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Abstract

Inula helenium L. (Elecampane) leaves are an underutilized source of natural antioxidants due to their rich phenolic content. Although most studies have investigated roots or other aerial parts, limited information is available on phenolic extraction from *I. helenium* leaves. This study aimed to optimize the solvent concentrations for phenolic compound extraction from *I. helenium* leaves using D-Optimal Mixture Design and to evaluate the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities of the extracts. Various solvent mixtures (ethanol, methanol, and water) were tested, and the optimal composition was determined as 42.40% ethanol, 0.00% methanol, and 57.60% water. Under these conditions, TPC and TFC were found to be 28.12 mg gallic acid equivalent (GAE)/g dry sample and 27.74 mg quercetin equivalent (QE)/g dry sample, respectively. Antioxidant activities measured by ABTS, DPPH, and FRAP assays were 19.73, 1.42, and 6.10 mg Trolox equivalent (TE)/g dry sample, respectively. These results indicate that *I. helenium* leaf extracts, with high phenolic content and potent antioxidant activity, represent a promising natural antioxidant source, particularly for applications in the food industry.

Keywords: Elecampane, Phenolic compound, D-Optimal mixture design, Extraction, Optimization

INTRODUCTION

The Asteraceae family is among the most prominent plant families, consisting of 1,400-1,700 genera and 24,000-35,000 species, representing about 10% of all known flowering plant species [1]. The genus *Inula* L., which is part of the Asteraceae family, comprises around 120 species, predominantly distributed across Europe, Africa, and Asia. In Türkiye, the *Inula* genus is represented by approximately 28 species, 33 subspecies, and varieties, eight of which are endemic. The endemism rate of Inula species in Turkey is 28.5%[2].

Inula helenium L., a perennial herb, belongs to the *Inula* L. genus; is widely distributed in East Asia, North America, and Europe, particularly in the Mediterranean region [3] [4]. *I. helenium* thrives in warm, humid, open, and calcareous areas, commonly found along riverbanks and streams in oak and coniferous forests. This species produces sessile, oval, and longitudinal leaves on its stems [5].

I. helenium (elecampane) is considered a medicinal herb due to its biologically active constituents. Different parts of the plant contain eudesmanolides, germacranolides, triterpenes, sterols, and inulin, making it highly significant for the pharmaceutical industry [6]. *I. helenium* is particularly rich in eudesmane-type sesquiterpene lactones, with isoalantolactone (IAL) and alantolactone (AL) as the major components. These compounds have garnered attention due to their antitumor, antidermatophytic, antifungal, anticancer, hepatoprotective, anti-inflammatory, and antibacterial properties. Furthermore, the European Food Safety Authority (EFSA) has classified alcoholic beverages made from *I. helenium* roots and rhizomes as natural additives and flavoring agents, while the U.S. Food and Drug Administration (FDA) approves this plant for use in food flavoring [4] [6].

Traditionally, *I. helenium* has been used for the treatment of acute respiratory diseases, pulmonary tuberculosis, diabetes, arthritis, and rheumatism [5]. *I. helenium* is utilized as an expectorant, antitussive, diaphoretic, and antibacterial agent. Recent studies have revealed that this plant exhibits various pharmacological activities, including antibacterial, antitumor, antiproliferative, antioxidant, anti-inflammatory, and antistress properties in traditional Chinese medicine [3] [7].

Extraction is a critical step for isolating biologically active compounds from medicinal plants, and selecting an appropriate solvent strongly influences both the phenolic profile and total phenolic content of the extract. Commonly used solvents for phenolic extraction include acetone, ethanol, methanol, ethyl acetate, water, propanol, and their combinations [3] [8] [9] [10]. The use of solvent mixtures plays a critical role in achieving efficient extraction of plant metabolites. While conventional approaches rely on manually adjusting solvent conditions, advanced statistical methods such as simplex-lattice, simplex-centroid, and D-optimal design have been increasingly applied [11]. Among these, the D-optimal design is advantageous because it requires fewer experimental runs, accommodates highly constrained conditions, and has been successfully employed in diverse areas, including food formulation and pharmaceutical applications. Therefore, it represents a practical and effective strategy for optimising metabolite extraction from plant materials [12]. For example, D-optimal mixture design has been applied for phenolic extraction from olive leaf [13], cornelian cherry [14], and *Phalaenopsis* leaves [12].

