Optimization of Ultrasound-Assisted Extraction of *Cyclamen purpurascens* Mill. Tubers: Box-Behnken Design and UHPLC-ESI-MS/MS Characterization

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Article

Optimization of Ultrasound-Assisted Extraction of *Cyclamen purpurascens* Mill. Tubers: Box-Behnken Design and UHPLC-ESI-MS/MS Characterization

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Abstract

In contemporary research on natural bioactive compounds, increasing emphasis is placed on the development of efficient and sustainable extraction technologies. This study aimed to develop and optimize an innovative extraction process for wild cyclamen (*Cyclamen purpurascens* Mill.) tubers to maximize the yield of total extractives using a Box-Behnken design. The effects of four extraction parameters were evaluated on the system response. A second-order polynomial model accurately described the extraction process, yielding a coefficient of determination of 0.919. The liquid-to-solid ratio was identified as the dominant factor affecting the extraction efficiency compared to the other factors investigated. The optimal extraction conditions were as follows: extraction time of 15.5 min, 13% (v/v) ethanol, liquid-to-solid ratio of 13.5 mL/g, and extraction temperature of 34 °C, resulting in a yield of 53.44%. The optimized process yielded a significant saponin content of 16.19 g/100 g, while the levels of phenolic compounds (132.52 mg GAE/100 g) and flavonoids (12.04 mg QE/100 g) were also quantified. UHPLC–ESI–MS/MS analysis confirmed the presence of triterpene saponins, flavonoids, and terpenoids. DPPH, $ABTS^{+}$, and CUPRAC assays indicated the antioxidant potential of the extract, while the minimum inhibitory concentration assay showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The established chemical profile and observed biological activities provide the basis for further evaluation of wild cyclamen tubers as a source of bioactive secondary metabolites.

Keywords: *Cyclamen purpurascens*; ultrasound-assisted extraction; optimization; Box-Behnken design; antioxidant activity; antimicrobial activity; UHPLC–ESI–MS/MS analysis

1. Introduction

In advanced industrial and scientific research, there is a growing interest in natural sources of bioactive compounds. This interest is driven not only by scientific exploration but also by market demand and consumer preferences for natural and sustainable products. The global botanical extracts market, which includes plant-derived bioactive compounds used in nutraceuticals, functional foods, cosmetics, and pharmaceuticals, was valued at approximately USD 32.3 billion in 2025. It is projected to surpass USD 64.6 billion by 2034, corresponding to a compound annual growth rate of approximately 8% [1]. Such projected growth underscores the urgent need for the development of innovative, efficient, eco-friendly, and reproducible extraction procedures.

The success of the extraction process largely depends on the extraction parameters, which directly affect the yield, chemical composition, and functional quality of the resulting extract [2]. In this context, ultrasound-assisted extraction (UAE), as an advanced extraction technique, significantly enhances extraction efficiency and improves extract quality compared to conventional extraction methods [3]. The UAE also results in lower consumption of organic solvents [4], making it particularly suitable for sustainable laboratory and industrial applications. The choice of the

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extraction technique is the key for the successful extraction of bioactive compounds. However, sustainable production also requires the systematic optimization of process parameters. It is essential to maximize the recovery of target compounds while preserving their chemical stability and bioactivity [5]. Contemporary research increasingly employs mathematical and statistical approaches to support process optimization. Among these, experimental design and numerical optimization methods have proven particularly effective. For instance, the Box-Behnken design (BBD) allows comprehensive evaluation of individual factors and their interactions using a reduced number of experimental runs [6].

Wild cyclamen (*Cyclamen purpurascens* Mill.) is an important endemic and medicinal plant species that naturally occurs in shady and humid forest areas of Central and Southeastern Europe, including the Balkans [7]. This perennial herbaceous plant, belonging to the Primulaceae family [8], is characterized by well-developed underground organs-tubers, which are not sufficiently investigated. Various secondary metabolites, primarily saponins, triterpenoids, and phenolic compounds, have been identified in these tubers [9]. These compounds exhibit pronounced antioxidant, anti-inflammatory, antibacterial, antiviral, and antitumor activities [10,11]. Despite its long-standing use in folk medicine [12], systematic research aimed at optimizing the extraction of bioactive compounds from wild cyclamen tubers remains limited, further highlighting the scientific significance of this study. Advanced separation techniques such as UHPLC-ESI-MS/MS are necessary for the accurate identification of complex saponin profiles in the *Cyclamen* genus, where traditional methods often fail due to structural similarities.

This study aimed to investigate the influence of key extraction parameters on the yield of total extractives (TE) from wild cyclamen tubers. The UAE procedure was developed and optimized to achieve maximal yield of TE using the BBD. The extract prepared under optimal conditions was further subjected to the identification of individual bioactive compounds, determination of the total content of major classes of bioactive compounds, and evaluation of its selected biological activities. The novelty of this study lies in the integrated application of UAE combined with the BBD for the systematic optimization of extraction parameters from wild cyclamen tubers. This approach provides quantitative insights into process efficiency and establishes a robust foundation for subsequent chemical and biological investigations.

2. Materials and Methods

2.1. Chemicals and Reagents

Acetonitrile and water (HPLC-MS grade), 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), α-tocopherol, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$), butylated hydroxytoluene (BHT), and gentamicin were purchased from Sigma Chemical (St. Louis, MO, USA). Formic acid was obtained from Carlo Erba Reagents (Val de Reuil, France), while 96% (*v*/*v*) ethanol and sulfuric acid were supplied by Zorka Pharma (Šabac, Serbia). Folin-Ciocalteu reagent, saponin, and gallic acid (purity 97%) were used as standards (AppliChem, Darmstadt, Germany). Copper (II) chloride ($CuCl_2$), neocuproine, and ammonium acetate (CH_3COONH_4), Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), and 2,3,5-triphenyltetrazolium chloride (TTC) were obtained from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2. Plant Material

Wild cyclamen (*Cyclamen purpurascens* Mill.) tubers were collected in Southeastern Serbia (Predejane, Leskovac; 42°49′58.22″ N, 22°07′44.9″ E). The plant material was thoroughly washed, cut into smaller pieces, and air-dried at room temperature. Moisture content of 6.13% (*w*/*w*) was determined by oven-drying at 105 °C until a constant weight was reached. The dried plant material was subsequently ground using a laboratory mill (Braun Aromatic KSM2, Kronberg im Taunus, Germany). The plant fraction of 0.4 mm was used for the extraction of bioactive compounds.

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2.3. Box–Behnken Design

The UAE of TE from wild cyclamen tubers was performed using an 8 L ultrasonic bath with ultrasound power of 300 W (Sonic, Niš, Serbia). The extractions were carried out in 250 mL round-bottom flasks equipped with reflux condensers. The effects of four independent variables, each investigated at three levels (low, medium, and high), on the defined system response were selected based on the preliminary investigation (Table 1) to ensure the response surface captures the optimal region.

Table 1. Factors analyzed in the extraction of total extractables with their actual and coded values according to the Box-Behnken design.

