, PR, Brazil); chloramphenicol from Vetec Química Fina (Duque de Caxias, RJ, Brazil); and sodium salt resazurin from Sigma-Aldrich Chemical Co. (St. Louis, MO). The bacterial strains used in this study were *Salmonella Typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 19111), and *Bacillus cereus* (ATCC 19637).

2.2. Plant Material and Sample Preparation

The *A. annua* L. samples used (genotype Artemis F2, MEDIPLANT, Switzerland) were cultivated in the experimental area of the Federal University of Technology—Paraná (UTFPR), Pato Branco Campus, PR, Brazil (26°07′S; 52°41′W−760 m altitude) during the 2022/2023 growing season.

The *A. annua* L. leaves were dried in a forced-air circulation oven (MyLabor, model SSD 30L, São Paulo, SP, Brazil) at 35 °C until they reached a constant weight. Subsequently, the leaves were ground using a knife mill (Tecnal, model R-TE-650/1, Piracicaba, SP, Brazil) equipped with a 16-mesh sieve, resulting in particle sizes ranging from 0.6 to 1.2 mm.

2.3. Central Composite Rotational Design (CCRD)

The methodology used for extract optimization was based on ref 12, with minor adjustments. The effects of two factors were evaluated: solvent concentration (ethanol/water ratio, v/v) and extraction time (seconds), each tested at five coded levels (−1.41, −1.00, 0.00, 1.00, and 1.41). The experimental design followed a Central Composite Rotational Design (CCRD), with a total of 11 runs (T1-T11), with three replicates at the central point for statistical analysis.

Ethanol was chosen as the solvent due to its low toxicity. The concentrations used (0, 35, 50, 85, and 100% ethanol/water v/v) and the extraction times (60, 95, 180, 265, and 300 s) were based on previous studies, with slight modifications to the extraction times due to the technical specifications of the extraction equipment (ultra-disperser).[14]

The response variables included the total phenolic content (TPC), antioxidant activity assessed by ABTS, DPPH, and FRAP assays, and total flavonoid content. These parameters provided a comprehensive evaluation of the antioxidant potential of the analyzed samples. Additionally, the identification and quantification of phenolic compounds, flavonoids, and three sesquiterpene compounds—artemisinin (ART), artemisinic acid (AA), and dihydroartemisinic acid (DHAA)—were carried out using high-performance liquid chromatography (HPLC), as detailed in Table 1.

2.4. Obtaining Extracts

To obtain the extracts, 500 mg of *A. annua* L. leaves were immersed in 20 mL of ethanol/water solutions prepared according to the levels and combinations of the factors established by the central composite rotational design (CCRD). Extraction was performed using a mechanical homogenizer (Ultraturrax, model T25 digital, IKA, Campinas, SP, Brazil) operating at 20,000 rpm. The extracts were centrifuged at 4000 rpm for 15 min (Kasvi, model K14−0815C, São José dos Pinhais, PR, Brazil). The resulting supernatants were collected and stored in an ultrafreezer at −80 °C until analysis.

2.5. Total Phenolic Compounds (TPC) and Total Flavonoids (TFC)

The quantification of total phenolic compounds was performed using the Folin-Ciocalteu spectrophotometric method, with absorbance measured at 740 nm, as described in the methodology proposed by Singleton.[15] The results were expressed as $g \cdot 100\,g^{-1}$ gallic acid equivalents (GAE). The total flavonoid content was determined following the method described by Park et al.,[16] with absorbance measured at 415 nm, using aluminum nitrate ($Al(NO_3)_3 \cdot 9H_2O$) and potassium acetate (CH_3COOK). The results were expressed as $g.100\,g^{-1}$ quercetin equivalents (QE).

2.6. Antioxidant Activity

Antioxidant activity was evaluated using spectrophotometric methods based on the scavenging of ABTS and DPPH radicals as well as the Ferric Reducing Antioxidant Power (FRAP) assay. The ABTS method (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) was performed with absorbance measured at 734 nm, following the methodology described by Re et al. in ref 17, and the results were expressed as mmol Trolox equivalents per gram of sample (TEA). Antioxidant activity was evaluated using the DPPH method (2,2-diphenyl-1-picrylhydrazyl) at 517 nm, according to the procedure described by Pulido et al. in ref 18. The FRAP assay was carried out according to Perin et al. ref 19 with an absorbance read at 517 nm, and the results were expressed as mmol Fe^{2+} per gram of sample.

2.7. High-Performance Liquid Chromatography (HPLC) Analysis

2.7.1. Quantification of ART, DHAA, and AA. For the identification and quantification of artemisinin (ART), dihydroartemisinic acid (DHAA), and artemisinic acid (AA), the methodology followed the procedures described by Ferreira and Gonzalez.[20] A high-performance liquid chromatography (HPLC) system (Model LC-920, Varian Inc., Walnut Creek, CA) coupled to a diode array detector (DAD) set at 195 nm was used. Analyses were carried out using an ACE C-18 column (250 mm × 4.6 mm i.d., 5 μm), maintained at 30 °C, with a sample injection volume of 10 μL. The mobile phase consisted of an isocratic mixture of 60% acetonitrile and 40% of 0.1% aqueous acetic acid solution (pH 3.2), at a flow rate of 1.0 $mL \cdot min^{-1}$. Quantification was based on calibration curves prepared from sesquiterpene standards (ART, DHAA, and AA; Sigma-Aldrich) (kindly provided by Jorge Ferreira, Agricultural Water Efficiency and Salinity Research Unit, US Salinity Laboratory, United States Department of Agriculture—USDA), with concentrations ranging from 0.05 to 0.5 $mg \cdot mL^{-1}$. The calibration curves showed excellent linearity within the tested range with a determination coefficient (R^2) (Figure S2 and Table S3). Results were expressed as $mg \cdot 100\,mg^{-1}$ of dried leaf.

Table 2. Optimized Conditions from the Resolutions of the Equations with Significant Regression Coefficients (p < 0.05) through the Multivariate Statistical Analysis Obtained for the Model, a Central Composite Rotational Design (CCRD)

	TPC	TFC	ABTS	DPPH	FRAP	ART	DHAA
ethanol/water (%)	50/50	59/41	48/42	48/42	92/8	73/27	76/24
time (S)	281	ns[a]	ns[a]	ns[a]	ns[a]	209	209

	AA	gallic acid	catechin	chlorogenic acid	caffeic acid	*p*-coumaric acid	ferulic acid
ethanol/water (%)	81/18	ns[a]	50/50	48/42	75/25	0/100	0/100
time (s)	217	ns[a]	ns[a]	ns[a]	184	138	180

[a]ns: not significant for the model (p > 0.05)). TPC: Total phenolic compounds; TFC: Total flavonoids compounds; ABTS and DPPH: radical scavenging activity; FRAP: ferric reducing antioxidant power; ART: artemisinin; DHAA: dihydroartemisinic acid; AA: artemisinic acid.

2.7.2. Quantification of Phenolic and Flavonoid Compounds. The characterization of the phenolic profile of the extracts was performed with the methodology adapted from Bordin et al.[21]

High-performance liquid chromatography (HPLC-DAD, Varian 900-LC) was performed with a reversed-phase C18 column (ACE-121, 250 × 4.6 mm, 5 μm) maintained at 30 °C with a flow rate of 1.0 mL·min^{-1}. The mobile phase consisted of two solvents: (A) an aqueous solution of phosphoric acid (H_2O:H_3PO_4, 99.8:0.2 v/v) and (B) pure methanol (CH_3OH, 100%). The gradient program started with 30% of solvent B, increasing to 64% at 15 min, 75% at 25 min, 95% at 27 min, and returning to 30% at 32 min, followed by a re-equilibration phase maintained until 42 min. The standards used for identification and quantification included phenolic acids (gallic, chlorogenic, caffeic, *p*-coumaric, ferulic, vanillic, and cinnamic acids) and flavonoids (myricitrin, isoquercetin, mearnsitrin, myricetin, catechin, epicatechin, quercetin, and kaempferol) (Figure 2 and Table S3).

2.8. Antibacterial Activity

The antibacterial activity was determined using the broth microdilution method in sterile 96-well plates following the protocol with adaptations. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were evaluated.

For MIC determination, 190 μL of BHI broth containing a bacterial suspension (1×10^8 CFU mL^{-1}) was treated with 20 μL of the extracts at different concentrations. After incubation at 37 °C for 24 h, 30 μL of resazurin (0.1 mg·mL^{-1}) was added. A color change from blue to pink indicated bacterial growth and was considered a negative result for the inhibitory activity (MIC). Chloramphenicol (1.2 mg·mL^{-1}) was used as a positive control, and the bacterial suspension without extract served as a negative control.