Although much research has been conducted on *I. helenium* plant roots [15] [16] [17], studies on *I. helenium* leaves are quite limited [5] [18]. There are no studies on the optimization of this herb. Therefore, this research is aimed to determine the total phenolic content and antioxidant activity of the extract obtained under optimal conditions, as well as to optimize the concentration of various solvents to be employed in the extraction of phenolic compounds from *I. helenium* leaves collected from Türkiye.

MATERIAL AND METHODS

Materials

In this study, dried *I. helenium* leaves were obtained from Süt Dünyası (Merkez, Tokat) for phenolic compound extraction. Before the extraction procedure, a grinder was used to roughly powder the dried *I. helenium* leaves (moisture content: 4.40 ±0.00%). After that, the powdered materials were sieved with a griddle having an area of 0.075 mm^2 pores. *I. helenium* leaf powder was stored at room temperature (25±2 °C) in a dark environment in glass jars.

Method

Phenolic Compound Extraction

The phenolic extraction process was carried out with a partial modification of the method applied by Bayram and Topuz [19]. Based on preliminary tests (solid:solvent ratio: 1:50; temperature: 30-100 °C; extraction time: 5-120 minutes), the solid:liquid ratio (1:25), temperature (50 °C), and extraction time (60 minutes) were determined and maintained constant during the extraction process. Firstly, *I. helenium* leaves (400 mg) were accurately weighed into extraction tubes. Then, ethanol/methanol/water mixtures (10 mL) at different concentrations (according to the .Table 1) were added to the samples. Solvent added samples were subjected to the extraction process in a shaking water bath at 50 °C for 60 minutes. Then, the extracts were centrifuged at 6000 rpm for 15 minutes and filtered through coarse filter paper before analysis (Fig. 1).

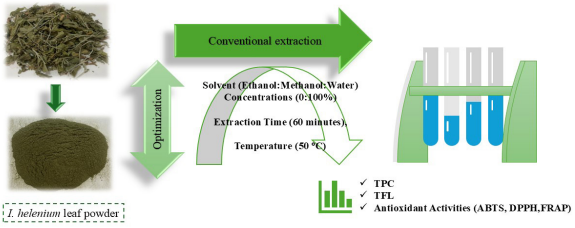

Fig 1 Flow chart of the phenolic extraction process from I. helenium leaf

Total Phenolic Content (TPC) Analyses

The (TPC) of the *I. helenium* extracts was determined using a slightly modified Folin–Ciocalteu (FC) method. 100 µL of the appropriately diluted extract was mixed with 200 µL of FC reagent and 2.0 mL of distilled water. The mixture was allowed to react at room temperature for 3 minutes, after which 1.0 mL of 20% (w/v) sodium carbonate (Na_2CO_3) solution was added and the mixture was thoroughly vortexed. The reaction mixture was then incubated at room temperature for 1 hour in the dark. Absorbance was measured at 765 nm using a UV–Vis spectrophotometer. A calibration curve was prepared with gallic acid standard solutions, and TPC values were calculated considering the dilution factor and expressed as mg gallic acid equivalents (GAE) per gram of dry sample. All measurements were performed in triplicate [20].