Extraction parameters	Factor	Unit	Low level (–1)	Intermediate level (0)	High level (+1)
Extraction time	A	min	5	15	25
Ethanol concentration	B	% (v/v)	0	40	80
Liquid-to-solid ratio	C	mL/g	5	10	15
Extraction temperature	D	°C	25	42.5	60

A second-order polynomial (quadratic) model was used to describe the extraction process, allowing the evaluation of linear and quadratic effects of the individual factors, as well as their interactions. The general equation form of the applied second-order polynomial model is presented in Eq. 1.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j}^{k} \beta_{ij} X_i X_j + \epsilon \quad (1)$$

where Y is the system response (TE); X_i, X_j are the factors; β_0 is the intercept; β_i is the coefficient of linear term of factor i, β_{ii} is the coefficient of the quadratic term of factor i; β_{ij} are the coefficient of the interaction between factors i and j; and ϵ is the experimental error of the model.

2.4. Determination of Total Phenolic and Total Saponin Contents

The spectrophotometric method with the Folin-Ciocalteu reagent was used to determine the total phenolic content in the extract [13]. For the analysis, 0.1 mL of the extract was mixed with 1 mL of ten-fold diluted Folin–Ciocalteu reagent in distilled water, followed by the addition of 1 mL of 7% (*w*/*v*) sodium carbonate solution. After incubation for 90 min, the absorbance was measured at 765 nm. The results were expressed as milligrams of gallic acid equivalents per 100 gram of dry weight (mg GAE/100 g d.w.).

The total saponin content in the extract was determined spectrophotometrically using the method described by Mora-Ocación et al. [14]. The stock solution (1 mg/mL) of standard saponin was prepared in 70% (*v*/*v*) ethanol and diluted in the concentration range of 5 – 1000 mg/mL. One milliliter of the sample was treated with 3.5 mL of Liebermann-Burchard reagent (16.7% (*v*/*v*) acetic anhydride in concentrated sulfuric acid) and incubated at room temperature for 30 min. The absorbance was measured using a double-beam UV-Vis spectrophotometer (Varian Cary-100 Conc, Mulgrave, Victoria, Australia). A negative control consisted of 1 mL of 70% (*v*/*v*) ethanol and 3.5 mL of Liebermann-Burchard reagent. The analyzed extract was prepared according to the same procedure. The saponin content was expressed as grams of saponin equivalent per 100 g dry weight (g SE/100 g d.w.).

2.5. UHPLC–ESI–MS/MS Analysis

The wild cyclamen tuber extract was analyzed using ultra-high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS) with slight modifications to the method described by Boškov et al. [15]. Analyses were performed on a Dionex Ultimate 3000 UHPLC+ system coupled to an LCQ Fleet Ion Trap (Thermo

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Fisher Scientific, San Jose, CA, USA). Xcalibur (2.2, SP1.48) and LCQ Fleet (v. 2.1) software were used for instrument control, data acquisition, and processing. Separation was achieved on a Hypersil Gold C_{18} column (50 × 2.1 mm, 1.9 µm) thermostated at 40 °C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.30 mL/min. The injection volume was 3 µL. The gradient program was as follows: 5% B for 0.8 min, 5–95% B over 10 min, 95% B for 2 min, 95–5% B over 0.1 min, and 5% B for 2.1 min. DAD detection was performed at 254, 280, 320, and 340 nm. ESI conditions were optimized for stable spray, efficient desolvation, and high sensitivity for *m*/*z* 100–1000. The electron spray ionization was carried out under the following conditions: the electrospray voltage was 5 kV; sheath gas 42 arbitrary units; auxiliary gas 11 arbitrary units; capillary temperature 275 °C; capillary voltage –/+46 V depending on mode; tube lens voltage 80 V (negative) and 115 V (positive). Tentative identification of compounds was performed based on retention times, mass spectra, and UV spectra, compared with data reported in the literature.

2.6. Determination of Antioxidant Activity

2.6.1. DPPH Assay

The spectrophotometric procedure for DPPH assay was previously described by Savić & Savić Gajić [13]. One milliliter of a DPPH radical solution (3×10^{-4} mol/L) was added to 2.5 mL of the extract sample. Instead of the extract solution, the negative control contained an equivalent volume of 96% (*v*/*v*) ethanol. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Varian Cary 100, Mulgrave, Victoria, Australia) after incubation for 30 min. The inhibition of DPPH radicals (I_{DPPH}) was calculated and subsequently plotted as a function of sample concentration. It was calculated according to Eq. 2:

$$I_{DPPH}(\%) = \frac{A_c - A_s}{A_c} \times 100 \quad (2)$$

where A_c is the absorbance for the negative control at 517 nm, A_s is the absorbance for the sample at 517 nm.

Based on the obtained relationship between DPPH radical inhibition and extract concentration, the half-maximal inhibitory concentration (IC_{50}) was determined by interpolation. The synthetic antioxidant BHT was used as a positive control.

2.6.2. Cupric Ion Reducing Antioxidant Capacity (CUPRAC)

Following the method given by Vasiljevic et al. [16], the sample was examined for its cupric ion reducing antioxidant capacity (CUPRAC). A serial dilution (0.156 to 20 mg/mL) of tuber extract (40 µL) prepared in DMSO was added to a mixture containing 50 µL of a 10 mM Cu(II), 50 µL of 7.5 mM neocuproine solution, and 60 µL of 1 M ammonium acetate buffer (pH = 7). The absorbance was measured at 450 nm (Elx 808, BioTek Instruments, Winooski, VT, USA) after incubation at 30 °C for 1 h. Cu(II) was exchanged with DMSO to form the blank. To enable comparison, two industrially important antioxidants, α-tocopherol and BHT, were also assayed (using the same range of concentrations).

2.6.3. $ABTS^{+}$ Free Radical Scavenging Activity

An aliquot of serial dilutions of the extract (0.078 – 20 mg/mL) prepared in 45% (*v*/*v*) ethanol was mixed with 200 µL of activated $ABTS^{+}$ solution. It was incubated at room temperature in the dark for 10 min. The absorbance was measured at 630 nm (Elx 808, BioTek Instruments, Winooski, VT, USA). Instead of the sample, the negative control contained the equivalent volume of 45% (*v*/*v*) ethanol. The results are expressed as the percentage of $ABTS^{+}$ neutralization (I_{ABTS}), while the calculation was done using Eq. 3:

$$I_{ABTS}(\%) = \frac{A_c - A_s}{A_s} \times 100 \quad (3)$$

where A_c is the absorbance of the negative control and A_s is the absorbance of the sample.