For the MBC, aliquots from wells without color change were plated on nutrient agar. The absence of growth indicated a bactericidal effect, while growth indicated only a bacteriostatic effect.

2.9. Data Analysis

The response variables from the Central Composite Rotatable Design (CCRD) were obtained in triplicate and are presented as means with their respective standard deviations. To assess the existence of significant differences between treatments, a Generalized Linear Model (GLM) with a γ probability distribution was applied, followed by Bonferroni's multiple comparison test using RStudio software. The data were also subjected to analysis of variance (ANOVA) (Table S1) at a significance level of p < 0.05, using Statistica software. The effects of the independent variables were estimated using a Pareto chart. The adequacy and optimization of the mathematical models were evaluated through Response Surface Methodology (RSM) (Figure 4), and the optimal condition was determined based on the equations generated by the models (eq 1 and Table 2). Model fit was expressed as the coefficient of determination (R^2) (Table S2).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where Y is the response of each variable, X_1 and X_2 present the independent variables of the model, and β_0, β_1, β_2, β_{11}, β_{22}, and β_{12} are the regression coefficients associated with the linear, quadratic, and interaction effects, respectively.

The chi-square (χ^2) test was used to assess the existence of statistical differences between the values predicted by the model and the experimentally observed data (Table S2).

Additionally, the global response (GR) was calculated using eq 2, with the aim of identifying the experimental condition that provided the best performance among the evaluated treatments.

$$\mathrm{GR} = \left[\frac{R_{(x1)}}{\mathrm{MR}_{(x1)}} + \frac{R_{(x2)}}{\mathrm{MR}_{(x2)}} + \ldots + \frac{R_{(xn)}}{\mathrm{MR}_{(xn)}} \right] \quad (2)$$

where R (x_n) represents the response obtained for each treatment, and MR (x_n) corresponds to the highest value observed in the data set n.

Multivariate analyses were also performed, including hierarchical cluster analysis (HCA), represented by a dendrogram, principal component analysis (PCA), and correlation networks based on Pearson correlation (HeatMap).

3. RESULTS AND DISCUSSION

3.1. Effects of Solvent Concentration (Ethanol/Water) and Extraction Time on the Yield of Bioactive Compounds

Ethanol was chosen as the solvent due to its low toxicity compared to other organic solvents, taking into account applications using either the pure solvent or residual solvent remaining after extract purification steps.[22] Given the wide range of compounds present in plant matrices and the rationale for choosing ethanol, it is necessary to determine which ethanol concentration is most efficient for extracting the greatest variety of compounds, as well as the ideal concentration of ethanol for each compound analyzed and biological activity.[23] Extraction time and solvent concentration were defined based on previous studies and also within a range that prevents sample heating during agitation, which could lead to the degradation of the plant's chemical compounds.

The data regarding the phytochemical composition of *A. annua* L. extracts, obtained under different ethanol and water concentrations (%) and extraction times (s) according to the CCRD design, are presented in Table 1.

The extraction efficiency of bioactive compounds was significantly affected by the predefined extraction conditions, influencing the recovery of different classes of compounds from the plant material, including total and individual phenolics and flavonoids, as well as bioactive sesquiterpenes.

3.2. Total Phenolic Compounds (TPC) and Total Flavonoids (TFC)

Longer extraction times and intermediate ethanol concentrations (50%) produced the highest concentrations of total flavonoids (TFC) and total phenolic compounds (TPC). The highest levels of TPC and TFC were found in treatment T8 (50% ethanol and 300 s), with values of 14.46 g of GAE·100 g^{-1} and 4.49 g of QE·100 g^{-1}, respectively. This difference was significant (p < 0.05) when compared to the other treatments,

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except for T9, T10, and T11 (all with 50% ethanol and 180 s extraction time), which did not differ significantly from each other ($p > 0.05$).

These results indicate that the combination of a moderate ethanol concentration and prolonged extraction times favors the extraction of these compounds, whereas highly polar (T5:0% ethanol) or nonpolar solvents (T6:100% ethanol) were less efficient. In T6, the contents were only 4.01 g GAE·100 g^{-1} for TPC and 0.62 g QE·100 g^{-1} for flavonoids, showing statistically significant differences compared to the best-performing treatments ($p < 0.05$). This result also highlights the importance of solvent polarity in the extraction of phenolic compounds and flavonoids present in the plant matrix.

Hydroalcoholic mixtures act as effective solvents for extracting compounds with varying degrees of polarity. This characteristic enables the efficient solubilization of phenolic compounds and flavonoids, which may exist in free form or be bound to the cell wall. The use of intermediate ethanol concentrations, as in treatments with 50% ethanol (T8, T9, T10, and T11), facilitates the disruption of interactions between these compounds and the plant matrix, enhancing their availability. Additionally, longer extraction times, such as in T8 (300 s), contribute to improved diffusion of the compounds into the solvent. The combination of appropriate solvent polarity and sufficient extraction time maximizes the availability of bioactive compounds in the extracts.[24]

3.3. Antioxidant Activity (ABTS, DPPH, FRAP)

The treatments with the highest levels of TPC and TFC also exhibited the greatest antioxidant activities. In the ABTS and DPPH assays, the highest values were observed in treatments T8 (50% ethanol, 300 s) and T11 (50% ethanol, 180 s), which, despite using the same solvent concentration, differed in extraction time. When compared to the values found in treatments T1 through T6, both displayed significant differences ($p < 0.05$). T6 (100% ethanol, 180 s) had the lowest values, 56.04 and 111.43 mM TE·g^{-1}, respectively, while treatment T11 had the highest levels, 118.71 mM TE·g^{-1} (ABTS) and 678.02 mM TE·g^{-1} (DPPH) (Table 1). These results indicate that extreme ethanol concentrations reduce the efficiency of the antioxidant compound extraction.

In the FRAP assay, T8 showed the highest value with 6087.11 mM Fe^{2+}·g^{-1}, followed by T4 (85% ethanol, 265 s) and T6, which presented intermediate values. The difference between the extracts obtained with 50% ethanol and the other treatments was statistically significant ($p < 0.05$), particularly when compared to extracts obtained with pure solvents (0% or 100% ethanol) and shorter extraction times (Table 1).

The results reinforce those extracts obtained using intermediate ethanol concentrations, and longer extraction times tend to exhibit higher contents of phenolics and flavonoids, resulting in greater antioxidant capacity against $ABTS^{+}$ and DPPH● radicals, as well as enhanced ferric reducing power, as evidenced by the FRAP assay. This is consistent with the well-established antioxidant potential of phenolic compounds and flavonoids.[25]

The ethanol concentrations determined for the optimization of TPC and AA (50−70%) are consistent with data reported in the scientific literature, which show that extracting solvents with moderate polarity are capable of extracting a greater amount of phenolic compounds and, consequently, a higher antioxidant potential compared to aqueous solutions or purely organic solvents such as 100% ethanol, dichloromethane, ethyl acetate, and hexane.[26] This characteristic is related to the solubility and polarity of phenolic acids and flavonoids in hydroalcoholic solvent mixtures, where the intermediate polarity of the solvent allows it to interact with a larger number of bioactive compounds with different chemical natures.[27]

3.4. Sesquiterpene Compounds (ART, DHAA, AA)

Among the metabolites in *A. annua* L., artemisinin is the most economically and scientifically relevant metabolite due to its well-established antimalarial activity.[28,30] This property was first reported by Klayman, who demonstrated the compound's effectiveness in treating patients in China.[28]

With the advancement of research, the biosynthetic pathway of artemisinin (ART) has been clarified, showing that dihydroartemisinic acid (DHAA) is its direct precursor. The artemisinic acid (AA) adduct is produced through a parallel route that competes for the same metabolic resources in the plant. Thus, ART, DHAA, and AA represent the central points of the biosynthetic pathway and reflect the metabolic balance that governs the artemisinin production.[7]

For this reason, analyzing these three compounds is essential to the study's objectives. Comparing different extraction methods allowed us to identify approaches that increase the yield of ART while minimizing the coextraction of DHAA and AA, resulting in a purer extract that offers clearer scientific insight. Thus, in turn, this supports a more direct evaluation of artemisinin's activity and opens opportunities to explore additional applications of this compound.