Total Flavonoid Content (TFC) Analysis

A method developed by Bayram and Topuz [19] was used to determine the (TFC) amount of *I. helenium* extracts. 500 µL of the appropriately diluted extract was mixed with 2 mL of distilled water and 150 µL of 5% (w/v) sodium nitrite ($NaNO_2$) solution. The mixture was allowed to stand at room temperature for 5 minutes, after which 150 µL of 10% (w/v) aluminum chloride ($AlCl_3$) solution was added. After reacting for 5 minutes, 1 mL of 1 M sodium hydroxide (NaOH) and 1.2 mL of distilled water were added, and the mixture was thoroughly vortexed. Absorbance was measured at 415 nm using a UV–Vis spectrophotometer. TFC values were calculated from a quercetin calibration curve and expressed as mg quercetin equivalents (QE)/g dry sample. All measurements were performed in triplicate.

Cation Radical Scavenging Activity ($ABTS^{\bullet+}$) Analysis

The ABTS radical scavenging activity of the *I. helenium* leaf extracts was determined using a slightly modified method of Re et al. [21]. 40 µL of each appropriately diluted extract was mixed with 4 mL of $ABTS^{\bullet+}$ solution. The mixture was vortexed and incubated in the dark at room temperature for 6 minutes. Absorbance was measured at 734 nm using a UV–Vis spectrophotometer. Antioxidant activity was calculated from a Trolox calibration curve and expressed as mg Trolox equivalents (TE) per gram of dry sample. All measurements were performed in triplicate.

Free Radical Scavenging Activity ($DPPH^{\bullet}$) Analysis

The DPPH radical scavenging activity of the *I. helenium* leaf extracts was determined using a slightly modified method of Blasi et al. [22]. 100 µL of each appropriately diluted extract was mixed with 3.9 mL of 0.06 mM DPPH solution and vortexed. The mixture was incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a UV–Vis spectrophotometer. Antioxidant activity was calculated from a Trolox calibration curve and expressed as mg (TE)/per gram of dry sample. All measurements were performed in triplicate.

Ferric Reducing Antioxidant Power (FRAP) Analysis

The FRAP value of the extract obtained under optimal conditions was determined using a slightly modified method of Benzie and Strain [23]. 100 µL of each appropriately diluted extract was mixed with 2.9 mL of FRAP working reagent and vortexed thoroughly. The mixture was incubated at room temperature for 30 minutes. Absorbance was measured at 593 nm using a UV–Vis spectrophotometer. Antioxidant activity was calculated from a Trolox calibration curve and expressed as mg (TE)/per gram of dry sample. All measurements were performed in triplicate.

Optimization and Statistical Analysis

The optimization of phenolic compound extraction from *I. helenium* leaves was carried out concerning solvent concentration (ethanol, methanol, water). Ethanol and methanol are polar solvents, but they have different polarities. Therefore, the amount of extracted phenolic compounds may differ for both solvents. The ranges of the mixture components used for the D-Optimal Mixture design applied for phenolic compound extraction are given in Table 1.

The D-Optimal Mixture design was used to optimize the solvent mixture that produced the highest TPC value. The "desirability" function technique was then used to improve the solvent mixture further. After extraction and processing, a one-sample t-test in SPSS 22.0 (IBM, USA) was used to

evaluate the difference between the experimentally acquired data and predicted values. With the use of the Design Expert 7.0 package program (Stat-Ease Inc., USA), statistical studies, regression analysis, optimization procedures, and response surface graphics were done to investigate the impact of solvent concentration on TPC values.

Table 1 Ranges of the mixture components used for the D-optimal Mixture design

Component	Minimum	Maximum
A: Ethanol (%)	0.00	100.00
B: Methanol (%)	0.00	100.00
C: Water (%)	0.00	100.00
	Total: 100.00	

RESULTS AND DISCUSSION

This study investigates the effect of different solvent mixtures (ethanol, methanol, and water) on the extraction of phenolic compounds from *I. helenium* leaves. Phenolic extract production from *I. helenium* leaves was carried out under 16 different conditions, employing a variety of solvent mixtures. The TPC values of the extracts produced by applying these conditions are presented in Table 2. TPC values for the extracts of *I. helenium* leaves varied from 2.95 to 28.12 mg GAE/g dry sample.