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2.7. Antibacterial Activity

The ability of the tuber's extract to inhibit the growth of pathogenic bacteria was assessed by the microdilution method with some modifications [17]. All tested bacterial strains (*Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Acinetobacter baumanii*, and *Salmonella enteritidis*) were American Type Culture Collection (ATCC) ones, except methicillin-resistant *Staphylococcus aureus*, which was a clinical isolate. Before testing, all strains were subcultured in TSB overnight at 37 °C. Starting concentration of bacterial suspension was adjusted to 10^6 CFU/mL based on optical density measured at 600 nm (Elx 808, BioTek Instruments, Winooski, VT, USA). Serial concentrations of extract (50 µL) in TSB, ranging from 0.005 to 10 mg/mL, were mixed with 50 µL of test microorganisms in a 96-well microplate (Sarstedt, Germany). Only TSB was used as a negative control, while 50 µL of bacterial suspension with 50 µL of TSB served as a positive control. In parallel, tested microorganisms were incubated with serial dilutions (0.002 to 5 mg/mL) of gentamicin (positive control). Plates were incubated at 37 °C for 24 h, after which an indicator of cellular respiration, TTC, was added to each well (its final concentration was 0.05%). Plates were incubated at 37 °C for 30 min. The appearance of color indicated cellular respiration, pointing out that bacteria were not suppressed by the presence of the sample. The lowest concentration at which no color was present was the minimal inhibitory concentration (MIC). Furthermore, each well without the color was subcultured on the plate with TSA and incubated for 24 h at 37 °C. The lowest concentration at which there was no growth of bacterial colonies presented the minimal bactericidal concentration (MBC).

2.8. Statistical Analysis

All experimental data are presented as mean ± standard deviation (SD) of three independent measurements. Statistical analysis of the experimental data was performed using Design-Expert software (version 13; Stat-Ease Inc., Minneapolis, MN, USA). Analysis of variance (ANOVA) was applied to evaluate the statistical significance of the developed model, as well as the effects of individual factors and their interactions. Model adequacy was assessed based on the coefficient of determination (R^2). The significance of the model terms was evaluated at a 95% confidence level ($p < 0.05$).

3. Results and Discussion

3.1. Modeling of Total Extractives from Wild Cyclamen Tubers

The extraction process of TE from wild cyclamen tubers was described using the BBD with four factors. The BBD enables the efficient evaluation of factor interactions and the identification of statistically significant parameters while requiring a relatively small number of experimental runs. The extractions were carried out under conditions representing combinations of different factor levels (extraction time, ethanol concentration, liquid-to-solid ratio, and extraction temperature) according to the BBD matrix, whose random order is presented in Table 2.

Table 2. BBD matrix for modeling the extraction of total extractives (TE) from wild cyclamen tubers. An asterisk represents the central point of the BBD. The numbers within the columns in the parentheses denote the low (–1), medium (0), and high (+1) levels of the corresponding factor.

No.	A: Extraction time (min)	B: Ethanol concentration (%)	C: Liquid-to-solid ratio (mL/g)	D: Extraction temperature (°C)	TE (%)	
					Experimental	Predicted
1	25 (+1)	40 (0)	15 (+1)	42.5 (0)	46.26	43.84
2	15 (0)	0 (–1)	10 (0)	60.0 (+1)	44.07	40.28
3	25 (+1)	40 (0)	5 (–1)	42.5 (0)	13.65	18.33
4	15 (0)	80 (+1)	15 (+1)	42.5 (0)	43.47	40.44

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5*	15 (0)	40 (0)	10 (0)	42.5 (0)	36.53	41.34
6	15 (0)	40 (0)	5 (–1)	60.0 (+1)	26.50	23.70
7	25 (+1)	80 (+1)	10 (0)	42.5 (0)	37.99	36.05
8	15 (0)	0 (–1)	5 (–1)	42.5 (0)	13.59	17.03
9	5 (–1)	40 (0)	5 (–1)	42.5 (0)	10.35	13.05
10	25 (+1)	40 (0)	10 (0)	60.0 (+1)	42.99	43.96
11	15 (0)	40 (0)	5 (–1)	25.0 (–1)	19.10	14.27
12	5 (–1)	40 (0)	10 (0)	25.0 (–1)	36.54	35.98
13	5 (–1)	0 (–1)	10 (0)	42.5 (0)	38.93	40.20
14	15 (0)	80 (+1)	10 (0)	25.0 (–1)	24.10	28.16
15	15 (0)	0 (–1)	15 (+1)	42.5 (0)	51.63	55.20
16	15 (0)	40 (0)	15 (+1)	25.0 (–1)	44.39	46.52
17	5 (–1)	40 (0)	10 (0)	60.0 (+1)	42.16	41.34
18	15 (0)	40 (0)	15 (+1)	60.0 (+1)	45.64	49.80
19*	15 (0)	40 (0)	10 (0)	42.5 (0)	39.98	41.34
20	5 (–1)	80 (+1)	10 (0)	42.5 (0)	34.61	36.43
21	15 (0)	80 (+1)	10 (0)	60.0 (+1)	48.65	50.92
22*	15 (0)	40 (0)	10 (0)	42.5 (0)	43.49	41.34
23*	15 (0)	40 (0)	10 (0)	42.5 (0)	37.12	41.34
24*	15 (0)	40 (0)	10 (0)	42.5 (0)	49.56	41.34
25	25 (+1)	40 (0)	10 (0)	25.0 (–1)	35.38	36.60
26	5 (–1)	40 (0)	15 (+1)	42.5 (0)	50.30	45.89
27	15 (0)	80 (+1)	5 (–1)	42.5 (0)	23.42	20.25
28	15 (0)	0 (–1)	10 (0)	25.0 (–1)	52.33	50.33
29	25 (+1)	0 (–1)	10 (0)	42.5 (0)	46.30	43.81

The response variable (TE) serves as a quantitative indicator of process efficiency, reflecting the outcome of the extraction under the specified experimental conditions. The central point was replicated five times to estimate experimental error, assess model reliability, and evaluate the presence of curvature in the response surface. The results of the ANOVA for the second-order polynomial model at a 95% confidence level (Table 3) showed that the model was statistically significant ($F = 11.4$, $p < 0.0001$). This confirms that the investigated factors and their interactions significantly contribute to TE variability. The high F-value, coupled with a p-value below 0.0001, underscores the statistical robustness of the model and confirms that the observed variations are genuinely attributable to the investigated extraction parameters. Among the individual factors, the liquid-to-solid ratio (factor C) has the most significant effect on the TE ($F = 110.18$, $p < 0.0001$). Its quadratic term (C^2) was also statistically significant ($F = 20.12$, $p = 0.0005$), indicating a non-linear relationship between the liquid-to-solid ratio and the response, with an optimal level present. Extraction temperature (factor D) also had a statistically significant effect on TE ($F = 5.24$, $p = 0.0382$). In addition, the interaction between ethanol concentration (factor B) and extraction temperature (factor D) was statistically significant ($F = 11.61$, $p = 0.0042$). In contrast, extraction time and ethanol concentration individually do not show statistically significant effects on the response ($p = 0.5703$ and $p = 0.0569$, respectively), although the latter approaches the threshold of significance. Other interaction terms, as well as the quadratic effects of factors A, B, and D, were not statistically significant ($p > 0.1$). The lack-of-fit was not significant ($F = 0.73$, $p = 0.6891$). It indicates that the model adequately fitted the experimental data and that the remaining variability can be attributed to random error rather than systematic inadequacy of the model.

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Table 3. Analysis of variance (ANOVA) for the second-order polynomial model used to model total extractives from wild cyclamen tubers.