The contents of ART, DHAA, and AA, quantified by HPLC-DAD (Figure 1 and Table S1), showed a strong dependence on the extraction conditions studied, particularly the solvent

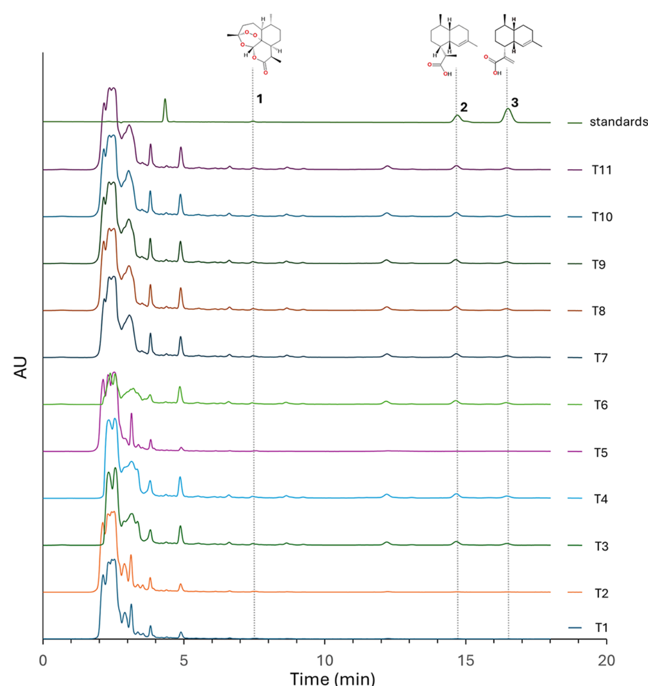

Figure 1. Chromatographic profile at 195 nm of different extracts based on Central Composite Rotatable Design (ethanol concentrations and extraction times): T1-T11. Sesquiterpene standard compounds: 1—artemisinin (ART), 2—dihydroartemisinic acid (DHAA), and 3—artemisinic acid (AA).

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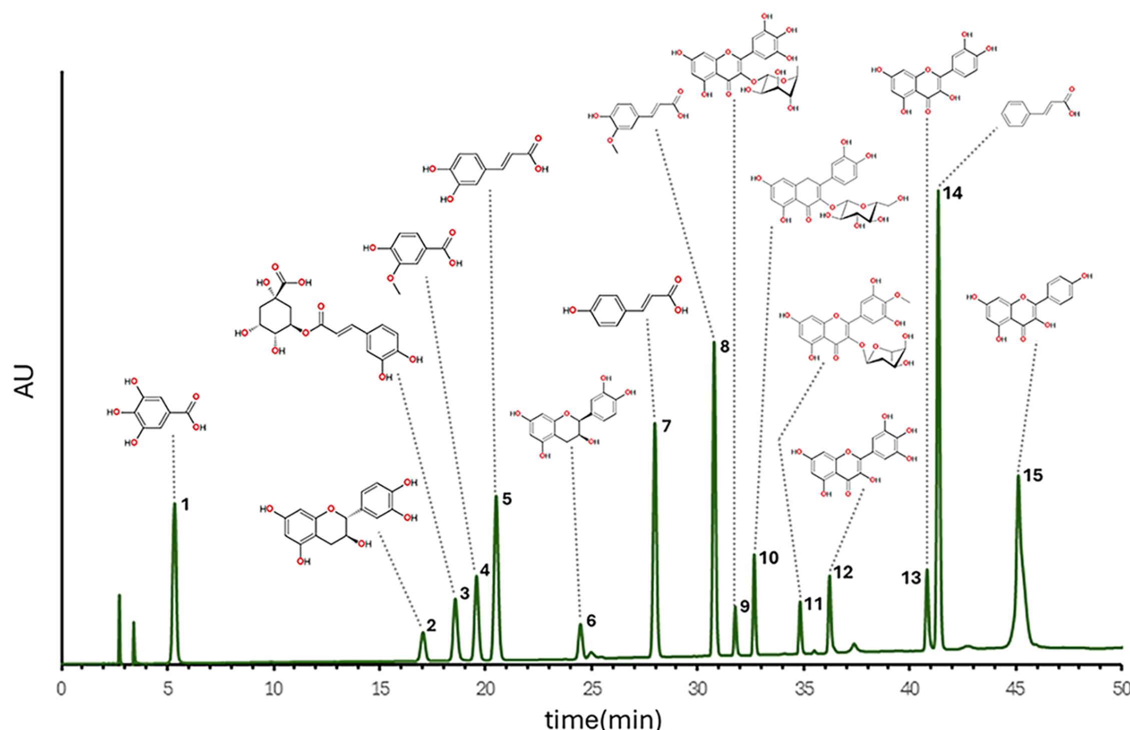


Figure 2. Standards mixture chromatographic profile at 280 and 370 nm of phenolic and flavonoid compounds: (1) gallic acid; (2) (+)-catechin; (3) chlorogenic acid; (4) vanillic acid; (5) caffeic acid; (6) (−)-epicatechin; (7) *p*-coumaric acid; (8) ferulic acid; (9) myricitrin; (10) isoquercetin, (11) mearnsitrin; (12) myricetin; (13) quercetin; (14) cinnamic acid; (15) kaempferol.

concentration (ethanol/water). Treatments with higher ethanol concentrations (85% and 100%) exhibited higher levels of ART, which can be attributed to the affinity between the solvent polarity and the chemical nature of sesquiterpene compounds.

The highest concentration of ART was obtained in T4 (85% ethanol, 265 s), with 6.47 mg·g^{-1}, followed by T6 (100% ethanol, 180 s), T8 (50% ethanol, 300 s), and the central point's T10 and T11 (50% ethanol, 180 s), all of which showed contents above 6.0 mg·g^{-1}. These treatments differed significantly ($p < 0.05$) from the other treatments with ethanol concentrations below 50% (Table 1).

For the compound DHAA, the highest contents were determined in T4 (2.45 mg·g^{-1}), T6 (2.23 mg·g^{-1}), and treatments T8 to T11, with slight variations in their levels, resulting in no significant differences among these treatments ($p > 0.05$). It can be observed that higher ethanol concentrations (≥50%) and intermediate to long extraction times (180 to 300 s) favor the extraction of this biosynthetic intermediate of artemisinin, which possesses biological activity.

In the extraction of AA, the highest contents were found in treatments with higher ethanol concentrations and longer extraction times, such as T4, which showed the highest AA content (0.84 mg·g^{-1}), as well as treatments from T3 to T8. We can conclude that for the extraction of these sesquiterpenes, higher ethanol concentrations and longer extraction times are important to break cellular structures and facilitate the diffusion of the chemical compound from the biomass into the solvent.

At lower ethanol concentrations (15% and 0%), the levels of ART, DHAA, and AA were minimized or even below the limit of detection (LOD), indicating that these solvent concentrations are unsuitable for the extraction of sesquiterpene compounds.

Sesquiterpene compounds have a less polar chemical nature; thus, increasing the proportion of ethanol in the extracting mixture produces a less polar solvent, which can better interact with the sesquiterpene structure and improve mass transfer from the plant matrix to the solvent.[29] Recent studies by Rodrigues et al.[30] investigated the influence of solvents and extraction methods on the recovery of ART, AA, and DHAA. Ethanol extraction delivered a high recovery rate and had the added advantage of being a solvent known for its low environmental toxicity. The authors evaluated different extraction methods and observed that the use of a dispenser (Ultraturrax) with ethanol was efficient for extraction, showing no significant difference compared to organic solvents such as petroleum ether and acetonitrile, which are frequently used in the extraction of ART, DHAA, and AA.

3.5. Individual Phenolic and Flavonoid Compounds

The concentrations of individual phenolic compounds, quantified by HPLC-DAD (Figure 2 and Table 1), varied significantly according to the extraction conditions, highlighting the direct influence of ethanol concentration and extraction time on the phytochemical profile of the extracts. Chlorogenic acid was the most abundant compound in most treatments, with the highest levels observed in T7 (298.91 mg·g^{-1}), T8 (289.95 mg·g^{-1}), T9 (298.91 mg·g^{-1}), and T10 (269.24 mg·g^{-1}), all with 50% ethanol and extraction times ranging from 60 to 180 s. These concentrations were significantly higher ($p < 0.05$) than those obtained in treatments with solvent concentrations at the extremes, such as T6 (100% ethanol, 20.75 mg·g^{-1}) and T5 (0% ethanol, 69.96 mg·g^{-1}).