The highest TPC value (28.12 mg GAE/g dry sample) was observed in experiment 15 (ethanol:methanol:water; 50:0:50). In the optimization study, the quadratic polynomial model obtained by regression analysis for TPC is given by Equation (Eq.) 1.

$$TPC^{*}= 0.08A+0.09B+0.17C-1.28\times10-3AB+6.08\times10-3AC+4.37\times10-3BC \quad \text{Eq. 1}$$

*TPC: Total Phenolic Content (mg GAE/g dry sample), A: ethanol fraction (%), B: methanol fraction (%), C: water fraction (%)

The analysis of variance (ANOVA) table (Table 3) illustrates how the solvent combinations affect TPC values. At the 99% confidence level, the quadratic models developed for TPC analysis are statistically significant (*P<0.01*), whereas the model inadequacy is found to be statistically significant (*P>0.05*) at the 95% confidence level.

Table 2 Experimental design for phenolic compound extraction and the corresponding TPC values

No	Ethanol (%) (A)	Methanol (%) (B)	Water (%) (C)	TPC (mg GAE/g dry sample)
1	0.00	50.00	50.00	24.28±0.82
2	80.80	19.20	0.00	2.95±0.35
3	50.00	50.00	0.00	6.78±0.47
4	31.00	34.60	34.40	21.83±1.24
5	0.00	0.00	100.00	18.36±0.12
6	0.00	0.00	100.00	16.36±1.65
7	16.80	16.70	66.50	23.86±0.47
8	0.00	100.00	0.00	8.28±0.71
9	0.00	50.00	50.00	24.78±0.82
10	16.40	66.60	17.00	15.95±1.18
11	0.00	100.00	0.00	10.74±0.88
12	50.00	0.00	50.00	26.99±1.00
13	62.10	19.00	18.90	19.16±0.29
14	100.00	0.00	0.00	8.33±0.06
15	50.00	0.00	50.00	28.12±1.18
16	100.00	0.00	0.00	8.37±0.94

Table 3 ANOVA table for TPC values

Source of Variation	Degree of freedom	Sum of squares	Average of squares	F-value	*P-value*
Model	5	953.59	190.72	74.20	*< 0.0001*
Linear Mixture	2	419.05	209.52	81.52	*< 0.0001*
AB	1	12.00	12.00	4.67	*0.0560*
AC	1	365.73	365.73	142.30	*< 0.0001*
BC	1	180.93	180.93	70.39	*< 0.0001*
Residual	10	25.70	2.57		
Lack of fit	5	19.92	3.98	3.45	*0.1003*
Pure Error	5	5.78	1.16		
Total	15	979.30			

The analysis of the collected data revealed that there was no statistically significant (*P>0.05*) impact of ethanol-methanol interaction (AB) on TPC (Table 3). In contrast, the ethanol-water interaction (AC) and methanol-water concentration (BC) were shown to significantly (*P< 0.01*) influence TPC at the 99% confidence level (Table 3). Both the regression coefficient (R^2) and the adjusted regression coefficient (Adj-R^2) for the model developed for TPC are fairly close to 1 (Table 4). The correctness and reliability of the experimental results are indicated by the low coefficient of variation (CV), which also suggests a strong correlation between the expected and actual values.

Table 4 Statistical parameters for TPC values

Parameter	TPC
R^2	0.97
Adj- R^2	0.96
Pred R^2	0.93
Adequate Precision	22.51
PRESS	62.42
C.V (%)	9.67