	Sum of squares	df	Mean square	F-value	p-value
Model	3698.28	14	264.16	11.40	< 0.0001
A – Extraction time	7.83	1	7.83	0.3378	0.5703
B – Ethanol concentration	99.79	1	99.79	4.31	0.0569
C – Liquid-to-solid ratio	2553.82	1	2553.82	110.18	< 0.0001
D – Extraction temperature	121.39	1	121.39	5.24	0.0382
AB	3.99	1	3.99	0.172	0.6846
AC	13.43	1	13.43	0.5796	0.4591
AD	0.9957	1	0.9957	0.043	0.8388
BC	80.86	1	80.86	3.49	0.0829
BD	269.11	1	269.11	11.61	0.0042
CD	9.46	1	9.46	0.4083	0.5332
A^2	43.27	1	43.27	1.87	0.1934
B^2	0.8911	1	0.8911	0.0384	0.8474
C^2	466.3	1	466.3	20.12	0.0005
D^2	3.32	1	3.32	0.1433	0.7107
Residual	324.5	14	23.18		
Lack-of-fit	209.5	10	20.95	0.7287	0.6891
Pure error	115	4	28.75		
Cor total	4022.78	28			

Standard deviation	4.81	R^2	0.9193
Mean value	37.21	Adjusted R^2	0.8387
Coefficient of variation (%)	12.94	Prediction R^2	0.6554
		Adequate precision	12.1733

The R^2 value was 0.9193, indicating that the model explains about 92% of the variability in TE. The adjusted coefficient of determination (Adjusted R^2) was 0.8387, indicating that the model is not overfitted and can be reliably used to interpret the results. The predicted R^2 of 0.6554 is reasonably consistent with the adjusted R^2, as the difference is less than 0.2. This suggests that the model also possesses satisfactory predictive capability for new data not included in the model training. The model's standard deviation was 4.81 and the coefficient of variation (C.V. %) was 12.94%. It is acceptable for most experimental systems, indicating moderate variability relative to the mean response value (37.21). Additionally, the adequate precision value of 12.17 exceeds the recommended minimum of 4, indicating an adequate signal-to-noise ratio and confirming that the model can be used to navigate the design space.

The polynomial regression model, expressed in terms of coded factors, provides a mathematical description of the TE extraction from wild cyclamen tubers (Eq. 4).

$$Y = 41.34 + 0.81A - 2.88B + 14.59C + 3.18D - 1.00AB - 1.83AC + 0.50AD - 4.50BC + 8.20BD - 1.54CD - 2.58A^2 + 0.37B^2 - 8.48C^2 + 0.72D^2 \quad (4)$$

where Y is the TE, A is the extraction time, B is the ethanol concentration, C is the liquid-to-solid ratio, and D is the extraction temperature.

These coded values enable the interpretation and comparison of the significance of individual effects, as well as their mutual interactions within the experimental range. The magnitude of the model coefficients indicates the extent to which each variable influences the response. The largest individual contribution was from factor C, suggesting that increasing the liquid-to-solid ratio has a strong positive effect on the response. The quadratic term of the same factor had a negative impact, resulting in a pronounced non-linear relationship. Beyond a certain point, further increases in the liquid-to-solid ratio may lead to a decrease in the response. Of all the interaction terms, the interaction between factors B and D contributes most significantly to the TE. Unlike factor B, the linear terms of factors A and D impact the increase in the system response. The interactions AB, AC, AD, and CD,

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as well as the quadratic terms B^2 and D^2, have smaller absolute coefficients, resulting in comparatively weaker influences on the response. The response reached a maximum at a specific extraction time due to the quadratic effect, while ethanol concentration and extraction temperature displayed a pronounced minimum.

Since the data points are close to the straight line, the residuals are normally distributed, as shown in the normal probability plot (Figure 1a). Ensuring the normality of residuals is essential, as it guarantees the validity of statistical tests (F-test and t-test) and, by extension, the accurate interpretation of the model. The normal probability plot shows no significant deviations from linearity, suggesting that heteroscedasticity, nonlinearity, or influential outliers are unlikely to affect model quality. Cook's distance analysis (Figure 1b) revealed no influential points, with all values falling below the standard threshold of 1. These findings demonstrate that the model is robust, and no individual data point disproportionately affects its estimates. Consequently, there was no need to remove any data or adjust the model for influential points.

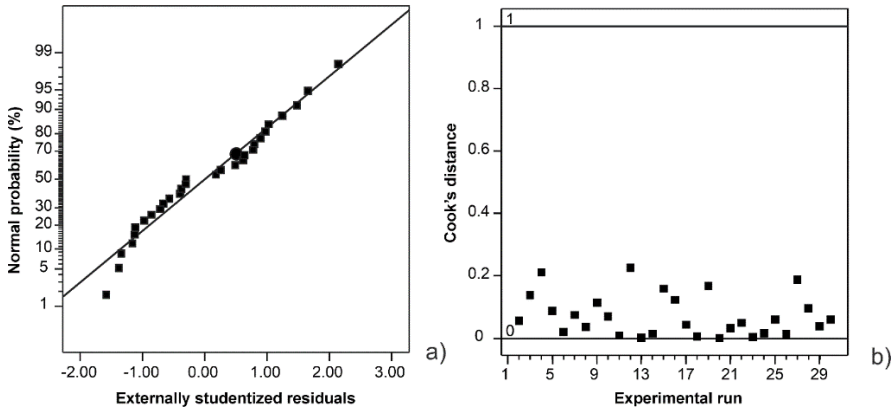

Figure 1. Diagnostic plots of the regression model: (a) normal probability plot for assessing the normality of residuals; (b) Cook's distance for identifying influential points.

Based on the three-dimensional plots illustrating the dependence of TE yield on various combinations of process parameters (extraction time, ethanol concentration, liquid-to-solid ratio, and temperature), the relative importance and influence of each factor can be assessed. A three-dimensional response, illustrating the combined effects of extraction time and ethanol concentration on the TE content at a liquid-to-solid ratio of 10 mL/g and an extraction temperature of 42.5 °C, is presented in Figure 2a. A pronounced non-linear interaction between these parameters is observed, and only a minor effect on TE yield is noted. In the initial extraction phase, increasing extraction time enhances TE yield due to improved solute diffusion and mass transfer. The TE yield plateaus after a specific time, reflecting solvent saturation and equilibrium in mass transfer. Further extraction beyond this stage does not increase yield and may cause partial degradation of the extracted bioactive compounds. Similar trends have been reported in the literature, where extraction time is identified as a critical factor influencing extraction efficiency [18]. Importantly, the present results demonstrate that equilibrium is attained substantially faster than in conventional extraction approaches. While TE yield increases with extraction time, UAE achieves equilibrium in only about one-quarter of the time needed for reflux extraction [19]. This accelerated equilibrium results from the enhanced mass transfer and cell disruption induced by ultrasonic cavitation, highlighting the superior efficiency and time-saving benefits of UAE.