Ferulic acid, on the other hand, was extracted in the highest amount in T5 (46.44 mg·g^{-1}), differing significantly ($p < 0.05$) from the other treatments, indicating that this compound is

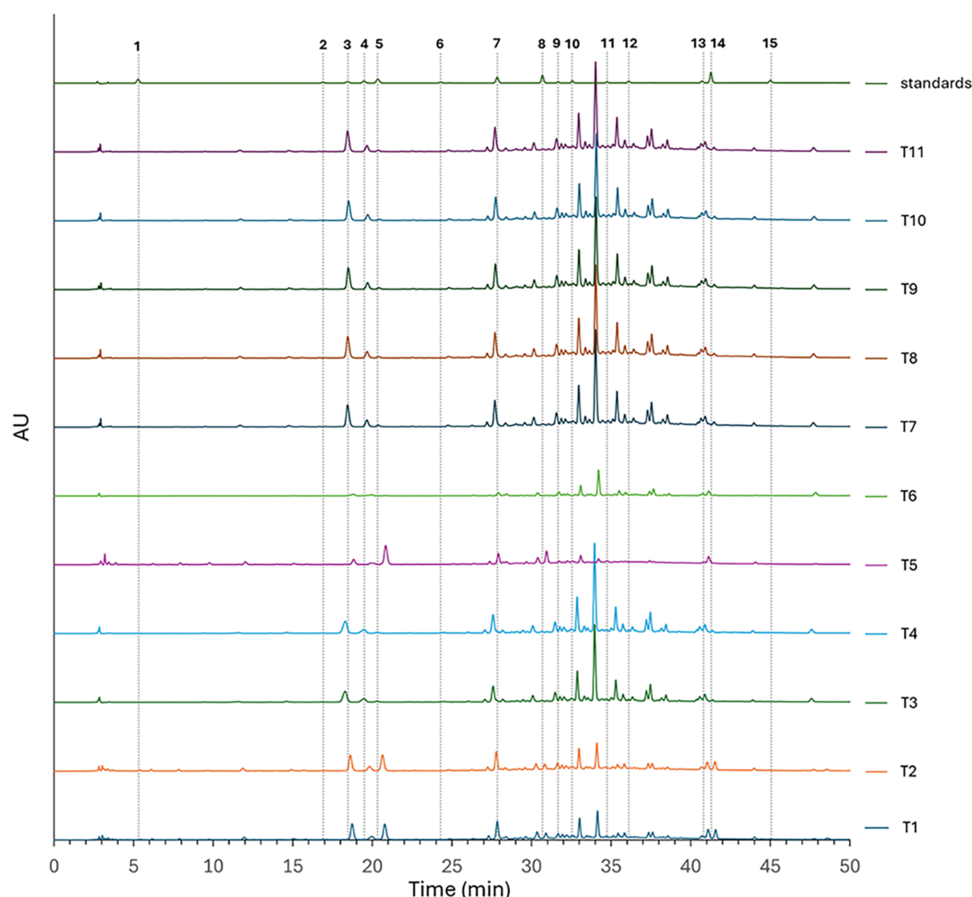


Figure 3. Chromatographic profile of extracts T1-T11 based on the Central Composite Rotatable Design (different ethanol concentrations and extraction times). Phenolic and flavonoid standard compounds: (1) gallic acid; (2) (+)-catechin; (3) chlorogenic acid; (4) vanillic acid; (5) caffeic acid; (6) (−)-epicatechin; (7) *p*-coumaric acid; (8) ferulic acid; (9) myricitrin; (10) isoquercetin, (11) mearnsitrin; (12) myricetin; (13) quercetin; (14) cinamic acid; (15) kaempferol.

more soluble in aqueous media. Gallic acid contents were quantified only in T1, T2, and T11, with concentrations ranging from 0.47 to 3.61 mg·g^{-1} and were absent (<LD) in the other treatments with higher alcohol content.

Catechin showed the highest levels in T7 (4.11 mg·g^{-1}), T8 (3.82 mg.g^{-1}), T9 (4.18 mg·g^{-1}), and T11 (3.57 mg·g^{-1}), all with 50% ethanol concentration. These values differed statistically ($p < 0.05$) from those obtained in T3, T4, and T5, where the levels were below 0.5 mg·g^{-1}.

Other compounds, such as caffeic, p-coumaric, and gallic acids, also showed variations among different treatments, with the highest levels generally found in solvents of intermediate polarity and extraction times between 180 and 300 s. These differences were statistically significant ($p < 0.05$), highlighting the importance of optimizing extraction conditions to maximize the recovery of specific phenolic compounds (Figure 3).

3.6. Global Response (GR) to Optimization

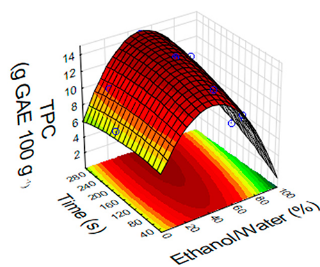
The global response for all treatments was calculated using eq 2, resulting in a value of 9.85, corresponding to treatment T11 (50% ethanol, 180 s), which represents the central point of the model. It was observed that intermediate ethanol concentrations and intermediate extraction times were more effective for extracting phenolic compounds and flavonoids, considering the polarity of the solvents (ethanol and water) and the chemical nature of the bioactive compounds. Although *p*-coumaric acid levels in treatment T11 were below the detection limit, this extraction condition, particularly the solvent concentration of 50% ethanol, was more efficient for most bioactive compounds, especially the total phenolic content (TPC) and antioxidant activity (as measured by ABTS and DPPH). These results are consistent with recent studies showing that hydroalcoholic solvents, such as 50% ethanol, are effective for extracting phenolic and flavonoid compounds due to their intermediate polarity, which facilitates the solubilization of a wide range of bioactive molecules.[31]

3.7. Application of Multivariate Analysis to Optimize Extraction Conditions

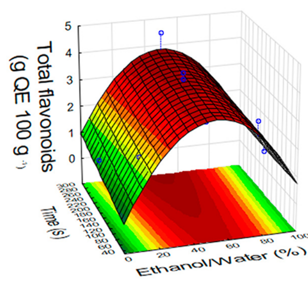
3.7.1. Optimization for TPC and TFC. For the TPC assay (Figure 4A), the Response Surface Methodology (RSM) indicates that the highest total phenolic compound yields were achieved with ethanol concentrations between 40% and 60%, representing the optimal range for extracting these metabolites. In contrast, extreme ethanol concentrations (0% and 100%) resulted in significantly lower yields. Extraction time had a positive effect up to approximately 200 s; beyond this point, increases in TPC levels were minimal, suggesting possible saturation of the extraction process.

Regarding the estimation of effects, all independent variables evaluated were statistically significant for the model ($p < 0.05$) (Figure S1A). The solvent factor contributed most significantly through its quadratic term, while time was more influential in