The effect of solvent combinations on the TPC values of extracts made from *I. helenium* is shown in Fig. 2 as a 3D response surface and contour plot. Upon examining the graph, it can be observed that a mixture of ethanol and water in similar volumes increases the TPC value. Similarly, a mixture of methanol and water in close volumes also increases the TPC value. Moreover, a mixture of all three solvents in equal volumes also leads to an increase in the TPC amount. According to the results of our study, extraction processes conducted at a 50% ethanol concentration yielded higher TPC values compared to extractions performed with 100% ethanol or methanol, as well as with water. These results imply that the ethanol-water mixture works better than single-component solvents and that the combination of organic solvents and water facilitates the extraction of both water-soluble and organic-soluble chemicals. Furthermore, it was determined that the ethanol-water mixture provided a higher yield compared to single-component solvents, facilitating the extraction of both organic and water-soluble compounds. Polyphenols, especially glycosides with many hydroxyl groups, are hydrophilic and more soluble in hydroalcoholic combinations than in solvents made entirely of alcohol [24]. In a study, Natolino et al. [25] used water, ethanol, and a 50% water-ethanol mixture (EtOH 50%) extraction of bioactive compounds from food by-products. It was determined that 50% water-ethanol mixture is more effective than pure components. In another study, Waszkowiak and Gliszczyńska-Świgło [26] reported that a 60:40 (v/v) ethanol-water mixture was the most effective for obtaining flaxseed extracts with high phenolic content and strong antioxidant activity.

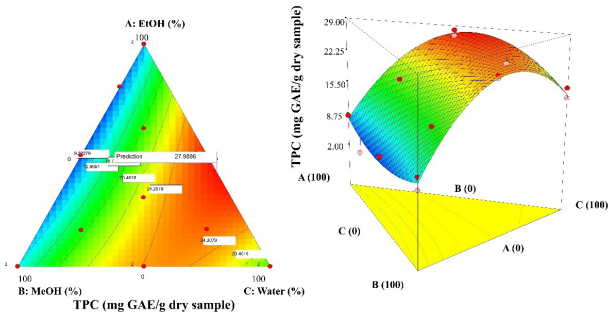

Fig 2 3D contour plot and response-surface showing the effect of solvent mixtures on TPC values

The predicted data from the polynomial models generated by the program for TPC values have been compared with the experimental data obtained in Fig 3. Upon examining the graph, it was observed that the predicted data tend to align along the 45° line with the experimental data. This suggests that the models developed are appropriate and well-suited for the data.

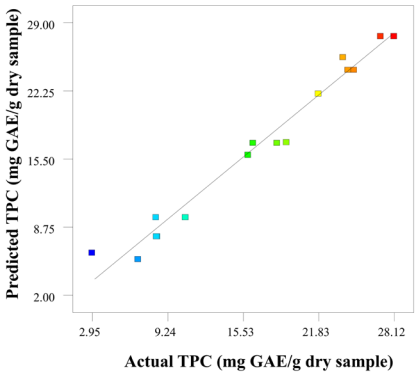

Fig 3 Relationship between actual data and predicted data in terms of TPC values

In the optimization study for phenolic compound extraction from *I. helenium*, two solution points were identified using the "desirability" function approach by the Design Expert Program. Among these solutions, the one selected by the program, with 42.40% ethanol, 0% methanol, and 57.60% water, was accepted as the optimum extraction conditions (Table 5).

In the phenolic compound extraction from *I. helenium* leaves carried out at 50 °C for 60 minutes, the optimum solvent mixture (ethanol: methanol: water; 42.40: 0: 57.60) predicted a TPC value of 27.99 mg GAE/g dry sample. Three repeated optimum point validation trials were conducted, and the TPC value was determined as 28.12±0.82 mg GAE/g dry sample. The experimental data and predicted values were analyzed using a one-sample t-test, revealing no statistically significant difference (*P>0.05*) between the two datasets.