The increase in the liquid-to-solid ratio leads to a pronounced enhancement of the TE yield (Figure 2b). This factor exerts the strongest effect by promoting mass transfer, facilitating solvent penetration into plant tissues and the release and dissolution of bioactive compounds until equilibrium is reached. The significant influence of the liquid-to-solid ratio on the extraction of bioactive compounds has been widely reported in the literature [20,21]. Several studies have demonstrated that the total yield of extracted bioactive compounds increases with an increasing

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liquid-to-solid ratio up to an optimal level [22]. Beyond this optimum, the extraction yield reaches a dynamic equilibrium and subsequently exhibits a marked decline. An appropriate liquid-to-solid ratio is critical for extraction optimization, since excessive solvent use increases costs and reduces sustainability. In Figure 2c, the extraction temperature exerts a stronger effect on TE yield than extraction time. The elevated temperatures accelerate dissolution kinetics and mass-transfer processes of bioactive constituents [18]. The interaction between ethanol concentration and liquid-to-solid ratio (Figure 2d) has an antagonistic effect on the TE yield. The effect of ethanol concentration becomes more pronounced at a higher liquid-to-solid ratio. The maximum TE yields are achieved when both parameters are at their higher levels. Singh et al. [23] reported a comparable influence of these parameters during the UAE of bioactive compounds from giloy. In Figure 2e, a strong synergistic interaction between ethanol concentration and extraction temperature can be noticed. Higher extraction temperatures improve solvent penetration and accelerate diffusion kinetics by reducing viscosity and surface tension. Additionally, higher temperatures facilitate the disruption of cell wall structures, promoting the release of intracellular bioactive constituents. A similar synergistic influence of ethanol concentration and temperature has been reported during the extraction of flavonoids from the stem and leaf waste of *Astragalus membranaceus* [24]. In Figure 2f, the effect of the liquid-to-solid ratio has a dominant influence on the extraction efficiency relative to the extraction temperature.

Summarily, the temperature and the liquid-to-solid ratio are the most critical factors affecting extraction efficiency, whereas extraction time has a minor impact. These results indicate that maximum TE yield can be achieved by performing the extraction at elevated temperatures, increasing the liquid-to-solid ratio, and optimizing ethanol concentration, while keeping the extraction time short without compromising yield. Ultrasonic treatment enhances mass transfer by generating cavitation effects, which disrupt plant cell walls and improve solvent penetration [25]. This leads to more efficient release of TE from wild cyclamen tubers.

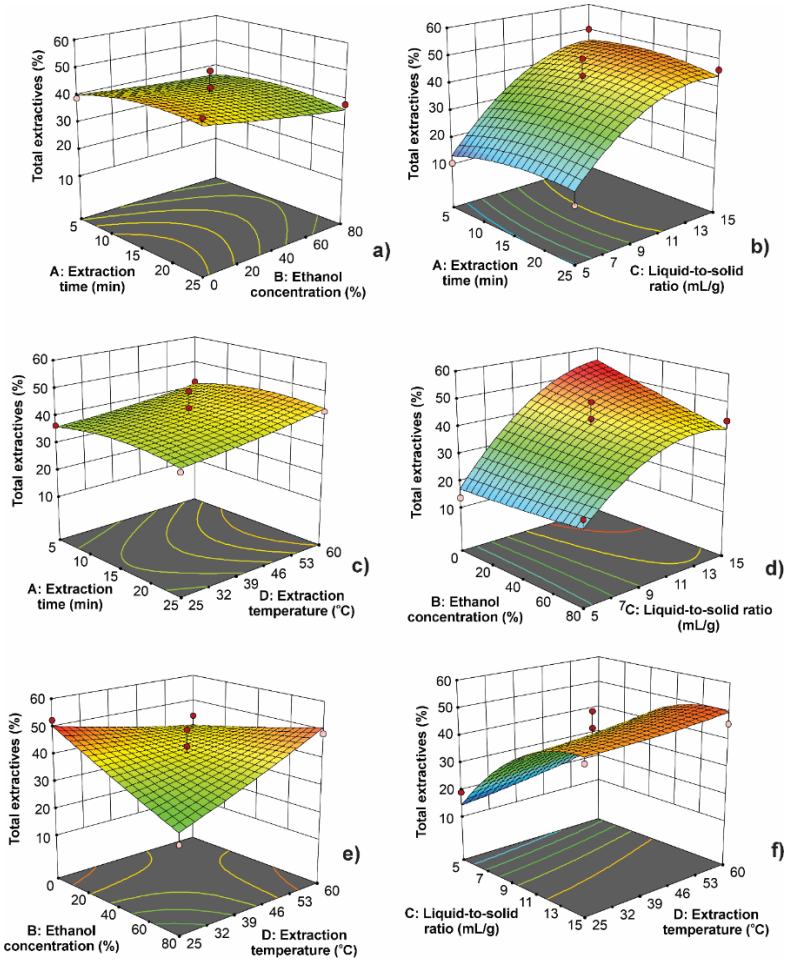

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Figure 2. Interaction effects between process variables: (a) extraction time and ethanol concentration (10 mL/g, 42.5 °C); (b) extraction time and liquid-to-solid ratio (40% (*v*/*v*) ethanol, 42.5 °C); (c) extraction time and extraction temperature (40% (*v*/*v*) ethanol, 10 mL/g); (d) ethanol concentration and liquid-to-solid ratio (42.5 °C, 15 min); (e) ethanol concentration and extraction temperature (15 min, 10 mL/g); and (f) liquid-to-solid ratio and temperature (15 min, 40% (*v*/*v*) ethanol).

3.2. Optimization of the Extraction Process of Total Extractives from Wild Cyclamen Tubers

The extraction process was optimized using the desirability function to determine the optimal combination of input parameters that maximizes TE yield. Based on the obtained results, the desirability value reached 1.000, indicating that the identified solution fully satisfies the defined objectives and constraints. The optimal extraction conditions were determined to be: an extraction time of 15.5 min, 13% (*v*/*v*) ethanol, a liquid-to-solid ratio of 13.5 mL/g, and an extraction temperature of 34 °C. Under these conditions, the predicted TE yield was 53.44%, while the experimentally obtained value was 52.87%, demonstrating a high level of agreement between the model and experimental data. Notably, the relatively low ethanol concentration suggests enhanced solubility of the target compounds in an aqueous medium. The optimal liquid-to-solid ratio is close to its upper limit within the design space. Furthermore, the optimal extraction time of approximately 15 min confirms that high extraction efficiency can be achieved within a relatively short processing time. Using this procedure, it is possible to improve process economy and energy efficiency. Overall, the identified optimal conditions represent a well-balanced compromise between extraction efficiency and rational resource utilization and can therefore be recommended for future laboratory-scale and potential industrial applications. To date, the available literature does not report optimized conditions for the extraction of TE from wild cyclamen tubers. The existing studies predominantly focus on the optimization of polyphenol extraction from this plant material [26].