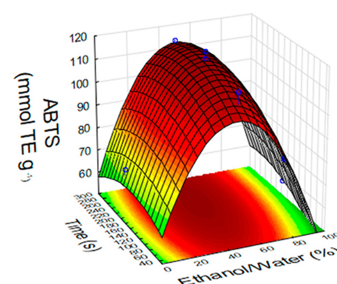
A - TPC



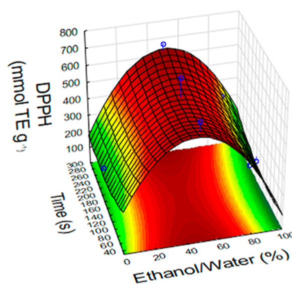
B - TFC



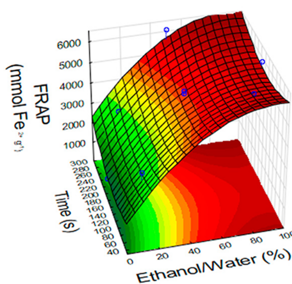
C - ABTS



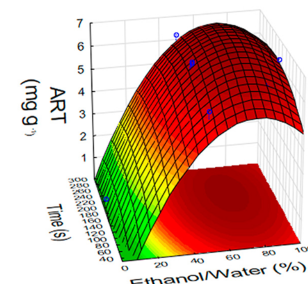
D - DPPH



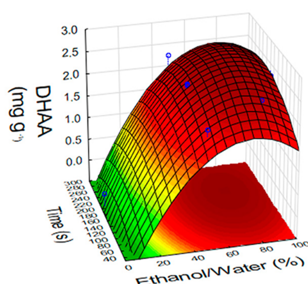
E - FRAP



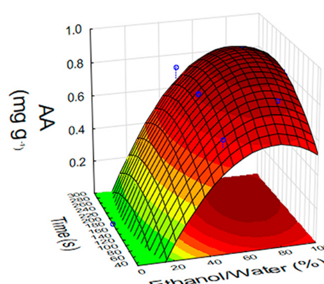
F - ART



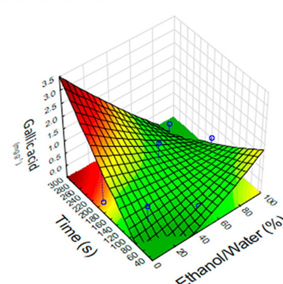
G - DHAA



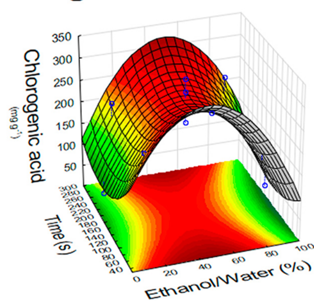
H - AA



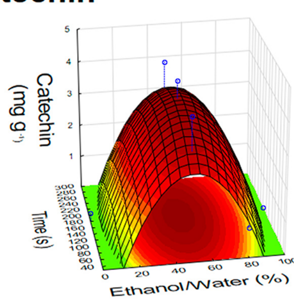
I - Gallic acid



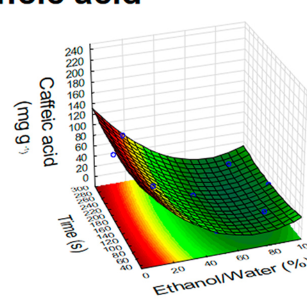
J - Chlorogenic acid



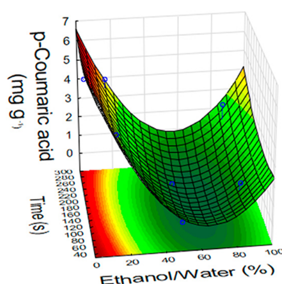
K - Catechin



L - Caffeic acid



M - p-Coumaric acid



N - Ferulic acid

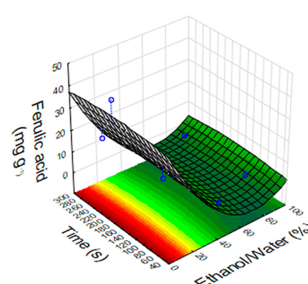


Figure 4. Response surface (RSM) for dependent variables optimized by the CCRD model. (A) TPC, (B) TFC, (C) ABTS, (D) DPPH, (E) FRAP, (F) ART, (G) DHAA, (H) AA, (I) gallic acid, (J) chlorogenic acid, (K) catechin, (L) caffeic acid, (M) *p*-coumaric acid, and (N) ferulic acid.

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its linear term. The interaction between these factors was also significant ($p < 0.05$), indicating that the TPC response is affected by changes in the levels of either variable.

The model evaluation for TPC is presented in Table S2 (see the Supporting Information). The coefficient of determination R^2 was 0.97, indicating excellent agreement between the experimental data and the proposed model. Additionally, the χ^2 test yielded $p > 0.05$, suggesting no significant difference between observed and predicted values. The Lack of Fit (LOF) (Table S1) test, in turn, showed $p < 0.05$, confirming that the model is adequate and does not require further adjustments.

For the TFC variable (Figure 4B), an ethanol concentration of approximately 55% and an extraction time of 200 s resulted in the highest total flavonoid content, likely due to the solvent's affinity with the extracted compounds. In contrast, ethanol concentrations below 30% or above 80%, as well as extraction times under 100 s, led to a significant decrease in extraction efficiency.

For the TFC model, the only factor that showed a significant effect was the solvent concentration in its quadratic term (Figure S1B), with a negative effect. This result indicates that excessive increases in the ethanol concentration may reduce the extraction efficiency, highlighting the presence of an optimal point within the studied range.

The model showed an R^2 of 0.89 and a χ^2 test with $p > 0.05$, indicating a good fit to the experimental data. However, the LOF value ($p > 0.05$) suggests that some adjustments to the model may be necessary (Tables S1 and S2).

3.7.2. Optimization for Antioxidant Activity by ABTS, DPPH, and FRAP Methods. The RSM for the ABTS assay (Figure 4C) indicates that ethanol concentrations between 40% and 50% and extraction times between 250 and 300 s are most effective for the model. Solvent concentration was the only significant factor (Figure S1C), showing a negative effect, which is consistent with previous findings.

In the DPPH assay (Figure 4D), ethanol concentrations between 35% and 60% and extraction times below 180 s demonstrated greater efficacy in extracting antioxidant compounds, whereas extreme concentrations reduced extraction efficiency. The solvent's quadratic effect was the only significant factor in the model (Figure S1D), showing a negative impact.

Finally, the FRAP assay (Figure S1E) shows that ethanol concentrations between 80% and 100% are more effective for extraction, with intermediate extraction times enhancing the yield. Both the linear and quadratic effects of solvent concentration were significant for the model ($p < 0.05$) (Figure 4E), with the quadratic term being the most relevant and showing a positive effect, indicating greater efficiency at higher solvent concentrations.

The models for the antioxidant assays showed R^2 values of 0.91 (ABTS), 0.90 (DPPH), and 0.97 (FRAP). The χ^2 test for ABTS yielded $p > 0.05$, indicating good agreement between observed and predicted values. However, for DPPH and FRAP, $p < 0.05$ suggested that the observed data did not adequately fit the models. Additionally, the LOF test showed $p > 0.05$ for all assays, indicating a need for adjustments to improve model accuracy (Tables S1 and S2).

3.7.3. Optimization for the Sesquiterpene ART, DHAA, and AA. The RSM for the sesquiterpenes artemisinin (ART), dihydroartemisinin (DHAA), and artemisinic acid (AA) (Figure 4F−H) indicates that the optimal ethanol concentration is between 70 and 80% for ART and DHAA, and 70 and 85% for AA, with extraction times around 200 s for all three compounds. Both extraction time and solvent concentration, in their linear and quadratic terms, were significant factors for compound extraction ($p < 0.05$) (Figure S1F−H). Solvent concentration showed a positive effect and was the main driver of the extraction efficiency. For AA, the solvent versus time interaction was also statistically significant.

The R^2 values were 0.97, 0.93, and 0.94 for ART, DHAA, and AA, respectively. The χ^2 test showed $p > 0.05$ for all compounds, indicating good agreement between experimental and predicted values. Although the Lack of Fit (LOF) test returned $p < 0.05$ (Tables S1 and S2), the experimental data adequately followed the proposed mathematical model without requiring further adjustments, supporting the model's reliability for optimizing the extraction of ART, DHAA, and AA.

3.7.4. Optimization of Individual Phenolic and Flavonoid Compounds Quantified by HPLC-DAD. Due to the structural diversity of phenolic compounds and flavonoids, the optimization of extraction variables does not follow a uniform pattern, unlike what is observed for TPC, TFC, and sesquiterpenes. These classes exhibit distinct solubility profiles, resulting in broader ranges of extraction time and solvent concentration and a less predictable response to the factors evaluated.

The polar compound gallic acid shows higher extraction efficiency in solvents with low ethanol concentration (<20%), as indicated by the RSM (Figure 4I). Extraction time also plays a role, evidenced by higher yields at T2 compared to T1 with the same solvent concentration. However, effect analysis (Figure S1I) revealed that none of the factors were statistically significant for the model ($p > 0.05$).

The R^2 value for gallic acid was 0.48, with both the χ^2 and LOF tests showing $p > 0.05$, indicating that the model is not adequately fitted—except for the χ^2 test. Therefore, adjustments are necessary to improve the agreement between the predicted and experimental data (Tables S1−S2).

Chlorogenic acid exhibits optimal extraction at ethanol concentrations between 30% and 50% (Figure 4J), with extraction times ranging from 160 to 180 s. Effect analysis showed that only the quadratic term of ethanol concentration was significant for the model ($p < 0.05$), with a slight negative interaction, indicating that moderately lower concentrations may maximize extraction (Figure S1J).

The model showed an R^2 of 0.89; however, the χ^2 test with $p < 0.05$ indicates poor fit of the data to the model. This is supported by the LOF test with $p > 0.05$, suggesting the need for minor model adjustments (Tables S1 and S2).

Catechin extraction is maximized at approximately 55% ethanol concentration and 200 s extraction time (Figure 4K). Only the quadratic term of solvent concentration was significant for the model (Figure S1K), exhibiting a weak negative interaction, suggesting that intermediate concentrations could favor extraction. The data indicate a need for model adjustment, as the model showed an R^2 of 0.79, with χ^2 and LOF tests presenting $p > 0.05$ (Tables S1 and S2).