Table 5 Optimization parameters and TPC, TFC, and antioxidant activity values of the extract obtained under optimal conditions

Optimum Conditions (for 50 °C, 60 minutes)		
Ethanol (%)	**Methanol (%)**	**Water (%)**
42.40	0.00	57.60
Predicted TPC (mg GAE/g dry sample)		
27.99		
Actual TPC (mg GAE/g dry sample)		
28.12±0.82		
TFC (mg QE/g dry sample)	27.74±0.61	
ABTS (mg TE/g dry sample)	19.73±2.61	
FRAP (mg TE/g dry sample)	1.42±0.38	
DPPH (mg TE/g dry sample)	6.10±0.77	

The TFC value of the extract obtained under optimal

conditions, together with its antioxidant activities measured using the ABTS, DPPH, and FRAP methods, is presented in Table 5.

According to Akthar [27], the TPC in *I. helenium* extracts (dried aerial parts) prepared using methanol, ethanol, water, and ethyl acetate ranged from 4.18 to 102.91 mg GAE/g dry weight. Gökbulut et al. [18] reported that the TPC in methanol extracts of *I. helenium* aerial parts was 189.6 ±3.9 mg GAE/g. The DPPH (IC_{50}) values of *I. helenium* leaf extracts were 0.23±0.03 mg/mL for methanol and 0.49±0.05 mg/mL for water extracts. For ABTS, the IC_{50} values were 0.39±0.06 mg/mL for methanol and 0.19±0.009 mg/mL for water extracts. Albayrak et al. [28] determined that TPC in aerial parts of four distinct *I. helenium* species ranged from 13.32 to 102.91 mg GAE/g for methanol, 17.30 to 37.31 mg GAE/g for ethanol, and 12.88 to 20.13 mg GAE/g for water extracts. Corresponding DPPH (IC_{50}) values ranged from 8.89 to 36.76 µg/mL for methanol, 12.28 to 61.70 µg/mL for ethanol, and 26.15 to 51.23 µg/mL for water extracts. In another study, the TPC, TFC, and DPPH (IC_{50}) values of ethanol leaf extracts of *I. helenium* from different regions ranged from 49.61 to 69.02 mg GAE/g, 191.20 to 376.22 mg rutin/g, and 338.83 to 865.32 µg/mL, respectively [5]. Moradi et al. [29] reported that the TPC and TFC in 15 distinct *I. helenium* populations (aerial parts) in Iran ranged from 53.86 to 100.27 mg GAE/g dry weight and 7.25 to 11.33 mg QE/g dry weight, respectively. Orhan et al. [30] determined that in methanol leaf extracts of five distinct *I. helenium* species, TPC ranged from 164.40 ±7.55 mg GAE/g, while TFC ranged from 43.78±2.58 mg QE/g extract. Sevindik et al. [31] reported that the TPC in ethanol leaf extracts of *I. helenium subsp. turcoracemosa* and subsp. *pseudohelenium* was 59.97±0.008 µg GAE/mL and 73.63±0.0006 µg GAE/mL, respectively.

Comparison of the results of this study with existing literature reveals that the phenolic content and antioxidant activity of phenolic extracts derived from *I. helenium* leaves can exhibit considerable variation. This variation can be attributed to several factors, including extraction parameters, cultural practices, geographical location, climatic conditions, harvest time, and plant species, all of which can influence both the quantity and composition of phenolic compounds in plants [10] [20].

CONCLUSION

The selection of the solvent system plays a crucial role in the efficient extraction of phenolic compounds from plant sources. This study aimed to determine the optimal conditions for maximizing TPC and to assess the impact of different solvent concentrations (ethanol, methanol, and water) on the extraction of phenolic compounds from *I. helenium* leaves. The effect of solvent mixtures on extraction efficiency was assessed using the D-Optimal Mixture design. The most efficient extraction condition was determined by optimization to be a solvent mixture of 42.40% ethanol, 0% methanol, and 57.60% water. According to the study's findings, phenolic compounds that are isolated from *I. helenium* leaves using the right solvent system have potent antioxidant properties, can making them a potential viable natural antioxidant source for the food industry. Future studies may focus on validating these findings with different plant species, exploring alternative extraction techniques, and investigating the bioavailability and functional applications of the extracted phenolic compounds in food systems.

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