3.3. Chemical Profile of the Wild Cyclamen Tuber Extract

In the wild cyclamen tuber extract obtained under optimal UAE conditions, a total phenolic content of 132.52 mg GAE/100 g d.w. was determined, indicating an efficient recovery of phenolic compounds under relatively mild processing conditions. Such conditions are particularly advantageous for minimizing the degradation of thermolabile substances and preserving the native phytochemical profile of the extract. The total phenolic content achieved by UAE was lower than that reported in our previous study employing conventional reflux extraction [26]. Despite this fact, it is important to note that the higher total phenolic content obtained previously (177 mg GAE/100 g d. w.) required an almost six-fold longer extraction time (97.7 min), a higher ethanol concentration (36.2%, *v*/*v*), and a markedly greater liquid-to-solid ratio (25 mL/g). These conditions increase the consumption of solvents and energy, thereby reducing the overall sustainability of the process. The enhanced performance of the UAE can be attributed to the acoustic cavitation phenomenon, which disrupts plant cell walls, improves solvent penetration, and intensifies mass transfer, leading to the rapid release of polyphenolic compounds [27]. Consequently, the slightly lower polyphenol yield obtained via UAE can be considered an acceptable and rational trade-off, given the substantial gains in extraction speed, energy efficiency, and adherence to the principles of green and sustainable extraction technologies. Aydin et al. [10] reported that ethanol is a more effective solvent for the extraction of polyphenols from *Cyclamen coum* tuber compared to methanol and acetone. In their study, an ethanolic extract prepared at 55 °C for 6 h exhibited the total phenolic content of approximately 4.70 mg GAE/mL of the extract. Mahomoodally et al. [28] reported that the methanolic extract of *Cyclamen coum* Mill. tubers contained a higher total phenolic content (approximately 7 mg GAE/g extract) than the corresponding aqueous extract (approximately 6 mg GAE/g extract).

The pronounced saponin content (16.19 g SE/100 g d.w.) confirms the dominance of this class of compounds in wild cyclamen tubers compared to the others. This level of saponins is consistent with previous phytochemical reports on the genus *Cyclamen* [29]. It provides a strong chemical basis for the biological effects traditionally attributed to this plant, including anti-inflammatory, antimicrobial,

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and cytotoxic properties [11]. The high saponin content may also explain the observed hemolytic and membrane-active properties of the extract [30]. The presence of polyphenols and saponins can potentially exert a synergistic effect on the biological activity of the extract.

UHPLC–ESI–MS/MS analysis of the tuber extract prepared under optimal conditions revealed an exceptionally complex and chemically diverse profile of secondary metabolites. Numerous bioactive compounds, including triterpene saponins, flavonoids, alkaloids, terpenoids, fatty acids, steroids, and phenolic compounds, were detected under both negative and positive ionization modes (Figure 3). In Table 4, tentatively identified bioactive compounds in the wild cyclamen tuber are presented. A significant fraction of the detected components could not be identified.

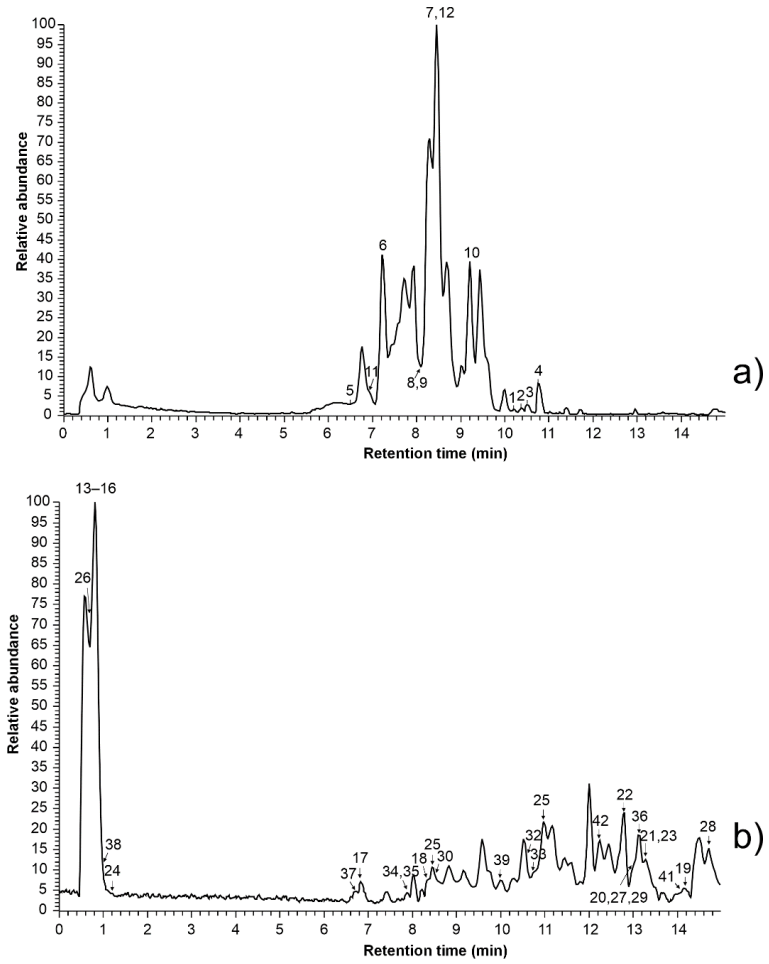

Figure 3. UHPLC–ESI–MS/MS chromatograms of cyclamen tuber extract obtained in (a) negative and (b) positive electrospray ionization modes, based on the base peak within the m/z range of 100–1500. The numbers in the chromatograms correspond to the reference numbers of tentatively identified bioactive compounds listed in Table 4.

Table 4. Bioactive compounds tentatively identified in wild cyclamen tuber extract by UHPLC–ESI–MS/MS in both electrospray ionization modes.

Ref. No.	Quasimolecular or adduct ion, m/z	t_R, min	Mode	Fragment ions, m/z	Compounds	Class of compound	Reference
1.	265.09	10.22	–	179, 97(100%)	oxidized fatty acid*	fatty acid	
2.	315.25	10.38	–	311, 297(100%),	isorhamnetin derivative	flavonol	[31]

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3.	557.36	10.49	–	279, 201, 172, 139 539(100%), 511, 300, 282, 274, 270, 256, 244, 226, 213	cucurbitacin B	tetracyclic triterpenoid	[32]
4.	559.15	10.74	–	513(100%), 483	dillapional-*O*-dihexoside	benzodioxol	[33]
5.	1075.54	6.49	–	1010, 943(100%), 914, 782, 763, 662, 619, 601, 488	cyclacoumin	triterpene saponin	[28]
6.	1105.48	7.17	–	1075, 1061, 973, 765, 736, 573, 442(100%)	cyclacoumin ismomer	triterpene saponin	[28]
7.	1203.91	8.54	–	1059(100%), 1045, 970, 927, 910, 897, 873, 765, 748, 568	cyclaminorin isomer	triterpenoid	[28]
8.	1219.89	8.18	–	1087(100%), 1075, 1058, 968, 943, 926, 907, 896, 871, 764, 746, 710, 637, 602, 584, 565	cyclacoumin isomer	triterpene saponin	[28]
9.	1223.35	8.11	–	1091, 1088, 1078(100%), 1060, 927, 765	cyclaminorin isomer	triterpenoid	[28]
10.	1239.79	9.18	–	1107	ginsenosides Ra3	saponin	[34]
11.	1255.37	6.94	–	1123(100%), 961	notoginsenoside A	saponin	[35]
12.	1267.18	8.57	–	1239, 1207, 1134, 1093(100%), 781	yesanchinoside H	saponin	[35]
13.	132.02	0.74	+	91, 86(100%)	*L*-leucine	amino acid	[36]
14.	166.03	0.77	+	149, 134, 131, 120(100%)	*L*-phenylalanine	alkaloid	[37]
15.	175.04	0.71	+	157(100%), 140, 130, 116, 70, 60	D-arginine	alkaloid	[37]
16.	205.02 $[M+H-OH]^+$	0.88	+	188(100%), 173, 159, 146, 93	*trans*-indole-3-acrylic acid	alkaloid	[37]
17.	246.97 $[M+H-OH]^+$	6.92	+	229(100%), 211, 205, 201, 187, 144, 130, 115	traumatic acid	fatty acid	[33]