For caffeic acid, lower ethanol concentrations (0 to 20%) combined with intermediate extraction times (120 to 200 s) favor extraction (Figure 4L). The factors such as solvent concentration (linear and quadratic), time (quadratic), and solvent versus time interaction were significant for the model ($p < 0.05$) (Figure S1L). Experimental data fit well with the predicted values, showing no need for model adjustments, as

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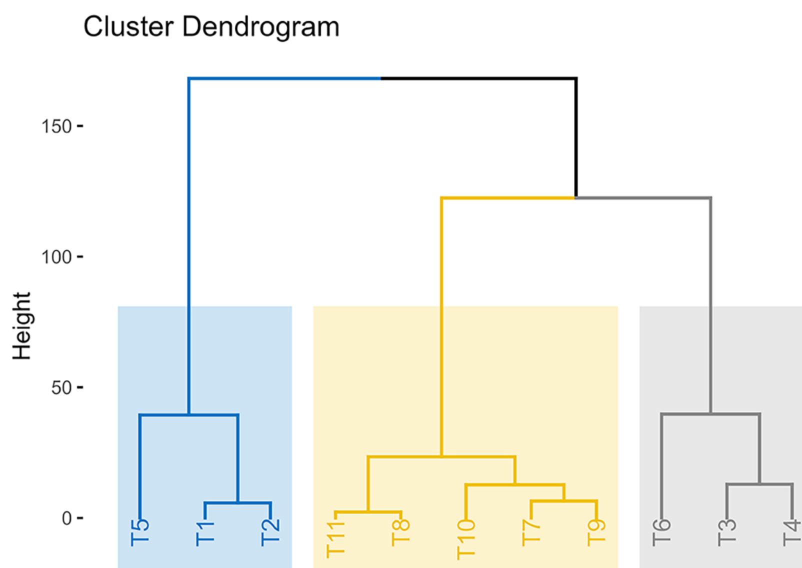

Figure 5. Hierarchical similarity analysis (HSA) dendrogram.

indicated by $R^2 = 0.96$, χ^2 with $p < 0.05$, and the LOF test with $p < 0.05$ (Tables S1 and S2).

The compounds *p*-coumaric and ferulic acids (Figure 4M,N) exhibited similar extraction profiles with optimal yields achieved at ethanol concentrations below 20%, combined with intermediate extraction times. As indicated by the RSM and Table 1, ethanol concentrations above 20% reduce the extraction efficiency, leading to minimum yields. Effect analysis (Figure S1M,N) showed that only the solvent factor, in both linear and quadratic terms, was significant ($p < 0.05$), with a negative interaction, confirming the patterns observed in the RSM.

The models showed R^2 values of 0.91 and 0.92 for *p*-coumaric and ferulic acids, respectively. For *p*-coumaric acid, the χ^2 test indicated $p > 0.05$, whereas for ferulic acid, the value was $p < 0.05$. However, there was a significant LOF test ($p < 0.05$) (Tables S1 and S2) for both compounds, indicating a good agreement between observed and predicted data, with no need for model adjustments.

3.7.5. Content of Solvent Concentration and Time Factors Optimized by Model Equations. The optimized factor values are presented in Table 2. Together with the previous results, it highlights that the chemical nature of the compounds exerts a significant and determining influence on the studied factors.

For TPC, TFC, and antioxidant activity measured by the ABTS and DPPH methods, the highest yields were obtained using solvents with intermediate polarity (48−59% ethanol), consistent with the chemical affinity of the compounds. In contrast, the FRAP method showed optimal results with a high ethanol concentration (92%), suggesting a preference for less polar compounds.

The sesquiterpenes (ART, DHAA, and AA) showed higher extraction efficiency at elevated ethanol concentrations (73−81%), consistent with their nonpolar nature, particularly the sesquiterpene lactones. Although nonpolar solvents are generally more effective for these compounds, ethanol is preferred for human and animal health applications due to its amphipathic properties and lower toxicity.[32]

The extraction of gallic acid could not be optimized by the model. For catechin and chlorogenic acid, only the solvent factor was significant, with optimal ethanol concentrations of 50% and 48%, respectively. Caffeic acid exhibited an extraction maximum at 75% ethanol and 184 s, while *p*-coumaric and ferulic acids achieved better extraction under 100% water, the other extreme of polarity (0% ethanol), and with intermediate extraction times of 138 and 180 s, respectively.

The optimization of extraction factors is essential for the efficient diffusion of compounds from the plant material into the solvent, especially in complex biomasses rich in various bioactive compound classes, such as *A. annua* L. Although extraction time holds some relevance, it plays a secondary role compared to the choice and proper concentration of the solvent, which proves to be the most decisive factor in the success of the extraction process.[13]

3.8. Hierarchical Similarity Analysis (HAS)

The hierarchical analysis was evaluated using a dendrogram and is presented in Figure 5, which shows the formation of three distinct clusters.

The first group (in blue), composed of treatments T5, T1, and T2, is associated with the lowest ethanol concentrations used in the extraction process (0% and 15%), indicating similarities in the chemical characteristics of the extracts obtained under these conditions. The second group (in yellow) includes treatments T11, T8, T10, T7, and T9, which used intermediate ethanol concentrations (∼50%). These treatments showed the most prominent responses in the evaluated dependent variables and contributed the most to the statistical model explanation (Table 1). Finally, the third group (in gray) consists of treatments T6, T3, and T4, which were subjected to high ethanol concentrations (100% and 85%, respectively). This group is more closely related to the extraction of less polar compounds, such as sesquiterpenes, and demonstrated better antioxidant activity, measured by the FRAP assay. In addition to HCA, principal component analysis (PCA) was also performed to identify patterns and reduce the dimensionality of the data, facilitating interpretation of the chemical variability among the samples.

3.9. Principal Component Analysis (PCA)

Principal component analysis (PCA) was used to explore the variability among treatments based on the profile of phenolic

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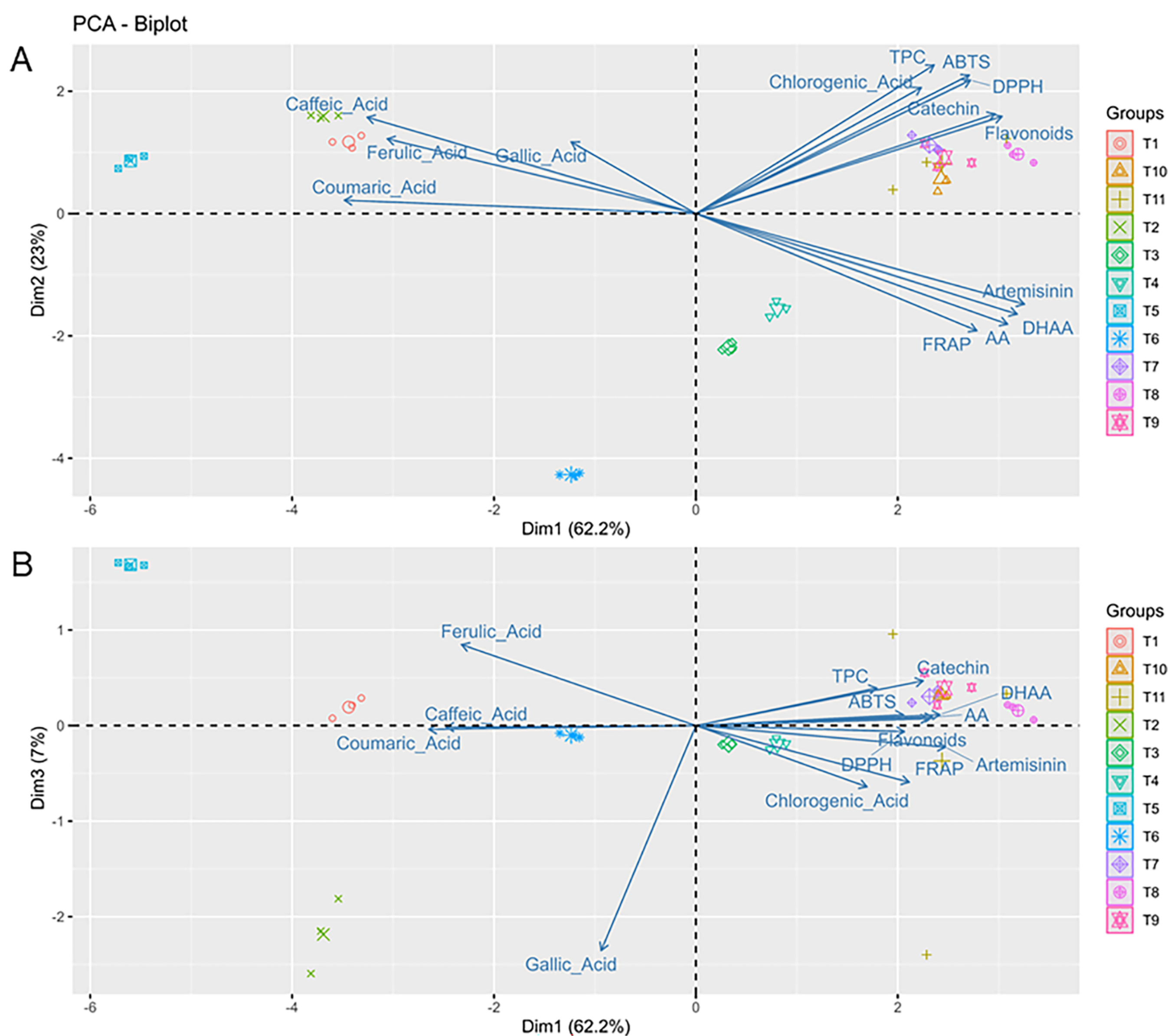

Figure 6. Principal components analysis (PCA), Dim 1 x Dim 2 (A) and Dim 1 x Dim 3 (B).

compounds, antioxidant parameters, and sesquiterpenic compounds with therapeutic potential (Figure 6).