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18.	279.9	8.38	+	263, 245(100%), 205, 149	benzenedicarboxylic acid, bis(2-methylpropyl) ester	fatty ester	[38]
19.	282.26	14.28	+	265, 247(100%), 198, 163, 151, 135, 121, 107, 93, 86	oleamide	amide	[39]
20.	295.1	13	+	277(100%), 259, 241, 221, 207, 195, 179, 169, 161, 151, 135, 119, 105	oxo-octadecadienonic acid	fatty acid	[33]
21.	297.09	13.23	+	279(100%), 261, 223, 183, 169, 155, 135, 123, 109	α-linolenic acid	fatty acid	[36]
22.	316.92	12.91	+	299(100%), 281, 263, 255, 245	*N*-methylcoclaurine *N*-oxide	alkaloid	
23.	321.21 [M+H-HOH]$^+$	13.35	+	303(100%), 275	herbacetin	flavonol	[40]
24.	341.97	1.18	+	325(100%), 310,304, 289, 271, 259, 253, 199, 185, 163, 145, 127	licocoumarone	2-arylbenzofuran flavonoid	[33]
25.	348.08 [M+H-HOH]$^+$	8.42	+	330, 312, 259, 227, 220, 209(100%), 202, 192, 180, 165, 151, 139, 131, 122, 117	reticuline	alkaloid	[41]
26.	363.12	0.65	+	514, 191, 145(100%)	3β,16β,17-trihydroxy-ent-kaurane 16,17-acetonide	diterpenoid	[42]
27.	387.29	12.97	+	369(100%), 351, 333, 309, 288, 115	cleomiscosin A	coumarinolignoid	[42]
28.	391.11	14.69	+	279, 261, 167, 149(100%), 113	7α-hydroxy-14,15-dinorlabd-8(17)-en-13-one	diterpenoid	[43]
29.	443.28 [M+H-HOH]$^+$	12.94	+	425(100%), 413, 395, 341, 261, 235, 229, 191, 163	lupenone	terpenoid	[37]
30.	446.07 [M+H-OH]$^+$	8.55	+	429, 411, 307(100%), 289, 271, 243, 205, 163	ergosterol endoperoxide	steroid	[39]

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31.	455.22	10.89	+	437(100%), 419, 411, 395, 375, 349, 323, 305, 295, 263, 247, 227, 201, 191, 159, 147	apigenin-6,8-di-C-*β*-*D*-glucoside	flavonoid	[44]
32.	471.22	10.6	+	453(100%), 435, 425, 407, 377, 348, 319, 301, 263, 245, 231, 199, 191, 173, 169, 135	hederagonic acid	triterpenoid	[45]
33.	489.28	10.74	+	471(100%), 453, 435, 423, 411, 362, 329, 309, 267, 227, 203, 185, 175	(3*β*,5*α*,9*α*)-3,6,19-trihydroxyurs-12-en-28-oic acid	terpenoid	[37]
34.	509.96	7.85	+	493(100%), 475, 457, 364, 348, 317, 301, 264, 213, 191	isorhamnetin 3-*O*-glucuronide	flavonoid	[46]
35.	510.05	7.88	+	493(100%), 475, 457, 348, 317, 226, 209	isorhamnetin 3-*O*-glucuronide	flavonoid	[46]
36.	537.28	13.1	+	457, 450, 432, 411, 368, 280(100%), 262, 229	lycopene or *β*-carotene	tetraterpene	
37.	575.37	6.75	+	557(100%), 539, 522, 485, 437, 391, 288, 271	unidentified saponin	saponin	
38.	654.29	1	+	636, 618, 602, 591(100%), 554, 537, 518, 482, 471, 454, 437, 422, 408, 392, 379, 337, 313, 307, 291, 269, 262, 226, 196	unidentified saponin	saponin	
39.	659.27	9.94	+	641, 615, 526, 455(100%), 437, 420, 217	apigenin-6,8-di-C-*β*-*D*-glucoside isomer	flavonoid	[44]
40.	677.5	14.08	+	659, 521, 395, 377(100%), 359, 263	4-*O*-caffeoylquinic acid isomer	phenolic acid	
41.	677.53	14.05	+	660, 395, 377(100%), 359, 331	4-*O*-caffeoylquinic acid isomer	phenolic acid	
42.	1212.24	12.19	+	937	unidentified saponin	saponin	

* https://massbank.eu/MassBank/ (available 1 February 2024).

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Triterpene saponins represented the predominant group of identified compounds, which is consistent with previous reports describing the genus *Cyclamen* as a rich source of this class of metabolites [47]. The presence of cyclacoumin (*m*/*z* 1075.54), its isomers (*m*/*z* 1105.48 and *m*/*z* 1219.89), and structurally related saponins were confirmed by characteristic fragmentation patterns observed in the negative ionization mode, involving successive losses of sugar moieties and the formation of stable aglycone ions. The detection of high *m*/*z* values, along with fragment ions at *m*/*z* 943, *m*/*z* 897, and *m*/*z* 765, indicates the presence of complex oligosaccharide chains linked to a triterpenoid backbone. In addition to cyclacoumin derivatives, ginsenosides Ra3 (*m*/*z* 1239.79), notoginsenoside A (*m*/*z* 1255.37), and yesanchinoside H (*m*/*z* 1267.18) were identified. Their presence in tubers may reflect structural convergence of saponin metabolites or conserved biosynthetic pathways across different plant taxa. These compounds are well known for their pronounced pharmacological properties, including anti-inflammatory, cytotoxic, and immunomodulatory activities [48].

The flavonoid profile of the extract comprised flavonols and flavonoid glycosides, including isorhamnetin derivative (*m*/*z* 315.25), herbacetin (*m*/*z* 303), and apigenin-6,8-di-*C*-*β*-*D*-glucoside (*m*/*z* 455.22) along with its isomer (*m*/*z* 659.27). These compounds are widely recognized for their strong antioxidant, anti-inflammatory, and cytoprotective properties [49]. Of particular interest is the detection of isorhamnetin-3-*O*-glucuronide, a metabolically stable flavonoid conjugate often associated with favorable bioavailability. The identification of 4-*O*-caffeoylquinic acid isomer further supports the phenolic nature of the extract and its contribution to antioxidant capacity. In the positive ionization mode, several nitrogen-containing compounds, including amino acids and alkaloids, were identified. The presence of *L*-leucine (*m*/*z* 132.02), *L*-phenylalanine (*m*/*z* 166.03), and *D*-arginine (*m*/*z* 175.04) reflects the primary metabolic status of the tubers, whereas the detection of alkaloids, such as reticuline (*m*/*z* 330), *N*-methylcoclaurine *N*-oxide (*m*/*z* 316.92), and *trans*-indole-3-acrylic acid (*m*/*z* 188), indicates active secondary metabolic pathways. The extract is also rich in terpenoids, including diterpenoids, triterpenoids, and tetraterpenes, which have also been previously reported in methanolic and aqueous tuber extracts of *Cyclamen coum* Mill. [28]. The identification of hederagonic acid (*m*/*z* 471.22), lupenone (*m*/*z* 425), and lycopene or *β*-carotene (*m*/*z* 537.28) suggests an active terpenoid metabolism, which is commonly associated with anti-inflammatory, antimicrobial, and cytotoxic effects [50]. The presence of ergosterol endoperoxide (*m*/*z* 429), a compound known for its antitumor and immunomodulatory activities [51], further enhances the biological relevance of the extract. In addition, several fatty acids and their oxidized derivatives, such as *α*-linolenic acid (*m*/*z* 297.09), oleamide (*m*/*z* 282.26), and oxo-octadecadienoic acid (*m*/*z* 295.1), were detected and may contribute to the regulation of inflammatory processes and cellular metabolism. The numerous components that were detected but unidentified, especially at higher m/z values, indicate the existence of previously uncharacterized secondary metabolites that may significantly contribute to the overall biological activity of the extract. These findings indicate the need for further detailed structural and biological investigations.