The first two principal components (Dim 1 and Dim 2) explained 85.2% of the total data variability (Figure 6A), while Dim 3 accounted for 7% of the variability, resulting in a cumulative explanation of 92.2% when all principal components are considered together.

Dimension 1, which accounted for 62.2% of the variation, provided the primary separation among treatments, grouping those associated with higher levels of total phenolic compounds (TPC), flavonoids (TFC), catechins, and chlorogenic acids, and strong antioxidant activity (ABTS and DPPH). On the other hand, the left quadrant of Figure 6A concentrated the treatments with higher levels of individual phenolic acids, such as gallic, ferulic, caffeic, and *p*-coumaric acids, indicating a chemical profile affinity within these treatment groups.

Treatments T8 and T9 stood out due to their strong association with bioactive compounds related to antioxidant activity. In contrast, T5 and T6 were isolated from the other groups as these treatments were subjected to extreme solvent concentrations (0% and 100%, respectively), which may have directly influenced their distinct chemical profiles. Treatments T1 and T2 clustered on the left side of Dimension 1, in the proximity of compounds of lower chemical complexity, with fewer functional groups or hydroxyl substituents. Meanwhile, T3 and T4 formed an intermediate group, located closer to vectors associated with sesquiterpenic compounds and the FRAP assay, reflecting a more efficient extraction of these metabolites at higher ethanol concentrations, consistent with the concentrations used in these treatments.

In Dimension 3 (Figure 6B), a greater separation of treatments T1, T2, T3, and T6 was observed on the left side of the plot. Although this dimension explains a smaller portion of the variance (7%), it effectively highlights relevant differences among treatments, with lower efficiency in extracting compounds with antioxidant potential. Gallic acid, strongly associated with this separation, stood out from the other compounds in contrast with the more complex metabolites. On the other hand, treatments T8, T9, T10,

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and T11 showed greater proximity to vectors related to antioxidant activity (ABTS and DPPH) and to sesquiterpenes (AA and DHAA). An association was also observed between artemisinin (ART) and total flavonoids (TFC), suggesting a similarity in the profiles extracted.

3.10. Pearson Correlation Using HeatMap

Pearson's correlation is represented through a heatmap (Figure 7), which illustrates the interrelationships among phenolic compounds, flavonoids, and sesquiterpenes extracted from *A. annua* L.

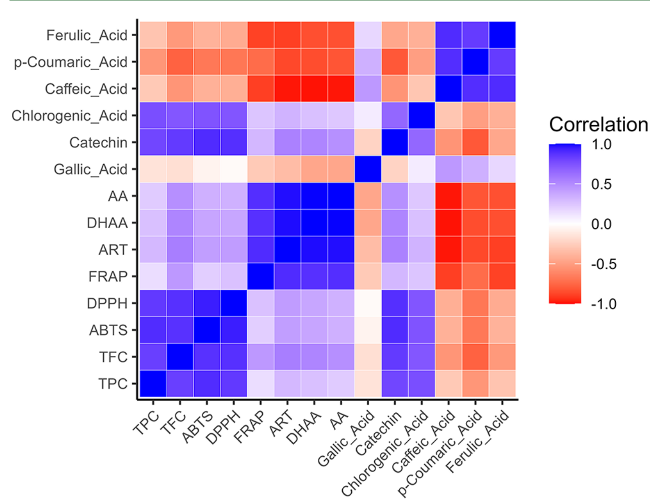

Figure 7. Pearson correlation analysis represented by a heatmap, illustrating the relationships among phenolic compounds, flavonoids, sesquiterpenes, and antioxidant activity assays in *A. annua* L. extracts.

A strong positive correlation was observed between the levels of total phenolic compounds (TPC), total flavonoids (TFC), catechin, and chlorogenic acid with antioxidant activity analysis ABTS and DPPH, indicating a significant contribution of these metabolites to the antioxidant capacity of the extracts. In contrast, ferulic, *p*-coumaric, and caffeic acids showed negative correlations with TPC, TFC, and the antioxidant assays, which suggests that although these compounds were present, they were not decisive contributors to the observed antioxidant activity.

The sesquiterpene compounds were shown to have a positive correlation with the FRAP antioxidant test. Although these compounds are less polar and show higher lipophilicity (Table S4), they significantly contribute to the antioxidant potential of the extracts, highlighting their relevance in the chemical composition of *A. annua* L. and their complementary role alongside the more polar fractions.

The sesquiterpene compounds artemisinin (ART), dihydroartemisinin (DHAA), and artemisinic acid (AA) showed positive correlations with TPC, TFC, ABTS, and DPPH. Although these correlations were low intensity, they indicate that extraction methods employing solvents with an affinity for phenolic compounds also favor, at least partially, the extraction of sesquiterpenes. This result suggests a potential synergy among these classes of compounds, contributing to the production of more complex extracts enriched with bioactive constituents.

Finally, the sesquiterpene compounds showed a negative correlation with ferulic, *p*-coumaric, caffeic, and gallic acids, suggesting that their extraction may be influenced by different conditions or mechanisms compared to those governing the extraction of phenolic acids. On the other hand, a positive correlation was observed between sesquiterpenes and the compounds catechin and chlorogenic acid, indicating that these specific phenolics may share physicochemical properties or affinities with sesquiterpenes that facilitate their coextraction.

The correlation analysis suggests that optimizing extraction parameters is key to obtaining extracts enriched with bioactive compounds, thereby enhancing their therapeutic potential through the synergistic interaction of different classes of metabolites.[33]

Multivariate analyses offered a comprehensive perspective of the relationship between the chemical and biological features of the different studied extracts. In the HCA analysis, treatments grouped with intermediate ethanol (approximately 50%) exhibited the highest TPC, TFC, and antioxidant activity (ABTS and DPPH), indicating that this cluster comprises extracts rich in bioactive compounds with biological potential.

This pattern can also be observed in the PCA analysis: in Dimension 1, which accounts for the majority of variability (62.2%), the vectors for TPC, TFC, catechin, and chlorogenic acid aligned with the 50% extract group, showing that these compounds are the main determinants of the separation between treatments.

In contrast, treatments extracted with a high ethanol concentration (≥85%) clustered along the vectors of ART, DHAA, and AA, reflecting their sesquiterpene composition. These same treatments showed the best performance in the

Table 3. Antimicrobial Activity by the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Methods of the Extracts Obtained in the CCRD Design

treatment (MIC—μg mL^{-1})	*E. coli*	*S. Typhimurium*	*B. cereus*	*L. monocytogenes*	treatment (MBC—μg mL^{-1})	*E. coli*	*S. Typhimurium*	*B. cereus*	*L. monocytogenes*
T1	>2.5	>2.5	>2.5	>2.5	T1	>2.5	>2.5	>2.5	>2.5
T2	>2.5	>2.5	>2.5	>2.5	T2	>2.5	>2.5	>2.5	>2.5
T3	0.039	0.039	0.039	0.039	T3	≥2.5	>2.5	0.039	>2.5
T4	0.039	0.039	0.039	0.039	T4	1.25	0.039	0.078	>2.5
T5	>2.5	>2.5	>2.5	>2.5	T5	>2.5	>2.5	>2.5	>2.5
T6	>2.5	≥2.5	1.25	1.25	T6	>2.5	≥2.5	≥2.5	>2.5
T7	0.625	0.039	0.039	0.039	T7	>2.5	1.25	>2.5	>2.5
T8	0.625	0.039	0.039	0.039	T8	>2.5	≥2.5	≥2.5	>2.5
T9	0.312	0.625	0.039	0.039	T9	>2.5	≥2.5	≥2.5	>2.5
T10	0.312	0.625	0.039	0.039	T10	>2.5	≥2.5	≥2.5	>2.5
T11	0.312	0.625	0.039	0.039	T11	>2.5	≥2.5	0.625	>2.5

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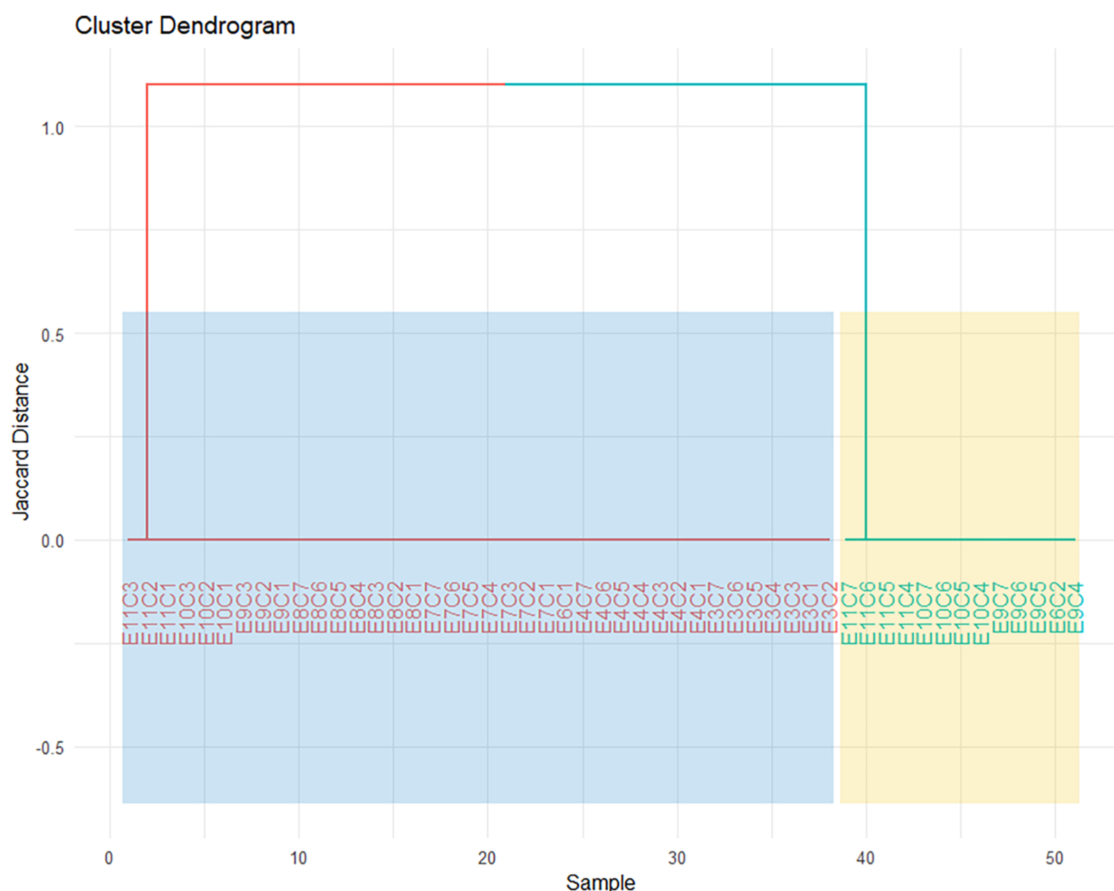

Figure 8. Similarity of responses for antimicrobial activity calculated by Jaccard distance. * E: extract = T: Treatment.

FRAP assay and antimicrobial analyses, indicating that the extraction of less polar compounds is directly associated with their biological activity, specifically their antimicrobial potential.

Finally, correlation analysis confirmed this interpretation by showing strong positive correlations among phenolics, flavonoids, and antioxidant activity as measured by ABTS and DPPH, while sesquiterpenes showed a more pronounced correlation between FRAP and sesquiterpene compounds. The multivariate results not only confirm the optimization of extraction using multivariate statistics but also demonstrate that the chemical composition is influenced by solvent polarity, which is the main factor responsible for response variability.

3.11. Antimicrobial Activity of Extracts

The results of the antimicrobial activity by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are presented in Table 3.

Treatments T3, T4, T7, and T8 stood out by showing the most successful responses against the tested strains. Notably, T3 and T4 exhibited inhibitory activity against all evaluated strains, with MIC values of 0.039 μg·mL^{-1}, indicating bacteriostatic effects, even at low extract concentrations. In addition to its inhibitory activity, treatment T4 also showed bactericidal effects, with MBC values of 0.156 μg·mL^{-1} for *B. cereus* and 0.625 μg·mL^{-1} for *L. monocytogenes*, reinforcing the antimicrobial potential of *A. annua* L. extracts against Gram-positive bacteria.

Treatments T7 and T8 also showed significant antimicrobial potential, with MIC values of 0.039 μg.mL^{-1} for *S. Typhimurium*, *B. cereus*, and *L. monocytogenes*. Notably, T7 demonstrated some bactericidal activity against *S. Typhimurium*, with an MBC of 1.25 μg·mL^{-1}. Treatments T9, T10, and T11, which correspond to the replication of the central point, showed MIC values of 0.312 μg·mL^{-1} for *E. coli*, 0.625 μg·mL^{-1} for *S. typhimurium*, and 0.039 μg·mL^{-1}, the lowest concentration tested for *B. cereus* and *L. monocytogenes*. Regarding bactericidal activity, these treatments presented MBC values of 2.5 μg·mL^{-1} for *S. Typhimurium*, *B. cereus*, and *L. monocytogenes*, except for T11, which showed greater efficacy against *B. cereus*, with an MBC of 0.625 μg·mL^{-1}.

Figure 8 shows the formation of two main groups based on the similarity of results from the antimicrobial activity assays. The first group (in blue) includes treatments with higher MIC values, starting with T11, T10, and T9 against *E. coli* and *S. Typhimurium* and progressing to T3, which showed the lowest MIC values across all tested strains. The second cluster (in yellow) comprises treatment T6, which exhibited the lowest MIC for *B. cereus* and *L. monocytogenes* as well as treatments T9, T10, and T11, which also showed the lowest tested MIC values for these two strains, indicating a similar pattern of bacterial inhibition.

Some studies have looked at *A. annua* L. potential to neutralize microbes, and the results are promising. Antecedent research shows that ethanol and aqueous extracts exhibit significant activity against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumoniae*, and even resistant strains.[34] For instance, Monirian et al.[35] showed that the aqueous extract has bactericidal and fungicidal effects, including against

Candida albicans. Kituyi et al.[36] reported similar results, finding a pronounced inhibition zone for *S. aureus* when using methanolic extracts. Moreover, essential oils derived from *A. annua* L. have exhibited antimicrobial activity against fish pathogens, as reported by Sakhaie et al.[37] The results show that *A. annua* L. is a good candidate to be considered for the development of antimicrobial agents that could be useful for applications in both human and animal health.

The antimicrobial activity of *A. annua* L. extracts showed a strong correlation with the levels of ART, DHAA, and AA. Treatments that exhibited the lowest MIC values, such as T3, T4, T7, and T8, also stood out for having the highest ART concentrations, ranging from 5.63 to 6.47 mg·g^{-1}.

Studies with *A. annua* L. have indicated antimicrobial potential against *E. coli* and *L. monocytogenes* strains using low-polarity solvents such as *n*-hexane and petroleum ether.[38] The inhibitory activity is often attributed to the presence of sesquiterpene compounds, especially ART. However, the presence of phenolic compounds, flavonoids, alkaloids, and other terpenes may also contribute significantly, acting synergistically to enhance the observed antimicrobial effects.[38,39]

Our study reveals that the Central Composite Rotational Design (CCRD) enables the isolation of the most bioactive compounds from *A. annua* L., thereby identifying ideal experimental conditions for maximizing yields of phenolics, flavonoids, and bioactive sesquiterpenes. The results showed that high concentrations of ethanol (≥70%) favored the extraction of sesquiterpenes, such as artemisinin, while hydroalcoholic solvents with intermediate ethanol levels (50−60%) showed to be better for the extraction of antioxidant compounds. The use of ethanol as the extraction solvent emphasizes the practical relevance of these findings, given its low toxicity and suitability for both therapeutic and food-related purposes.

The extracts obtained under optimized conditions showed high antioxidant and antimicrobial activity, especially against Gram-positive bacteria, indicating their potential for future applications as food preservatives or molecules of pharmaceutical interest. However, this study is limited to *in vitro* analyses and does not include tests in living organisms, which prevents the assessment of essential properties such as biological efficacy, toxicity, and bioavailability. Future research should include *in vivo* experiments, studies in food matrices, industrial-scale evaluations, and exploration of more sustainable extraction methods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.5c00629.

> Results of the analysis of variance (ANOVA) for the dependent variables of the CCRD model (Table S1); equations of the model generated for the dependent variables, coefficient of determination (R^2) and chi-square test (χ^2) (Table S2); bioactive chemical compounds identified and quantified by HPLC-DAD (Table S3); lipophilicity values (octanol−water partition coefficient) for followed compounds (Table S4); estimation of effects for the CCRD model (Figure S1); standard mixture chromatographic profile at 195 nm of Sesquiterpene compounds (Figure S2); standard mixture chromatographic profile at 280 e 370 nm of phenolic and flavonoid compounds (Figure S3) (PDF)

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