3.4. Antioxidant Activity of Wild Cyclamen Tuber Extract

This pronounced chemical diversity reflects the complexity of the metabolic network in the wild cyclamen tubers and provides a solid basis for a wide range of potential biological activities. The antioxidant potential of the analyzed extract was evaluated using three in vitro assays (DPPH, $ABTS^{+}$, and CUPRAC), enabling a broader interpretation of its redox behavior. As demonstrated by the DPPH assay, the extract exhibited considerably weaker radical-scavenging activity (IC_{50} = 4.60 mg/mL) compared to the synthetic antioxidant BHT (IC_{50} = 0.036 mg/mL). This marked difference is expected given that BHT is a pure, low-molecular-weight phenolic antioxidant with high hydrogen-donating efficiency. The extract represents a chemically complex matrix dominated by triterpene saponins. Such compounds, although biologically active, are generally characterized by limited direct hydrogen atom transfer capacity toward stable free DPPH radicals.

In the ABTS assay, a concentration-dependent increase in radical scavenging was observed, reaching approximately 50% inhibition at 4.95 mg/mL. Compared to DPPH, the higher

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responsiveness in the ABTS system may be attributed to differences in reaction mechanisms and solvent compatibility. $ABTS^{\bullet+}$ is soluble in both aqueous and organic media and can react with a broader spectrum of antioxidants, including moderately polar constituents [52]. Nevertheless, the concentrations required to achieve substantial $ABTS^{\bullet+}$ scavenging remain considerably higher than those typical for potent phenolic-rich extracts.

The CUPRAC assay results provide additional insight into the reducing capacity of the extract. A gradual increase in cupric ion reducing activity was observed with rising concentrations. However, the absorbances remained markedly lower than those recorded for α-tocopherol and BHT across all tested concentrations. While reference antioxidants exceeded the upper detection limit (>4.00 AU) at higher concentrations, the cyclamen extract reached only 1.23 AU at 20 mg/mL. Since the CUPRAC method primarily reflects electron transfer capacity, these findings suggest a limited abundance of strong electron-donating compounds within the extract.

Flavonoids and phenolic acids are well-established antioxidants capable of both hydrogen atom donation and electron transfer [53]. Their apparently low contribution to the overall antioxidant profile of the extract can likely be attributed to the optimized extraction conditions. The relatively low ethanol concentration (13.02%, *v*/*v*) favors the extraction of highly polar glycosidic saponins over less polar phenolic compounds. These findings are consistent with previous reports demonstrating significantly enhanced antiradical activity in cyclamen tuber extracts prepared with higher ethanol concentrations (≥70%, *v*/*v*), which more efficiently recover polyphenolic constituents regarded as the principal contributors to radical-scavenging activity [9,10]. Moreover, the UAE under mild temperature and short extraction time did not ensure exhaustive extraction of bound or less accessible phenolics.

The combined DPPH, ABTS, and CUPRAC results indicate that the antioxidant potential of the wild cyclamen tuber extract is moderate and primarily concentration-dependent, reflecting its specific phytochemical composition.

3.5. Antibacterial Activity of the Wild Cyclamen Tuber Extract

The antibacterial activity of the prepared extract and the antibiotic gentamicin as a positive control was evaluated based on the MIC values (Table 5). The ethanol extract showed selective inhibitory activity against *S. aureus* and *E. coli*, with MIC values of 10 mg/mL for both strains. Although these values are higher than those of the standard antibiotic, they demonstrate the functional potential of the crude extract against both Gram-positive and Gram-negative bacteria. Gentamicin showed stronger antibacterial activity against almost all tested bacterial strains (except *A. baumannii*), with MIC values ranging from 0.01 mg/ml to 5.00 mg/ml. Aydin et al. [10] reported the different antibacterial potential of the methanolic extract of cyclamen (*Cyclamen coum*), which can be attributed to interspecific variations in saponin and phenolic profiles, as well as the influence of environmental factors and extraction efficiency. In the range of concentrations tested, the TE showed bacteriostatic rather than microbicidal activity, which provided the basis for further fractionation to isolate the most active antimicrobial compounds.

Table 5. Minimal inhibitory concentrations of wild cyclamen tuber extract and reference antibiotic gentamicin against the various bacterial strains.

Bacterial strain	Minimum Inhibitory Concentration, MIC (mg/mL)	
	Wild cyclamen tuber extract	Gentamicin
S. aureus ATCC 25923	10.00	1.25
B. cereus ATCC 10876	>10.00	2.50
Methicillin-resistant *S. aureus*, clinical strain	>10.00	0.63
E. faecalis ATCC 29219	>10.00	0.01
E. coli ATCC 25922	10.00	0.16
P. aeruginosa ATCC 35032	>10.00	5.00

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P. mirabilis ATCC 7002	>10.00	0.01
A. baumanii ATCC 19606	>10.00	>10.00
S. enteritidis ATCC 13078	>10.00	0.31

Standard deviations are not presented since the results of the triplicate experiment showed no variations.

4. Conclusion

In this study, a UAE procedure for the recovery of TE from wild cyclamen tubers was successfully developed and optimized using a Box-Behnken design. Among the parameters analyzed, the liquid-to-solid ratio and extraction temperature had the most pronounced effect on the yield. The optimized parameters (15.5 min, 13% (v/v) ethanol, and 34 °C) provide a rapid and cost-effective protocol for the industrial isolation of cyclamen saponins, minimizing solvent and energy consumption in accordance with the principles of green chemistry.

Chemical characterization by UHPLC–MS/MS confirmed the predominance of triterpene saponins (16.19 g/100 g), establishing these tubers as a highly valuable raw material compared to other secondary metabolites, such as phenols and flavonoids. The observed antioxidant and antibacterial activities are directly related to this specific saponin-rich profile, rather than to the high phenolic content. The combination of UAE and response surface methodology represents an efficient, reproducible, and sustainable approach to standardizing extracts from under-studied plant sources. Future research will focus on the isolation of individual saponins on a large scale and the evaluation of their specific pharmacological potential in the pharmaceutical and cosmetic industries.

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